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Rozprawa doktorska

**Rola białek RBMXL3 oraz DDX53 w regulacji
metabolizmu RNA w komórkach linii płciowej człowieka**

mgr Agata Jolanta Barszcz

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Promotor:

dr hab. n. med. Natalia Rozwadowska, prof. IGC

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1. Wykaz skrótów

2D dwuwymiarowy (ang. *two-dimensional*)

3D trójwymiarowy (ang. *three-dimensional*)

ALI granica powietrze–ciecz (ang. *air–liquid interface*)

ARs receptory androgenowe (ang. *androgen receptors*)

AS alternatywny splicing (ang. *alternative splicing*)

BTB bariera krew–jądro (ang. *blood-testis barrier*)

ChIP immunoprecypitacja chromatyny (ang. *chromatin immunoprecipitation*)

ChIP-seq immunoprecypitacja chromatyny połączona z sekwencjonowaniem (ang. *chromatin immunoprecipitation followed by sequencing*)

Co-IP-MS koimmunoprecypitacja sprzężona ze spektrometrią mas (ang. *co-immunoprecipitation coupled with mass spectrometry*)

CTAs antygeny nowotworowo-jądrowe (ang. *cancer-testis antigens*)

DEGs geny o zróżnicowanej ekspresji (ang. *differentially expressed genes*)

DSGs geny o zmienionym splicingu (ang. *differentially spliced genes*)

EBs ciała embrioidalne (ang. *embryoid bodies*)

ECM macierz zewnątrzkomórkowa (ang. *extracellular matrix*)

eCLIP-seq wzmocniona metoda sieciowania i immunoprecypitacji połączona z sekwencjonowaniem (ang. *enhanced crosslinking and immunoprecipitation followed by sequencing*)

FSH hormon folikulotropowy (ang. *follicle-stimulating hormone*)

GO ontologia genów (ang. *gene ontology*)

hESCs ludzkie embrionalne komórki macierzyste (ang. *human embryonic stem cells*)

hiPSCs ludzkie indukowane pluripotencjalne komórki macierzyste (ang. *human induced pluripotent stem cells*)

hPGCLCs komórki przypominające pierwotne komórki linii płciowej człowieka (ang. *human primordial germ cell-like cells*)

hPGCs pierwotne komórki płciowe człowieka (ang. *human primordial germ cells*)

hPSCs ludzkie pluripotencjalne komórki macierzyste (ang. *human pluripotent stem cells*)

HPA atlas białek człowieka (ang. *Human Protein Atlas*)

IHC immunohistochemia (ang. *immunohistochemistry*)

IVG gametogeneza *in vitro* (ang. *in vitro gametogenesis*)

LH hormon luteinizujący (ang. *luteinizing hormone*)
NOA azoospermia nieobturacyjna (ang. *non-obstructive azoospermia*)
PGCs pierwotne komórki płciowe (ang. *primordial germ cells*)
PTMs okołokanalikowe komórki mioidalne (ang. *peritubular myoid cells*)
RBDs domeny wiążące RNA (ang. *RNA-binding domains*)
RBPs białka wiążące RNA (ang. *RNA-binding proteins*)
RNA-seq sekwencjonowanie RNA (ang. *RNA sequencing*)
RNP kompleks rybonukleoproteinowy (ang. *ribonucleoprotein complex*)
RRM domena RRM (ang. *RNA recognition motif*)
SCOS zespół komórek Sertolego (ang. *Sertoli cell-only syndrome*)
SLCs komórki podobne do spermatogonii (ang. *spermatogonium-like cells*)
snRNPs jądrowe rybonukleoproteiny (ang. *small nuclear ribonucleoproteins*)
SSCs spermatogonialne komórki macierzyste (ang. *spermatogonial stem cells*)
TEs elementy transpozycyjne (ang. *transposable elements*)
ToC jądro na chipie (ang. *testis-on-a-chip*)
TOs organoidy jąder (ang. *testicular organoids*)
WES sekwencjonowanie całego egzomu (ang. *whole-exome sequencing*)
WGS sekwencjonowanie całego genomu (ang. *whole-genome sequencing*)

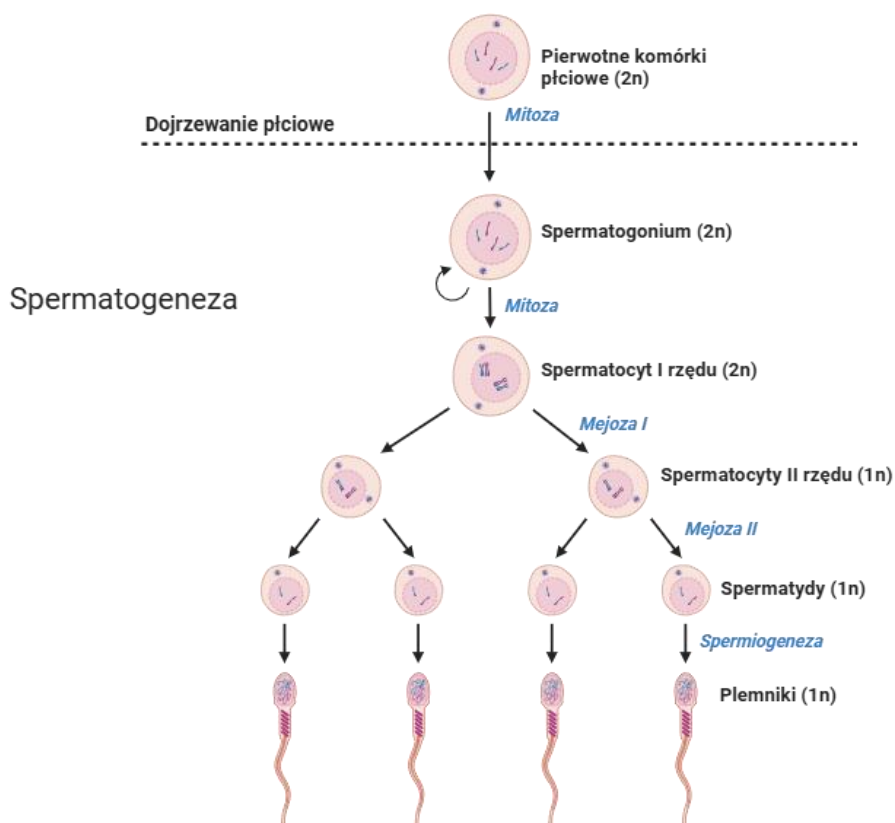
2. Wstęp

Rozwój komórek linii płciowej i proces spermatogenezy człowieka

U podstaw rozwoju każdego organizmu leży precyzyjnie skoordynowany proces, który od pierwszych podziałów zarodkowych prowadzi do powstania wyspecjalizowanych tkanek i narządów oraz wyodrębnienia komórek odpowiedzialnych za przekazanie informacji genetycznej kolejnym pokoleniom. Komórki linii płciowej pełnią wyjątkową funkcję biologiczną, zapewniając zarówno ciągłość gatunku, jak i zmienność stanowiącą podstawę ewolucji. Rozwój ludzkiej linii płciowej rozpoczyna się od specyfikacji pierwotnych komórek płciowych (ang. *human primordial germ cells*, hPGCs), będących embrionalnymi prekursorami gamet. Komórki te nie są jeszcze zdeterminowane płciowo i powstają na wczesnym etapie embriogenezy, około 2.-3. tygodnia (Saitou, Nagano, and Mizuta 2025; Tang et al. 2016). Następnie hPGCs migrują do rozwijających się gonad, gdzie pod wpływem sygnałów molekularnych różnicują się w gonocyty płodowe. Gonocyty płodowe, konkretnie prospermatogonia w przypadku męskiej linii płciowej, stanowią prekursorową populację, z której po urodzeniu wykształca się pula spermatogonialnych komórek macierzystych (ang. *spermatogonial stem cells*, SSCs). Proces spermatogenezy jest inicjowany dopiero w okresie dojrzewania płciowego i przebiega przez całe życie dorosłe mężczyzny (Rycina 1).

Spermatogeneza jest wysoce zorganizowanym i dynamicznym procesem, prowadzącym do przekształcenia diploidalnych spermatogonii w haploidalne plemniki. Proces ten przebiega etapowo i wymaga precyzyjnej regulacji ekspresji genów, ścisłej kontroli sygnalizacji molekularnej oraz rozległego przeprogramowania epigenetycznego, co warunkuje powstawanie prawidłowych i funkcjonalnych gamet męskich (Griswold 2016). Istotną rolę w tym procesie odgrywa regulacja hormonalna, która koordynuje procesy różnicowania męskich komórek płciowych. Prawidłowa spermatogeneza zachodzi w wyspecjalizowanym mikrośrodkowisku, w którym kolejne etapy różnicowania komórek germinalnych są wspierane przez komórki somatyczne, w szczególności komórki Sertolego, komórki Leydiga i komórki mioidalne okołokanalikowe. Procesy dynamicznej przebudowy epigenetycznej gamet, w tym globalne zmiany metylacji DNA, modyfikacje histonów oraz reorganizacja chromatyny są niezbędnym elementem mejozy oraz różnicowania postmeiotycznego, a także kształtowania znaków epigenetycznych, które są przekazywane potencjalnemu potomstwu (McSwiggin and O'Doherty 2018).

Proces spermatogenezy u człowieka przebiega w kanalikach nasiennych zlokalizowanych w jądrach i trwa około 74 dni, a inicjacja różnicowania w danym obszarze kanalika odbywa się cyklicznie w odstępach około 16 dni (Amann 2008).



Rycina 1. Schemat przedstawiający proces spermatogenezy człowieka. Wykonano w BioRender.com, na podstawie (Eisenberg et al. 2023).

Spermatogeneza obejmuje proliferację spermatogonialnych komórek macierzystych (SSCs), ich różnicowanie do spermatocytów, a następnie podziały meiotyczne spermatocytów, które prowadzą do powstania haploidalnych spermatyd, które przekształcają się w dojrzałe plemniki (Amann 2008; Eisenberg et al. 2023). Komórki linii płciowej męskiej utrzymywane są przez populację SSCs zlokalizowaną wzdłuż błony podstawnej nabłonka plemnikotwórczego, wykazując zróżnicowanie stanów funkcjonalnych - od spermatogonii w stanie spoczynku (ang. *quiescence*) po zaangażowanie w proces różnicowania (Guo et al. 2020; Kaltsas et al. 2025; Kubota and Brinster 2018). W wyniku różnicowania spermatogonia typu B dają początek spermatocytom I rzędu poprzez ostatni podział mitotyczny. Powstałe spermatocyty I rzędu przekraczają barierę krew-jądro (ang. *blood-testis barrier*, BTB), uzyskując

izolację od układu immunologicznego, co umożliwia ochronę komórek postmejotycznych przed odpowiedzią ze strony układu immunologicznego (Mruk and Cheng 2015). Bariera krew-jądro zachowuje swoją integralność i funkcję ochrony immunologicznej, ponieważ proces „przechodzenia” spermatocytów polega na ścisłym remodelowaniu połączeń między komórkami Sertolego i opiera się na mechanizmie „śluzu” (Wanjari and Gopalakrishnan 2024). Po przejściu do przedziału adluminalnego spermatocyty wchodzi w pierwszy podział mejotyczny (mejozę I), podczas którego chromosomy homologiczne ulegają parowaniu oraz wzajemnej wymianie materiału genetycznego w procesie crossing-over, co prowadzi do powstania unikalnych kombinacji alleli rodzicielskich, istotnych dla różnorodności genetycznej. W wyniku mejozy I powstają dwa spermatocyty II rzędu, które następnie przechodzą drugi podział mejotyczny (mejozę II), prowadząc ostatecznie do utworzenia czterech haploidalnych, okrągłych spermatyd, z których każda zawiera pojedynczy zestaw chromosomów. Przekształcanie haploidalnych spermatyd w plemniki o pełnej dojrzałości morfologicznej i funkcjonalnej nie wymaga dalszych podziałów komórkowych, lecz polega na złożonych procesach przebudowy wewnątrzkomórkowej. Proces ten jest określany jako spermiogeneza, natomiast uwalnianie dojrzałych plemników z komórek Sertolego do światła kanalików nasiennych określane jest jako spermiacja (Eisenberg et al. 2023). U człowieka spermiogeneza trwa około trzech tygodni (Hermo et al. 2010; Teves and Roldan 2021). Powstawanie dojrzałych, funkcjonalnych plemników wymaga skoordynowanego przebiegu licznych procesów komórkowych i molekularnych, takich jak przebudowa jądra komórkowego i wymiana histonów na protaminy, formowanie akrosomu, rozwój witki oraz redukcja cytoplazmy prowadząca do wytworzenia ciał resztkowych, podlegających następnie fagocytozie przez komórki Sertolego (Skinner 2018).

Każdy etap spermatogenezy wymaga precyzyjnie kontrolowanych sygnałów regulacyjnych dostarczanych przez komórki somatyczne jądra, w tym komórki Sertolego, komórki mioidalne oraz komórki Leydiga, a także wsparcia ze strony układu naczyniowego i specyficznego mikrośrodowiska gonady męskiej.

Spermatogeneza człowieka różni się istotnie od przebiegu tego procesu u innych gatunków, w tym szeroko wykorzystywanych organizmów modelowych, takich jak muszka owocowa (*Drosophila melanogaster*), danio pręgowany (*Danio rerio*) czy nawet mysz domowa (*Mus musculus*). Różnice te obejmują zarówno czas trwania i dynamikę procesu, jak i architekturę tkanki, gatunkowo specyficzne programy ekspresji genów oraz

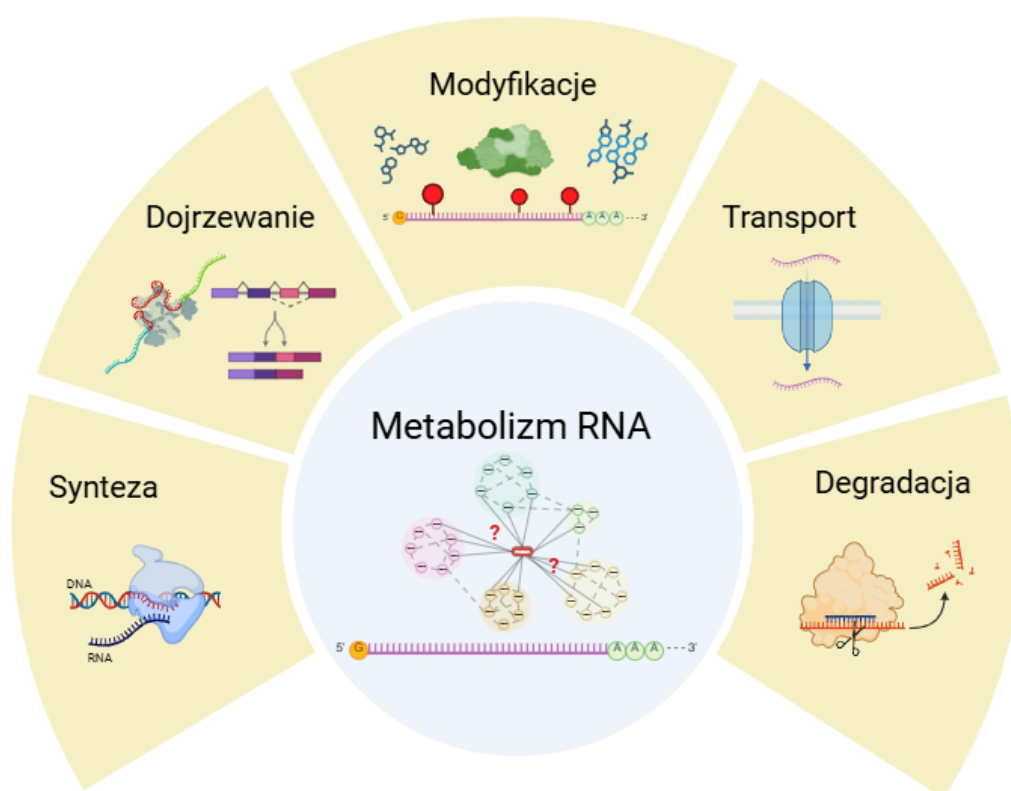
odmienne mechanizmy regulujące różnicowanie komórek płciowych. Badania transkryptomyczne i epigenetyczne wykazały, że liczne geny oraz szlaki regulacyjne kluczowe dla spermatogenezy człowieka są nieobecne, zmienione lub odmiennie regulowane w modelach mysich, co podkreśla ograniczenia w bezpośredniej ekstrapolacji wyników badań z organizmów modelowych na biologię rozrodu człowieka (Hermann et al. 2018; Soumillon et al. 2013; Tharmalingam, Jorgensen, and Mitchell 2018). Ponadto dla wielu genów potencjalnie zaangażowanych w spermatogenezę nadal brakuje jednoznacznych danych potwierdzających ich funkcję w tym procesie, co odzwierciedla zarówno ograniczenia dostępnych modeli badawczych, jak i wysoką złożoność mechanizmów molekularnych towarzyszących różnicowaniu męskich komórek germinalnych (Siebert-Kuss et al. 2022).

Metabolizm RNA jako istotny mechanizm regulacyjny podczas spermatogenezy

Podstawowym założeniem biologii molekularnej jest tzw. centralny dogmat, opisujący przepływ informacji genetycznej od DNA przez RNA do białka. Informacja zapisana w DNA ulega transkrypcji do RNA, które następnie - w przypadku mRNA - jest tłumaczona na sekwencję aminokwasów tworzących białko. Choć schemat ten stanowi fundament rozumienia procesów komórkowych, obecnie wiadomo, że ekspresja genów zapisanych w genomie podlega złożonej i wielopoziomowej regulacji. W trakcie rozwoju organizmu oraz różnicowania komórek ekspresja genów jest kontrolowana przez złożoną sieć mechanizmów epigenetycznych, transkrypcyjnych oraz potranskrypcyjnych, które wspólnie zapewniają odpowiedni poziom, czas oraz tkankową i komórkową specyficzność ekspresji genów. W tym wielopoziomowym systemie szczególną rolę odgrywa metabolizm RNA, obejmujący procesy syntezy, dojrzewania, modyfikacji, transportu oraz degradacji cząsteczek RNA (Rycina 2). Na każdym etapie dochodzi do precyzyjnej regulacji liczby, stabilności oraz różnorodności izoform i modyfikacji chemicznych RNA, co bezpośrednio wpływa na poziom oraz właściwości syntetyzowanych białek w komórce. Z tego względu metabolizm RNA ma fundamentalne znaczenie dla prawidłowego funkcjonowania komórek.

Metabolizm RNA odnosi się do procesów obejmujących cały cykl życia cząsteczki RNA (Choi, Ham, and Hwang 2022). Procesy takie jak modyfikacje końców transkryptów, alternatywne składanie (splicing) pre-mRNA, transport jądrowo-cytoplazmatyczny, regulacja stabilności mRNA oraz kontrola translacji są ze sobą ściśle

powiązane i zachodzą w sposób skoordynowany, tworząc wielopoziomową sieć regulacyjną. Niezwykle istotny udział we wszystkich tych procesach mają białka wiążące RNA (ang. *RNA-binding proteins*, RBPs), które bezpośrednio wpływają na los transkryptów (Dassi 2017). Zaburzenia któregośkolwiek z etapów metabolizmu RNA mogą prowadzić do znaczących zmian w ekspresji genów i mogą być powiązane z rozwojem chorób, w tym nowotworów, schorzeń neurodegeneracyjnych (Tao et al. 2024) oraz zaburzeń płodności (Morgan et al. 2021a), co dodatkowo podkreśla znaczenie tych procesów dla utrzymania homeostazy komórkowej. Metabolizm RNA nie jest zatem jedynie pasywnym etapem pośrednim, lecz aktywnym architektem fenotypu komórkowego, gdzie każda modyfikacja chemiczna (Gehring and Roignant 2021) czy zmiana partnera białkowego w kompleksie rybonukleoproteinowym (składającym się z RNA oraz białek wiążących RNA, ang. *ribonucleoprotein complex*, RNP) ma fundamentalne znaczenie dla regulowanego procesu.



Rycina 2. Schemat przedstawiający procesy związane z metabolizmem RNA. Wykonano w BioRender.com.

Jak wspomniano powyżej, spermatogeneza należy do jednego z najbardziej złożonych procesów różnicowania komórkowego w organizmie człowieka (Komeya, Sato, and Ogawa 2018). Wymaga niezwykle precyzyjnej i wielopoziomowej regulacji ekspresji genów, a kontrola metabolizmu RNA stanowi jeden z kluczowych mechanizmów regulacyjnych. Kolejne etapy dojrzewania komórek rozrodczych (od SSCs stanowiących rezerwuar macierzystych komórek germinalnych, przez spermatocyty wchodzące w mejozę, aż po haploidalne spermatydy) wiążą się z intensywnymi zmianami transkryptomicznymi (Hermann et al. 2018). Charakterystyczną cechą spermatogenezy jest także przestrzenne i czasowe rozdzielanie transkrypcji i translacji. W trakcie spermiogenezy dochodzi do postępującej kondensacji chromatyny oraz wymiany histonów na białka przejściowe, a tych na protaminy, co prowadzi do niemal całkowitego wygaszenia aktywności transkrypcyjnej (Morgan et al. 2021a). Wiele mRNA niezbędnych na późnych etapach różnicowania - w tym transkrypty białek przejściowych i protamin - musi być syntetyzowanych w spermatydach z wyprzedzeniem i przez wiele dni pozostawać w stanie represji translacyjnej, zanim zostaną aktywowane w odpowiednim momencie spermiogenezy. Te specyficzne transkrypty mRNA są w tym celu magazynowane w kompleksach mRNP, składających się z mRNA oraz białek wiążących RNA (RBPs), które chronią je przed degradacją oraz utrzymują je w stanie czasowej represji translacyjnej. Regulacja losu cząsteczek RNA stanowi zatem jeden z centralnych mechanizmów zapewniających prawidłowy przebieg spermatogenezy.

Alternatywny splicing i jego znaczenie w regulacji spermatogenezy

Splicing stanowi fundamentalny etap obróbki potranskrypcyjnej RNA, polegający na usuwaniu intronów z pre-mRNA oraz precyzyjnym łączeniu egzonów, co prowadzi do powstania ciągłej sekwencji kodującej w dojrzałym mRNA. Chociaż splicing klasycznie jest zaliczany do grupy procesów potranskrypcyjnych, znaczna część składania pre-mRNA zachodzi kotranskrypcyjnie, pozostając ściśle sprzężona z przebiegiem transkrypcji. Splicing pre-mRNA jest katalizowany przez spliceosom – dynamiczny i wysoce złożony kompleks rybonukleoproteinowy, złożony z pięciu małych jądrowych rybonukleoprotein (ang. *small nuclear ribonucleoproteins*, snRNPs) oraz licznych białek pomocniczych, które współdziałają w ściśle kontrolowanej sekwencji zdarzeń (Wilkinson, Charenton, and Nagai 2020). Prawidłowe rozpoznanie miejsc donorowych 5' (ang. *5' splice site*) i akceptorowych 3' (ang. *3' splice site*) stanowi istotne wyzwanie

w komórkach ludzkich, szczególnie ze względu na dużą długość intronów oraz złożoną strukturę genów. W genomie człowieka przeciętny gen zawiera liczne introny, często znacznie dłuższe niż egzonowe regiony kodujące. Najważniejszym mechanizmem zwiększającym złożoność transkryptomu jest alternatywny splicing (ang. *alternative splicing*, AS), któremu podlega około 95% ludzkich genów wieloegzonowych (Wang et al. 2008; Wilkinson et al. 2020). Proces ten umożliwia generowanie wielu izoform mRNA z pojedynczego genu, różniących się strukturą oraz potencjalną funkcją, co stanowi kluczowy element regulacji ekspresji genów i w konsekwencji także różnorodności funkcjonalnej proteomu. Wybór alternatywnych miejsc splicingu jest regulowany przez sekwencje regulatorowe w pre-mRNA oraz złożone mechanizmy molekularne, w których kluczową rolę odgrywają białka RBPs.

Alternatywny splicing znacząco zwiększa złożoność transkryptomu i proteomu bez konieczności zwiększania liczby genów. Proces ten odgrywa szczególnie istotną rolę w komórkach męskiej linii płciowej, ponieważ geny zaangażowane w spermatogenezę często wykazują tkankowo i etapowo specyficzną ekspresję izoform mRNA - różniących się pomiędzy poszczególnymi subpopulacjami komórek germinalnych. Umożliwia to dynamiczne przeprogramowanie proteomu podczas różnicowania komórek męskiej linii płciowej. W konsekwencji precyzyjna regulacja alternatywnego splicingu jest niezbędna dla prawidłowego przebiegu spermatogenezy, a gonada męska należy do narządów o najwyższym poziomie tego procesu spośród tkanek ssaków (Mazin et al. 2021; Song et al. 2020). Tak wysoki stopień zależności spermatogenezy od precyzyjnej regulacji alternatywnego splicingu sprawia, że zaburzenia czynników splicingowych mogą prowadzić do defektów rozwoju komórek germinalnych i męskiej niepłodności. Badania z wykorzystaniem modeli zwierzęcych wykazały, że taką zależność obserwuje się dla wielu czynników splicingowych. Przykładowo, inaktywacja PTBP2 prowadzi u myszy do postępującego zaniku komórek germinalnych związanego ze zwiększoną apoptozą spermatocytów oraz zahamowaniem dojrzewania spermatyd (Zagore et al. 2015). Utrata SRSF1 zaburza natomiast regulację alternatywnego splicingu genów związanych z przebiegiem mejozy i różnicowaniem komórek germinalnych, prowadząc do zatrzymania różnicowania oraz nasilonej apoptozy męskich komórek płciowych (Lei et al. 2023). Podobnie dysfunkcja RBM5, innego czynnika splicingowego wiążącego RNA, skutkuje zatrzymaniem spermatogenezy na etapie okrągłych spermatyd i całkowitą bezpłodnością samców myszy (O'Bryan et al. 2013).

Defekty w obrębie aparatu splicingowego mogą manifestować się szerokim spektrum fenotypów związanych z zaburzeniami spermatogenezy, w zależności od tego, które czynniki splicingowe wykazują nieprawidłową aktywność oraz na jakim etapie różnicowania komórek germinalnych ich działanie jest najbardziej krytyczne. Niezwykle istotna jest także pula transkryptów mRNA, na które wpływają RBPs w sposób bezpośredni (poprzez wiązanie) bądź pośredni (poprzez interakcje z innymi białkami), ponieważ różne transkrypty mogą być kluczowe na różnych etapach procesu spermatogenezy (Corley, Burns, and Yeo 2020). Warto także zaznaczyć, że regulacja alternatywnego splicingu ma charakter sieciowy - wiele czynników splicingowych oddziałuje ze sobą, tworząc kompleksy i modyfikując wzajemnie swoje aktywności.

Kontrola długości ogona poly(A) a stabilność RNA

Ogon poli(A) to szereg nukleotydów adeninowych, który jest dodawany podczas potranskrypcyjnej obróbki pre-mRNA niemal wszystkich eukariotycznych cząsteczek mRNA i jest wymagany dla ich eksportu z jądra komórkowego do cytoplazmy. Ten region transkryptu odgrywa kluczową rolę w potranskrypcyjnej regulacji ekspresji genów, wpływając na stabilność, lokalizację oraz translację. Długość ogona poli(A) regulowana jest przez dynamiczną równowagę pomiędzy poliadenylacją a deadenylacją, kontrolowaną przez polimerazy poli(A), liczne białka RBPs, w tym białka PABP, oraz kompleksy deadenylujące PAN2-PAN3 i CCR4-NOT (Passmore and Collier 2021). W wielu komórkach somatycznych wydłużenie ogona poli(A) zwiększa stabilność transkryptu i sprzyja efektywnej translacji poprzez ułatwienie tworzenia kompleksu inicjującego translację. Deadenylacja może stanowić natomiast jeden z pierwszych etapów degradacji mRNA i odgrywa istotną rolę w kontroli czasu życia transkryptów (Passmore and Collier 2021).

W komórkach męskiej linii płciowej regulacja długości ogona poli(A) może przebiegać według odmiennego modelu niż w większości komórek somatycznych. Podczas spermiogenezy wiele kluczowych transkryptów pozostaje przez długi czas w stanie represji translacyjnej, a ich aktywacji translacyjnej może towarzyszyć skrócenie ogona poli(A) (Ozturk and Uysal 2018). Klasycznym przykładem tego mechanizmu są wspomniane już wcześniej transkrypty protamin i białek przejściowych chromatyny, które ulegają intensywnej transkrypcji w okrągłych spermatydach, a następnie są magazynowane w postaci kompleksów mRNP do późniejszych etapów spermiogenezy.

Aktywacja translacji tych transkryptów zachodzi dopiero na etapie wydłużających się spermatyd, równoległe z postępującą wymianą histonów na białka przejściowe, a tych na protaminy oraz wygaszaniem aktywności transkrypcyjnej (Ozturk and Uysal 2018). Badania przeprowadzone z wykorzystaniem modelu mysiego wykazały, że całkowita utrata funkcji białka YBX2 - o zdolności wiązania DNA i RNA, oddziałującego z regionami 3'UTR mRNA protamin i uczestniczącego w utrzymaniu ich represji translacyjnej - prowadzi do przedwczesnej aktywacji translacji tych transkryptów w okrągłych spermatydach oraz ciężkich zaburzeń spermatogenezy (Yang et al. 2005).

Ponieważ w późnych etapach spermiogenezy aktywność transkrypcyjna ulega niemal całkowitemu wygaszeniu, cytoplazmatyczna regulacja długości ogona poli(A) stanowi istotny mechanizm modulowania dostępności translacyjnej zmagazynowanych mRNA (Ozturk and Uysal 2018). Narastająca liczba doniesień naukowych wskazuje również, że prawidłowa kontrola metabolizmu mRNA w dojrzałych plemnikach może wpływać na stabilność zachowanych transkryptów oraz ich potencjalną funkcję po zapłodnieniu, sugerując, że dojrzałe męskie gamety zachowują funkcjonalnie istotny repertuar RNA (Sendler et al. 2013).

Regulacja długości ogona poli(A) odgrywa istotną rolę również we wcześniejszych etapach różnicowania komórek germinalnych. Badania z wykorzystaniem modelu mysiego wykazały, że podjednostka CNOT3 kompleksu deadenylującego CCR4-NOT ulega wysokiej ekspresji w nieodróżnicowanych spermatogoniach, a utrata jej funkcji prowadzi do wyczerpania puli spermatogonialnych komórek macierzystych oraz niepłodności samców (Chen et al. 2025; Miao and Hu 2025). Mechanizm ten związany jest z deregulacją stabilności transkryptów kodujących czynniki różnicowania, których degradacja zależna od deadenylacji jest niezbędna dla utrzymania nieodróżnicowanego stanu spermatogoniów.

Udział kompleksów mRNP w regulacji losu mRNA

Los cząsteczek mRNA w komórce regulowany jest przez złożoną sieć oddziaływań obejmujących białka wiążące RNA (RBPs), inne czynniki metabolizmu RNA oraz interakcje z różnymi klasami cząsteczek kwasów rybonukleinowych. W wyniku tych oddziaływań powstają kompleksy mRNP, odgrywające kluczową rolę w regulacji cyklu życia mRNA (Corley et al. 2020; Mitchell and Parker 2014). Skład kompleksów mRNP jest dynamiczny i może zmieniać się w zależności od typu komórki,

stanu fizjologicznego czy etapu różnicowania komórkowego. Zmiana któregokolwiek z komponentów należących do mRNP może wpływać na funkcjonalny status związanych z nim transkryptów, oddziałując na przykład na ich stabilność, lokalizację oraz dostępność translacyjną. Co więcej, kompleksy mRNP mogą organizować się w wyspecjalizowane bezbłonowe struktury komórkowe, takie jak ciała P (ang. *P-bodies*), granule stresu (ang. *stress granules*) czy szczególnie istotne w kontekście komórek płciowych granule germinalne (ang. *germ granules*) (Pamula and Lehmann 2024; Pham-Bui and Lee 2025). Struktury te, bogate w RNA oraz białka RBPs, uczestniczą w regulacji stabilności mRNA, jego czasowym magazynowaniu, kontroli translacji oraz, w zależności od typu granuli, także w procesach degradacji RNA. Pełnią rolę dynamicznych centrów regulacyjnych RNA, dlatego są niezwykle ważne w kontekście różnicowania męskich komórek płciowych.

U ssaków najlepiej poznaną strukturą należącą do granuli germinalnych jest ciało chromatoidalne (ang. *chromatoid body*) - wyspecjalizowana cytoplazmatyczna granula obecna głównie w okrągłych spermatydach i uczestnicząca w potranskrypcyjnej regulacji ekspresji genów podczas spermiogenezy (Anbazhagan et al. 2022). Ciało chromatoidalne pełni rolę centrum organizacji wielu kompleksów mRNP oraz metabolizmu RNA w męskich komórkach germinalnych, integrując procesy magazynowania transkryptów i kontroli translacji. W skład kompleksów mRNP obecnych w ciele chromatoidalnym wchodzi między innymi helikazy typu DEAD-box (np. DDX4/VASA) oraz członkowie rodziny PIWI - białka uczestniczące w regulacji stabilności transkryptów, kontroli translacji oraz szlaku piRNA (ang. *PIWI-interacting RNA*), zaangażowanego m.in. w potranskrypcyjne wyciszenie i degradację wybranych transkryptów, w tym transpozonów (Anbazhagan et al. 2022; Meikar et al. 2014). W tym kontekście ciało chromatoidalne należy postrzegać nie tylko jako strukturę zaangażowaną w magazynowanie i regulację translacji wybranych transkryptów mRNA, lecz także jako ważne centrum posttranskrypcyjnej kontroli RNA w komórkach germinalnych. Szczególne znaczenie ma tu regulacja aktywności elementów transpozycyjnych (ang. *transposable elements*, TEs), czyli ruchomych elementów DNA, których niekontrolowana aktywność stanowi istotne zagrożenie dla integralności genomu. Do najliczniejszych elementów tego typu u ssaków należą retrotranspozony LINE1 (L1), stanowiące około 19% genomu człowieka, których ekspresja i mobilizacja w komórkach linii płciowej muszą podlegać ścisłej kontroli (Yang and Wang 2016; Zhou et al. 2022). W okresach intensywnego przeprogramowania epigenetycznego aktywacja

L1 może prowadzić do niestabilności genomu, uszkodzeń DNA oraz zaburzeń różnicowania komórek germinalnych, a tym samym stanowić zagrożenie dla prawidłowego przebiegu spermatogenezy (Zamudio and Bourc'his 2010). Z tego względu mechanizmy kontrolujące aktywność elementów transpozycyjnych, w tym szlaki zależne od małych RNA i białek wiążących RNA, są integralnym elementem regulacji posttranskrypcyjnej zachodzącej podczas spermatogenezy. Pomimo rosnącej liczby badań mechanizmy te pozostają słabo scharakteryzowane w ludzkich komórkach linii płciowej, a ich złożoność, wraz z innymi warstwami regulacji RNA niezbędnymi dla prawidłowej spermatogenezy, sprawia, że odtworzenie tego procesu w warunkach *in vitro* nadal stanowi istotne wyzwanie. Badania z wykorzystaniem modeli zwierzęcych wykazały, że zaburzenia funkcji białek związanych z ciałkiem chromatoidalnym prowadzą do nieprawidłowej organizacji tych struktur, a także do deregulacji translacji, zaburzeń spermiogenezy oraz upośledzenia męskiej płodności (Vasileva et al. 2009; Xu, Cao, and Bao 2021).

Znaczenie kompleksów mRNP nie ogranicza się do późnych etapów spermiogenezy. Badania przeprowadzone w ostatnich latach wskazują, że dynamiczna organizacja kompleksów mRNP odgrywa istotną rolę również we wcześniejszych etapach spermatogenezy, obejmujących utrzymanie populacji spermatogoniów oraz regulację przebiegu mejozy (Gao et al. 2025; Morgan et al. 2021b; Voronina et al. 2011). W spermatogoniach kompleksy mRNP uczestniczą w kontroli stabilności i translacji transkryptów związanych z utrzymaniem komórek macierzystych oraz rozpoczęcia procesu różnicowania. Przykładem może być białko NANOS2, które tworzy kompleksy mRNP uczestniczące w represji translacyjnej wybranych transkryptów i utrzymaniu nie zróżnicowanego stanu spermatogoniów w modelu mysim (Zhou et al. 2015). Istotną rolę odgrywają również kompleksy mRNP zawierające DAZL - białko RBP niezbędne dla prawidłowego przebiegu mejozy, regulujące translację licznych transkryptów zaangażowanych w rozwój męskich komórek płciowych (Li et al. 2018). Ciekawą obserwacją jest fakt, że alternatywny splicing genu *Dazl* prowadzi do powstania dwóch izoform białka o odmiennych właściwościach funkcjonalnych, a utrata jednej izoformy skutkuje ciężkimi zaburzeniami spermatogenezy i niepłodnością (Li et al. 2025). Ten przypadek podkreśla, jak precyzyjnie kontrolowane procesy potranskrypcyjne determinują przebieg spermatogenezy oraz jak istotną rolę w utrzymaniu prawidłowego rozwoju komórek germinalnych odgrywa funkcjonalna różnorodność białek RBPs. Złożona organizacja kompleksów mRNP oraz ich dynamiczne remodelowanie stanowią

jeden z kluczowych mechanizmów regulujących ekspresję genów podczas spermatogenezy i warunkujących prawidłowy przebieg tego procesu.

Białka wiążące RNA jako centralne regulatory spermatogenezy

Białka wiążące RNA, określane także jako białka RBPs, stanowią liczną i funkcjonalnie zróżnicowaną grupę potranskrypcyjnych regulatorów ekspresji genów, które uczestniczą w metabolizmie RNA (Gerstberger, Hafner, and Tuschl 2014; Hentze et al. 2018). RBPs są najczęściej klasyfikowane na podstawie obecności specyficznych domen wiążących RNA (ang. *RNA-binding domains*, RBDs), których budowa i właściwości funkcjonalne dostarczają informacji dotyczących preferencji wiązania oraz potencjalnych celów oddziaływania tych białek (Gerstberger et al. 2014). Wśród wyspecjalizowanych domen wiążących RNA można wyróżnić, między innymi, domenę RRM (ang. *RNA recognition motif*), domenę KH (ang. *K homology domain*), domeny RG/RGG (ang. *arginine-glycine-rich domains*) czy też domenę charakterystyczną dla helikaz RNA typu DEAD-box (ang. *Asp-Glu-Ala-Asp box domain*). Domena RRM należy do najczęściej występujących domen wiążących RNA u eukariontów i uczestniczy w rozpoznawaniu krótkich sekwencji jednoniciowego RNA, odgrywając istotną rolę między innymi w regulacji splicingu, stabilności oraz translacji transkryptów mRNA (Corley et al. 2020; Schmeing and Hart 2024). Domena KH odpowiada natomiast za oddziaływanie z jednoniciowym RNA lub DNA i występuje w licznych białkach uczestniczących w regulacji transkrypcji oraz translacji mRNA (Corley et al. 2020; Valverde, Edwards, and Regan 2008). Domeny RG/RGG są bogate w reszty argininy i glicyny, co umożliwia im oddziaływanie zarówno z RNA, jak i innymi białkami, a także uczestnictwo w tworzeniu dynamicznych kompleksów mRNP i granuli RNA-białkowych (Chong, Vernon, and Forman-Kay 2018; Corley et al. 2020). Z kolei helikazy RNA typu DEAD-box pełnią kluczową rolę w remodelowaniu struktury RNA oraz reorganizacji kompleksów RNA-białko, uczestnicząc między innymi w splicingu, translacji, degradacji RNA i regulacji powstawania granuli (Putnam and Jankowsky 2013). Różnorodność domen RBDs oraz ich kombinacji w obrębie pojedynczych białek umożliwia precyzyjną i wielopoziomą kontrolę metabolizmu RNA w komórkach eukariotycznych (Gerstberger et al. 2014).

Dotychczasowe badania wykazały, że ekspresja oraz aktywność wielu RBPs podlega ścisłej regulacji czasowo-komórkowej podczas spermatogenezy,

odzwierciedlając wysoką specjalizację mechanizmów potranskrypcyjnych zachodzących w męskich komórkach germinalnych (Li et al. 2024; Xu et al. 2025). Podejścia atlasowe oparte na wysokoprzepustowych analizach ujawniły istnienie specyficznych dla poszczególnych populacji komórek germinalnych sieci RBPs, których dynamiczna ekspresja koreluje z kolejnymi etapami różnicowania męskich komórek płciowych, podkreślając centralną rolę RBPs. Dlatego zaburzenia funkcji białek wiążących RNA mogą prowadzić do deregulacji procesu spermatogenezy i być przyczyną niepłodności. Do dobrze scharakteryzowanych białek RBPs uczestniczących w spermatogenezie należą między innymi białka RBMY - kodowane na chromosomie Y, zawierające domenę RRM i uczestniczące w regulacji splicingu transkryptów istotnych dla rozwoju komórek germinalnych (Elliott 2000). Delecje regionów AZF (ang. *Azoospermia Factor*) obejmujących geny *RBMY* należą do najlepiej udokumentowanych genetycznych przyczyn ciężkich zaburzeń spermatogenezy i azoospermii u ludzi. Istotną rolę odgrywa również *RBMXL2*, którego ekspresja jest szczególnie wysoka w spermatocytach, a badania z wykorzystaniem modelu mysiego wykazały, że utrata funkcji tego białka prowadzi do zaburzeń splicingu transkryptów kluczowych dla procesu mejozy i w konsekwencji niepłodności u samców (Ehrmann et al. 2019). Istotną rolę w regulacji spermatogenezy odgrywa również *DDX4/VASA* - helikaza RNA typu DEAD-box, będąca jednym z najlepiej scharakteryzowanych markerów komórek germinalnych (Xu et al. 2021). Białko to uczestniczy w regulacji metabolizmu RNA, organizacji kompleksów mRNP oraz funkcjonowaniu granuli germinalnych. Badania z wykorzystaniem modelu mysiego wykazały, że utrata funkcji *DDX4* prowadzi do całkowitej utraty płodności u samców, natomiast płodność samic pozostaje zachowana (Tanaka et al. 2000).

Podsumowując złożoność oraz etapową specyfikę regulacji metabolizmu RNA podczas spermatogenezy, wskazano, że prawidłowość tego procesu zależy nie tylko od aktywacji określonych programów transkrypcyjnych, lecz także od precyzyjnej kontroli losu RNA przez białka wiążące RNA, kompleksy mRNP oraz mechanizmy regulujące splicing, stabilność i translację transkryptów. W tym kontekście szczególnie istotna staje się identyfikacja nowych czynników i zrozumienie funkcji regulatorów metabolizmu RNA. Do takich białek należą *RBMXL3* i *DDX53*, będące przedmiotem badań przeprowadzonych w ramach pracy doktorskiej.

RBMXL3 i DDX53 jako białka potencjalnie związane ze spermatogenezą oraz z rozwojem męskich komórek płciowych

RBMXL3 jest genem specyficznym dla ssaków naczelnych, zlokalizowanym na chromosomie X, którego funkcja pozostaje słabo poznana. Wyniki analiz transkryptomicznych dostępnych w Atlasie Białek Człowieka (ang. *Human Protein Atlas*, HPA) wskazują, że *RBMXL3* ulega ekspresji głównie w męskiej gonadzie, szczególnie w spermatogoniach i spermatocytach. Białko *RBMXL3* zawiera domenę wiążącą RNA (RRM), a badania innych członków rodziny RBMX wykazały ich udział w procesach potranskrypcyjnej regulacji ekspresji genów.

DDX53 jest genem zlokalizowanym na chromosomie X, kodującym białko należące do rodziny helikaz RNA typu DEAD-box, uznawanych za kluczowe regulatory metabolizmu RNA. Wyniki analiz transkryptomicznych dostępnych w bazie HPA wskazują, że *DDX53* ulega ekspresji głównie w męskiej gonadzie, szczególnie w spermatogoniach, spermatocytach i spermatydach.

W kontekście niniejszej rozprawy szczególnie istotne są badania, w których zidentyfikowano geny *RBMXL3* oraz *DDX53* jako potencjalnie związane z procesem spermatogenezy człowieka na podstawie analiz sekwencjonowania całogenomowego (WGS) oraz sekwencjonowania całego egzomu (WES) wykonanych w grupie niepłodnych mężczyzn (Hardy et al. 2022; Malcher et al. 2022). Wyniki tych prac, w połączeniu ze specyficzną ekspresją *RBMXL3* oraz *DDX53* w męskich komórkach linii płciowej, stanowiły przesłankę do podjęcia badań nad rolą białek *RBMXL3* i *DDX53* w regulacji procesów związanych z powstawaniem gamet męskich. Dotychczas nie badano *RBMXL3* oraz *DDX53* w tym kontekście, a poznanie roli tych białek podczas spermatogenezy człowieka pozostaje utrudnione z uwagi na ograniczoną dostępność odpowiednich modeli badawczych. W związku z tym w niniejszej rozprawie doktorskiej wykorzystano ludzką linię komórkową TCam-2, uzyskaną od prof. Sohei Kitazawy (Kobe University, Japonia), wywodzącą się z linii komórek germinalnych nowotworu jądra (nasieniaka) (De Jong et al. 2008; Looijenga, Stoop, and Biermann 2014). Komórki TCam-2 wykazują profil ekspresji zbliżony do hPGCs i stanowią cenny model wykorzystywany w badaniach dotyczących rozwoju męskiej linii płciowej (Irie et al. 2015; Nettersheim et al. 2016; Smialek et al. 2020).

3. Hipoteza i cele rozprawy doktorskiej

Niepłodność jest globalnym, istotnym problemem zdrowotnym, dotyczącym pary na całym świecie, a wiedza dotycząca genetycznych podstaw rozwoju komórek płciowych oraz precyzyjnej regulacji spermatogenezy nadal pozostaje ograniczona.

W niniejszej rozprawie doktorskiej postawiono hipotezę badawczą, że *RBMXL3* oraz *DDX53* uczestniczą w regulacji procesów zachodzących w męskich komórkach płciowych człowieka. W związku z tym celem niniejszej rozprawy doktorskiej była identyfikacja sieci powiązań oraz molekularnych interaktorów białek *RBMXL3* i *DDX53* w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka.

Cel ten został zrealizowany poprzez:

1. Systematyczne opracowanie aktualnego stanu wiedzy na temat modeli *in vitro* stosowanych w badaniach rozwoju ludzkiej linii płciowej oraz spermatogenezy człowieka

2. Identyfikację sieci powiązań oraz molekularnych interaktorów *RBMXL3* w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka

1. Uzyskanie ludzkiej linii komórkowej TCam-2 z nadekspresją *RBMXL3-FLAG* oraz *GFP-FLAG* (jako kontroli).
2. Zbadanie lokalizacji białka *RBMXL3* w ludzkiej linii komórkowej TCam-2.
3. Analiza zmian transkryptomicznych wywołanych nadekspresją *RBMXL3-FLAG* z wykorzystaniem sekwencjonowania RNA (RNA-seq).
4. Identyfikacja celów RNA oraz DNA bezpośrednio oddziałujących z białkiem *RBMXL3* metodą eCLIP-seq (wzmocniona metoda sieciowania i immunoprecypitacji połączona z sekwencjonowaniem) oraz ChIP-seq (immunoprecypitacja chromatyny połączona z sekwencjonowaniem).
5. Identyfikacja interaktorów białkowych *RBMXL3* metodą ko-immunoprecypitacji sprzężonej ze spektrometrią mas (Co-IP-MS) oraz walidacja wybranych kandydatów białkowych z użyciem podwójnego barwienia immunofluorescencyjnego.

3. Identyfikację sieci powiązań oraz molekularnych interaktorów DDX53 w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka

1. Uzyskanie ludzkiej linii komórkowej TCam-2 z nadekspresją *DDX53-FLAG* oraz *GFP-FLAG* (jako kontroli).
2. Zbadanie lokalizacji białka DDX53 w ludzkiej linii komórkowej TCam-2.
3. Analiza zmian transkryptomicznych wywołanych nadekspresją *DDX53-FLAG* z wykorzystaniem RNA-seq.
4. Identyfikacja celów RNA oraz DNA bezpośrednio oddziałujących z białkiem DDX53 metodą eCLIP-seq oraz ChIP-seq.
5. Identyfikacja interaktorów białkowych DDX53 metodą Co-IP-MS oraz walidacja wybranych kandydatów białkowych z użyciem podwójnego barwienia immunofluorescencyjnego.

4. Omówienie publikacji wchodzących w skład cyklu prac stanowiących rozprawę doktorską

4.1. Artykuł 1: „*In vitro* models for human germ cells and spermatogenesis research: concepts and perspectives”

Spermatogeneza człowieka stanowi fundamentalny i wysoce złożony proces biologiczny, którego pełne odtworzenie w warunkach *in vitro* pozostaje dotychczas nieosiągnięte. Mimo znaczącego postępu naukowego, rozbudowany charakter regulacji genetycznej i epigenetycznej tego procesu sprawia, że wiele pytań dotyczących molekularnych podstaw dla zachowania męskiej płodności wciąż pozostaje bez odpowiedzi. W niniejszej publikacji przedstawiono kompleksowy przegląd aktualnych strategii badawczych zmierzających do odtworzenia procesu powstawania męskich gamet w warunkach laboratoryjnych, a także do badania mechanizmów regulujących rozwój linii płciowej i spermatogenezę człowieka oraz identyfikacji nowych białek zaangażowanych w te procesy.

W rozdziale pierwszym, który stanowi wstęp do dalszej części publikacji, opisano biologiczną złożoność spermatogenezy człowieka, podkreślając, że rozwój gamet wymaga precyzyjnej koordynacji ekspresji genów, sygnalizacji hormonalnej oraz rozległego przeprogramowania epigenetycznego. Podkreślono również fundamentalne różnice między procesami związanymi z rozwojem męskich komórek linii płciowej u ludzi i zwierząt (np. myszy), co ogranicza możliwość bezpośredniej translacji wyników badań oraz wskazuje na potrzebę opracowania modeli specyficznych dla człowieka. Zwrócono również uwagę na istnienie licznych, wciąż słabo poznanych regulatorów molekularnych oraz unikalnych izoform genów, których identyfikacja z zastosowaniem konwencjonalnych metod jest utrudniona, a których funkcje pozostają często niewyjaśnione.

W kolejnej części publikacji omówiono proces spermatogenezy człowieka oraz jego dynamikę, od proliferacji spermatogonialnych komórek macierzystych (SSCs), przez podziały mejotyczne spermatocytów, aż po proces spermiogenezy prowadzący do powstania dojrzałych plemników. Podkreślono kluczową rolę bariery krew–jądro (BTB) oraz znaczenie wsparcia fizycznego i sygnałowego zapewnianego przez komórki somatyczne, takie jak komórki Sertolego, Leydiga oraz komórki mioidalne (PTMs).

Następnie omówiono podstawowe populacje ludzkich komórek jądra, klasyfikowane jako komórki rozrodcze lub somatyczne, które są stosowane w badaniach nad spermatogenezą. Wśród modeli opartych na komórkach płciowych uwzględniono pierwotne spermatogonie, spermatocyty i spermatydy izolowane z tkanki, podkreślając ich istotność dla badań przy jednoczesnych ograniczeniach związanych z niską żywotnością *in vitro*. Przedstawiono również bardziej zdefiniowane modele badawcze, takie jak długoterminowe hodowle komórek podobnych do spermatogonialnych komórek macierzystych (SSC-like) oraz stabilne linie nowotworowe, w tym linię TCam-2, która wykazuje profil ekspresji zbliżony do ludzkich pierwotnych komórek płciowych (hPGCs) i umożliwia prowadzenie analiz molekularnych o większej skali i powtarzalności. Zwrócono też uwagę na wykorzystanie somatycznych komórek jądra – Sertolego, Leydiga i komórek mioidalnych (PTMs). Podkreślono także, że pierwotne hodowle ludzkich komórek jądra lepiej odzwierciedlają warunki fizjologiczne, jednak ich zastosowanie jest ograniczone przez zmienność między dawcami, krótszy czas życia oraz dostępność materiału. Unieśmiertelnione linie komórkowe stanowią bardziej powtarzalne modele badawcze, lecz mogą nie w pełni odzwierciedlać właściwości komórek *in vivo*, dlatego wymagają ostrożnej interpretacji wyników.

Kolejny rozdział został poświęcony tradycyjnym strategiom hodowli dwuwymiarowych (2D), a w szczególności systemom kohodowli komórek płciowych z somatycznymi. Za pomocą tych modeli dąży się do rekonstrukcji niszy komórek SSC poprzez zapewnienie bezpośrednich kontaktów międzykomórkowych oraz sygnalizacji molekularnej, choć ich skuteczność w indukowaniu kompletnej mejozy pozostaje ograniczona. W rozdziale opisano przykłady takich badań i zwrócono uwagę na to, że klasyczne systemy 2D kohodowli odtwarzają jedynie wybrane aspekty tkanki jądra, a kluczowe znaczenie dla spermatogenezy może mieć także trójwymiarowa organizacja komórek, co uzasadnia rozwój bardziej zaawansowanych modeli hodowlanych.

W dalszej części przeglądu omówiono innowacyjne podejście polegające na różnicowaniu komórek macierzystych *in vitro*, które mogłyby stanowić potencjalnie nieograniczone źródło komórek męskiej linii płciowej. Przytoczono przykłady takich badań, podkreślając istnienie powtarzalnych protokołów różnicujących ludzkie komórki macierzyste do komórek przypominających pierwotne komórki linii płciowej człowieka (hPGCLCs). Pomimo postępów w opracowywaniu modeli opartych na komórkach pluripotentnych oraz uzyskiwaniu populacji przypominających różne etapy rozwoju linii płciowej, ich funkcjonalna równoważność z komórkami *in vivo*, w tym zdolność do pełnej

spermatogenezy, nie została dotychczas jednoznacznie potwierdzona, a tożsamość i funkcjonalność uzyskiwanych komórek wymaga pogłębionej weryfikacji.

W następnej części artykułu skupiono się na trójwymiarowych (3D) systemach hodowlanych używanych w badaniach spermatogenezy człowieka, których celem jest bliższe odwzorowanie architektury jądra. Przedstawiono metody hodowli oparte na rusztowaniach (ang. *scaffold-based*) oraz metody wolne od rusztowań (ang. *scaffold-free*), wykorzystujące zdolność komórek do samoorganizacji w wielokomórkowe agregaty, wraz z przykładami takich badań. Konwencjonalne systemy hodowli 3D odtwarzają jedynie wybrane elementy mikrośrodowiska jądra, co ogranicza ich zastosowanie głównie do analiz dotyczących wczesnych etapów spermatogenezy i interakcji komórkowych. W odpowiedzi na te ograniczenia rozwijane są modele organoidowe oraz bardziej zaawansowane platformy hodowli *in vitro* opisane w końcowej części niniejszej publikacji.

W kolejnym rozdziale opisano organoidy jąder (TOs) oraz trójwymiarowe systemy organotypowej hodowli jąder. Podczas gdy organoidy powstają w wyniku ukierunkowanej samoorganizacji różnego typu komórek, modele organotypowe polegają na utrzymywaniu nienaruszonych fragmentów tkanki *ex vivo*, co pozwala na zachowanie naturalnych interakcji w obrębie kanalików nasiennych. Podkreślono, że choć oba podejścia zwiększają trafność fizjologiczną badań, żadne z nich nie umożliwiło dotąd uzyskania funkcjonalnych ludzkich plemników *in vitro*.

W końcowej części pracy przedstawiono nowoczesny kierunek badań, jakim są platformy mikroprzepływowe typu testis-on-a-chip (ToC). Układy te umożliwiają dynamiczną kontrolę mikrośrodowiska poprzez ciągłą perfuzję, co poprawia dostarczanie składników odżywczych i usuwanie produktów przemiany materii, przybliżając warunki hodowli do warunków fizjologicznych. W pracy uwzględniono przykłady zastosowania tych technologii do hodowli ludzkich kanalików nasiennych, fragmentów tkanki jądra, a także kohodowli komórek płciowych z somatycznymi. Pomimo że ludzkie modele typu testis-on-a-chip pozostają na wczesnym etapie rozwoju i rzadko umożliwiają osiągnięcie późnych etapów mejozy, uznaje się je za istotny postęp technologiczny w kierunku bardziej wiernego odwzorowania warunków panujących *in vivo*. Dalszy rozwój tych platform może przyczynić się do lepszego modelowania spermatogenezy *in vitro* oraz wspierać jej bardziej zaawansowane etapy.

Wnioski: W publikacji stanowiącej zarazem wstęp do niniejszej rozprawy doktorskiej przedstawiono modele *in vitro* stosowane w badaniach rozwoju linii płciowej

oraz spermatogenezy człowieka. W pracy omówiono zarówno proste hodowle komórkowe, jak i bardziej zaawansowane modele *in vitro* oparte na różnicowaniu komórek macierzystych, systemy 3D oraz platformy bioinżynieryjne. Ostatni rozdział artykułu prezentuje wnioski wskazujące, że pomimo znaczącego rozwoju licznych modeli laboratoryjnych do badania spermatogenezy człowieka, pełne odtworzenie tego złożonego procesu nie zostało dotąd osiągnięte, a istniejące systemy odwzorowują jedynie wybrane aspekty gametogenezy. Za istotny warunek dalszego postępu uznano stosowanie rzetelnych kryteriów walidacji komórek przypominających komórki męskiej linii płciowej. Ważnym kierunkiem rozwoju są systemy mikroprzepływowe oraz warunki hodowli wolne od komponentów zwierzęcych, zwiększające powtarzalność i potencjał kliniczny. Podkreślono, że wiedza na temat spermatogenezy człowieka wciąż w dużej mierze opiera się na prostszych modelach *in vitro*, które stanowią dostępne systemy badawcze umożliwiające analizę procesów komórkowych oraz identyfikację kluczowych regulatorów specyficznych dla człowieka. Konieczne pozostaje dalsze doskonalenie modeli oraz uwzględnianie wyzwań etycznych towarzyszących ich rozwojowi.

Mój wkład w powstanie publikacji:

- współudział w opracowaniu koncepcji i stworzenie planu pracy
- przegląd i wybór prac omówionych w publikacji
- napisanie oryginalnej wersji publikacji oraz przygotowanie abstraktu w formie graficznej
- przygotowanie ostatecznej wersji publikacji

***In vitro* models for human germ cells and spermatogenesis research: concepts and perspectives**

Agata J. Barszcz¹, Natalia Rozwadowska¹

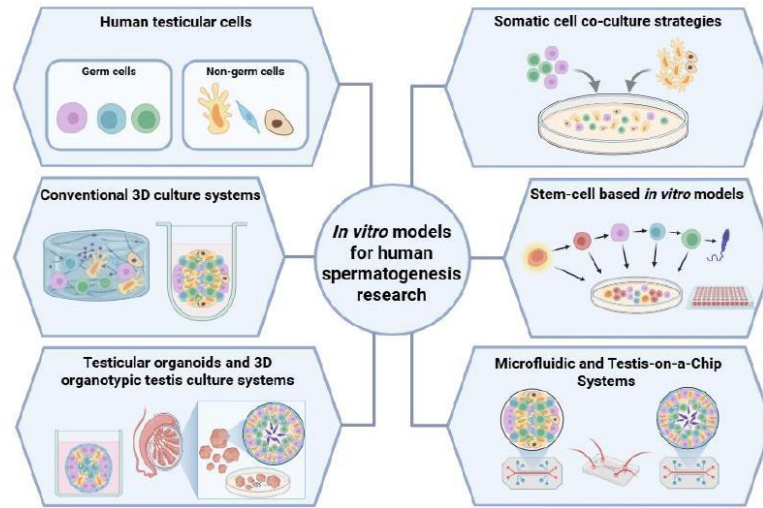
¹ Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

Abstract

Spermatogenesis is a continuous, highly regulated biological process that transforms spermatogonia into mature sperm cells. It is crucial for successful reproduction, playing a major role in maintaining population and ensuring the survival and continuity of the species across generations. Despite its importance, we are still unable to fully recapitulate functional human spermatogenesis *in vitro*. Species-specific differences in germ cell development limit the direct translation of scientific findings from model organisms, highlighting the need for further research focused on human-specific regulatory mechanisms. Here, we provide an overview of human 2D and 3D models that may be used to address research questions concerning human male gamete generation and to facilitate progress towards its eventual achievement. From simple cellular model systems used in human spermatogenesis research, through advanced multifactorial approaches, to complex bioengineered platforms, this review discusses an increasingly sophisticated and rapidly expanding field that continues to evolve alongside advances in stem cell biology, reproductive medicine, and tissue engineering. It presents an overview of available non-animal models for studying human spermatogenesis, with the aim of helping researchers identify those best suited to their experimental aims.

Keywords: *in vitro* spermatogenesis, human germ cell models, testicular organoids, 2D culture systems, 3D culture systems

Graphical abstract



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Introduction

Spermatogenesis in humans is a highly organised and dynamic developmental process that transforms diploid spermatogonia into haploid spermatozoa. It proceeds through successive phases requiring precise temporal and spatial regulation of gene expression, tightly controlled molecular signaling, and extensive epigenetic reprogramming to ensure the production of functionally competent sperm [1]. In parallel, precise hormonal regulation orchestrates stage-specific signaling events that coordinate germ cell development with the supportive functions of somatic cells within the testis. Moreover, spermatogenesis involves dynamic epigenetic remodeling, including DNA methylation, histone modifications, and large-scale chromatin reorganization, processes that are essential for meiotic progression, post-meiotic differentiation, and the establishment of paternal epigenetic information transmitted to the embryo [2]. Collectively, these multilayered regulatory mechanisms underscore the complexity of human spermatogenesis and highlight persistent gaps in our understanding of the genetic and epigenetic factors governing male fertility.

Human spermatogenesis differs in several fundamental aspects from that of other species, including widely used animal models such as the mouse. These differences encompass the timing and organization of germ cell development, testicular architecture, species-specific gene expression programs, and distinct regulatory mechanisms governing germ cell differentiation. Comparative

transcriptomic and epigenetic studies have demonstrated that numerous genes and regulatory pathways critical for human spermatogenesis might be absent, divergent, or differentially regulated in the mouse and other model organisms, underscoring the limitations of extrapolating findings from animal models to human male reproductive biology [3–6].

At the molecular level, spermatogenesis is governed by highly coordinated gene-expression programs that encompass a large repertoire of protein-coding genes (including stage-specific transcript isoforms) and non-coding transcripts. Despite extensive efforts to identify candidate genes implicated in human spermatogenesis, important regulatory factors are likely to remain undiscovered. Moreover, many proposed candidate genes still require experimental validation, since a substantial proportion remain poorly characterized at the functional level [7].

This complexity is further compounded by pervasive alternative splicing and the generation of testis-specific gene isoforms, which are often difficult to detect with conventional approaches and whose precise roles across distinct spermatogenic stages remain poorly defined. The use of experimentally approachable male germ cell *in vitro* model would enable the functional validation of molecular candidates and support the development of a complete *in vitro* human spermatogenesis model.

Spermatogenesis

As outlined above spermatogenesis is a complex and highly coordinated biological process and as a fundamental component of male reproduction, it enables the generation of vast numbers of sperm cells daily. In humans, each spermatogenic wave lasts ~74 days with the ~16 days interval between commitment of spermatogonia at a specific site in a tubule [8]. Spermatogenesis occurs continuously, therefore with ageing, the number of total chromosome replication events (and the risk of errors) increases [9].

Spermatogenesis involves the maintenance and proliferation of spermatogonial stem cells (SSCs) and their differentiation into spermatocytes, the meiotic divisions of spermatocytes to form haploid spermatids, and finally the transformation of spherical spermatids to a sperm-like mature spermatids that give rise to spermatozoa (motile mature sperm cells) [8, 9]. The spermatogenic lineage is sustained by SSCs which are located along the basement membrane of the seminiferous epithelium and encompass several states, ranging from quiescence to differentiation commitment [10, 11]. As a result of differentiation, type B spermatogonia give rise to primary spermatocytes through a final mitotic division. Produced primary spermatocytes pass the blood-testis barrier (BTB) and become immunologically isolated so that the post-meiotic germ cells could be protected from immune cell recognition and responses [12, 13]. The BTB remains functionally intact, as “passing” is highly regulated remodeling process in which Sertoli cells form new intercellular junctions ahead of migrating cells while disassembling old junctions behind them, thereby preserving barrier integrity [14]. After passing from the basal to adluminal compartment of the seminiferous epithelium, primary spermatocytes

enter meiosis I, during which homologous chromosomes pair and exchange genetic material through crossing over, generating unique combinations of parental genes that are essential for genetic diversity. As a result of meiosis I, two secondary spermatocytes are produced that should enter meiosis II to ultimately generate four round haploid spermatids, with each cell harboring a single copy of each chromosome. The maturation of round haploid spermatids into morphologically and functionally mature spermatozoa does not involve any cellular divisions and is an intracellular remodeling process. This differentiation of round spermatids into spermatozoa is termed spermiogenesis, and the release of a mature spermatozoon by the Sertoli cell into the lumen of the seminiferous tubule is called spermiation [9]. In humans, spermiogenesis extends over ~3 weeks [15, 16]. The formation of motile mature sperm also requires many well-coordinated cellular and molecular events, such as nuclear remodeling and histone-to-protamine transition, acrosome formation, flagellum development, and cytoplasmic reduction into residual bodies, which are phagocytosed by Sertoli cells [17].

It is important to emphasize that each stage of spermatogenesis depends on distinct regulatory cues provided by the testicular somatic microenvironment, including Sertoli cells, peritubular myoid cells, and Leydig cells, as well as on support from the surrounding vascular network and the unique spatial organization of the seminiferous tubule.

Sertoli cells are responsible for intimate physical contact with germ cells, supporting them throughout their development. Sertoli cells are essential for spermatogenesis *in vivo*, not only by providing structural support, but also by maintaining the blood–testis barrier, secreting growth factors and hormones, and orchestrating the endocrine signaling of hormones such as testosterone, as they express functional androgen receptors (ARs), in contrast to germ cells [9, 18]. Additionally, Sertoli cells are the primary targets of follicle-stimulating hormone (FSH, secreted by the pituitary gland), which regulates their metabolic and secretory activity essential for spermatogenesis. Peritubular myoid cells (PTMs) are somatic, smooth-muscle type cells with contractile properties that surround the wall of the seminiferous tubules. They are also adjacent to Leydig cells and express ARs. PTMs actively participate in testicular signaling networks by secreting molecules that modulate Sertoli cells' activity and influence germ cell development [19–21]. Leydig cells are specialized cells located in the interstitial compartment of the human testis. In response to luteinizing hormone (LH) secreted by the pituitary gland, they produce and secrete testosterone, maintaining high intratesticular concentrations of this hormone, essential for the initiation and progression of spermatogenesis. Leydig cells also secrete a range of growth factors and cytokines that contribute to the regulation of the testicular microenvironment and facilitate intercellular communication critical for coordinated spermatogenic function of the testis [22, 23].

Human testicular germ and somatic cell populations, cultured either individually or in combination, provide valuable insight into human spermatogenesis and represent experimentally accessible models for functional studies. However, the dynamic and interdependent nature of spermatogenesis-related processes underscores the difficulty of generating a fully representative model

of human spermatogenesis *in vitro* and highlights the need for systems that progressively capture the complexity of the male testis tissue. Major challenges include coordinating stage-specific signaling and supporting epigenetic reprogramming required for germ cell differentiation, while preserving key features of testicular architecture and microenvironment. Collectively, these considerations emphasize the inherent complexity of human spermatogenesis and provide a biological framework for understanding the challenges associated with its faithful reconstruction *in vitro*.

Human testicular cells for spermatogenesis studies

Human testicular cells can be maintained as heterogeneous cultures, enriched subpopulations or purified cell types and constitute widely used two-dimensional (2D) and three-dimensional (3D) *in vitro* models of human spermatogenesis. When cultured, researchers typically distinguish between germ and non-germ (somatic) cells.

Germ cell-based models comprise primary human spermatogonia, spermatocytes, and spermatids isolated directly from testicular tissue, as well as more standardized *in vitro* experimental models, including spermatogonial stem cell (SSC)-like cultures and human germ cell-derived tumor cell lines, such as the seminoma-derived TCam-2 cell line [24, 25]. Primary human germ cells are typically obtained from adult testicular tissue samples, but they can also be derived from prepubertal testes, which are particularly relevant for the isolation of early spermatogonial populations. Cells are obtained through mechanical dissociation and/or enzymatic digestion, followed by enrichment and isolation strategies such as differential plating, density gradient centrifugation, or sedimentation-based methods [26], depending on the protocol or specific research objective. When highly purified cell populations are required, flow cytometry-based sorting may be applied. Primary human germ cells serve as gold-standard physiological models for studying individual stages of human spermatogenesis; however, their limited viability and restricted progression *in vitro* constrain long-term culture and the ability to achieve complete gametogenesis. In conventional 2D culture systems, primary human spermatogonia can be maintained for several weeks to months under optimized conditions, whereas spermatocytes and spermatids are typically sustained for shorter periods. Consequently, these cells are primarily used to study their proliferation and maintenance *in vitro*, differentiation capacity, meiotic progression, and stage-specific molecular and functional analyses. They are also invaluable for constructing discrete transcriptomic atlases of human spermatogenesis [27]. To overcome the limited lifespan and differentiation capacity of primary germ cell cultures, several *in vitro* systems supporting spermatogonial stem cell (SSC)-like populations have been developed. These include long-term SSC-like cultures as well as germ cell-derived tumor cell lines. Human SSC-like cultures have been established from testicular tissue and can be maintained for extended periods under defined growth factor conditions, enabling expansion of spermatogonial populations with stem-like properties [28–31]. Although these cultures do not fully recapitulate spermatogenesis *in vivo*, they retain key molecular

markers of undifferentiated spermatogonia and provide valuable systems for studying self-renewal, differentiation potential and responses to environmental or genetic perturbations. A simpler and more stable experimental system can be provided by germ cell-derived tumor cell lines, for example the seminoma-derived TCam-2 cells. These cells retain gene expression profiles and germline-specific markers characteristic of primordial germ cells, the embryonic precursors of the male germline, and may serve as useful models for studying human germ cell biology and tumorigenesis [32, 33]. Furthermore, compared with short-term primary germ cell cultures, they provide a more stable and experimentally tractable platform that enables prolonged maintenance, genetic manipulation, and reproducible large-scale analyses. In parallel, germ cell-derived tumor cell lines, most notably the seminoma-derived TCam-2 line, represent experimentally tractable *in vitro* models that preserve transcriptional and epigenetic features of primordial germ cells and early gonocytes. These systems collectively complement short-term primary germ cell cultures by offering more stable and scalable platforms for investigating human germ cell biology, germ cell tumorigenesis and mechanisms regulating germline fate.

Non-germ (somatic) cell-based models of human spermatogenesis include primary cultures derived from patient testicular tissue as well as commercially available or laboratory-generated [34] somatic cell preparations. Primary cultures typically comprise Sertoli, peritubular myoid, and Leydig cells, and are widely used to study paracrine signaling, steroidogenesis, and somatic support of germ cells *in vitro* [19, 35, 36]. Several vendors provide cryopreserved primary human Sertoli and Leydig cells, and selected immortalized derivatives, which reduce dependence on fresh clinical material. Although several immortalized human testicular somatic cell lines are finally available, they have not yet been widely adopted as standardized reference models, and their long-term functional stability relative to primary cells remains insufficiently characterized. Consequently, most studies continue to rely on primary human somatic cells, which are considered to more faithfully preserve native phenotypic and functional properties. Nevertheless, systematic validation and wider adoption of immortalized human somatic cell lines could improve experimental reproducibility and accessibility, and their application should be considered in controlled and characterized experimental settings.

It is worth to note that although germ and somatic primary human testicular cell cultures more closely reflect physiological properties of the native tissue, their use is constrained by donor-to-donor variability and restricted proliferation and lifespan *in vitro*. They also depend on access to suitable donor material and appropriate ethical approval, which can restrict availability and complicate experimental planning. As a result, studies relying on such cultures can be challenging to conduct and interpret, and their outcomes may vary depending on donor age, health status, or prior exposures. In contrast, a limited number of immortalized and, in some cases, commercially available human testicular cell lines may provide renewable and standardized systems that support reproducible assays, facilitate genetic manipulation, and enable selected screening applications. However, immortalization can alter key phenotypic traits, including cell-cycle regulation, differentiation state, and signaling responses.

Consequently, these models may only partially recapitulate the functional properties of cells *in vivo*, and findings derived from them require careful validation.

Finally, human testicular cells can also be cultured as 3D models by recreating, to varying degrees, the structural organization and microenvironment of the seminiferous tubules. In contrast to conventional 2D monolayers, 3D systems allow cells to establish spatial interactions that more closely resemble *in vivo* conditions. Commonly employed approaches include scaffold-based 3D cultures, scaffold-free spheroids or organoid-like structures, as well as more advanced systems discussed in the following sections.

Somatic cell co-culture strategies

Co-culture systems combining germ cells with testicular somatic cells represent one of the earliest and most widely used *in vitro* strategies to study human spermatogenesis. These models attempt to recreate key aspects of the spermatogonial stem cell (SSC) niche by providing direct cell–cell interactions and partially recapitulating the paracrine signaling present *in vivo* [10]. In the human testis, germ cell development depends strongly on signals from surrounding somatic cells, particularly Sertoli cells, but also peritubular myoid cells and other stromal components that contribute structural support and molecular regulatory mechanisms. For example, Leydig cells, although less frequently, have also been incorporated into certain co-culture systems as they provide testosterone and additional signaling factors that indirectly support germ cell differentiation. These supportive somatic components might either be cultured in a mixed cells population or as a feeders layer in 2D systems, or incorporated into more advanced 3D models [12, 37]. The co-culture systems could also be categorized based on the composition of the somatic supporting cells, ranging from germ cells cultured with a single somatic cell type - most commonly Sertoli cells [38, 39] - to more complex systems incorporating different testicular somatic populations or mixed testicular residual somatic cells isolated from patient [28, 40]. Example 2D co-cultures are spermatogonia or spermatogonial stem cells (isolated from testicular biopsies) seeded on a Sertoli cell feeder layer. These cells are used as they can adapt to *in vitro* conditions and, importantly, proliferate (unlike meiotic/postmeiotic germ cells), making them a promising starting population for attempts to propagate them toward mature sperm *in vitro*. However, pure human testicular feeder systems are uncommon in the literature. Protocols for human spermatogonial stem cells (hSSCs) often rely on mouse Sertoli feeder layers - immortalized mouse cell lines or primary mouse Sertoli cells - to support short-term hSSC proliferation and identity. Human Sertoli feeders show limited long-term success, while mouse conditions yield poor long-term hSSC proliferation due to species-specific signaling differences [40, 41].

Although conventional 2D co-culture systems have potential to advance our understanding of germ cell-somatic cell interactions, they still reproduce only selected aspects of the complex microenvironment of the human testis. Early models primarily focused on recreating the cellular

composition of the SSCs niche by combining germ cells with supportive somatic populations. However, increasing evidence indicates that proper spermatogenic progression depends on both cell identity and their 3D spatial organization, which has driven the development of more structurally representative culture systems that recapitulate the seminiferous epithelium and testicular niche architecture. Nevertheless, key challenges associated with the somatic cell co-culture approaches include maintaining physiologically relevant ratios and spatial organization of germ and somatic cells, recapitulating the dynamic signaling cues required at successive stages of spermatogenesis, and preserving the stability of somatic cell populations during prolonged cell culture [10].

Taken together, besides *in vitro* culture difficulties, it should be emphasized that approaches based on primary or non-immortalized human testicular cells provide valuable and physiologically relevant experimental systems, but they remain limited by restricted tissue availability, ethical and logistical constraints, variable sample quality, and substantial donor-to-donor heterogeneity. In this context, stem-cell-based *in vitro* models have emerged as an important strategy for generating human germ cell-like populations from human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). These systems have become an important experimental platforms for investigating the mechanisms of human germ cell development.

Stem-cell based *in vitro* models

Among emerging experimental strategies to model human spermatogenesis, stem-cell-based systems have attracted considerable attention, as they provide a potentially unlimited source of germ-cell-like populations for *in vitro* studies of human germ cells development. These approaches are typically described within the framework of *in vitro* gametogenesis (IVG), in which human pluripotent stem cells (hPSCs) are differentiated toward germ cell-like states by inducing developmental programs that recapitulate key stages of human germline specification [42, 43]. These strategies could help to clarify the mechanisms that govern human spermatogenesis *in vivo* and would enable the generation of experimentally tractable germ cell-like populations for studying specific stages of human gametogenesis *in vitro*.

Such differentiation protocols primarily aim to generate human primordial germ cell-like cells (hPGCLCs) corresponding to early stages of the human germline lineage [44, 45]. Primordial germ cells (PGCs) are the embryonic precursors of gametes that arise during early embryogenesis and subsequently migrate to the developing gonads, where they differentiate into fetal gonocytes (prospermatogonia), which ultimately establish the postnatal spermatogonial stem cell pool. Differentiation of hPSCs to hPGCLCs constitutes the most reproducible strategy. Landmark study by Irie et al. demonstrated that SOX17 acts as a key regulator of human germ-cell fate, enabling specification of PGC-like cells from hPSCs under defined signaling conditions [44]. These cells express canonical germline markers and exhibit transcriptional and epigenetic features resembling early human PGCs. This strategy has since

become a commonly used approach for generating hPGCLCs. Another important protocol was developed shortly after by Sasaki et al., who also established a stepwise differentiation strategy for generating hPGCLCs from hiPSC [45]. Using defined cytokine signals, this system enabled efficient and reproducible induction of hPGCLCs *in vitro* and has since also become a commonly used platform for studying early human germline development. Several modifications of hPGCLC differentiation protocols have subsequently been reported, including feeder-free induction systems and long-term expansion cultures that maintain germline potential [46–48]. More recent protocols have modified the original hPGCLC induction strategy by adapting culture formats or developmental contexts, including monolayer differentiation platforms or embryo-like culture systems [49, 50]; however, most still rely on the core BMP-driven germline specification program established in the landmark studies by Irie and Sasaki in 2015. A recent scoping review by Santos et al. [51] summarized *in vitro* methods for generating human primordial germ cell-like cells (hPGCLCs).

Several studies have reported differentiation toward prospermatogonial-like states as well as spermatogonial stem cell-like (SSC-like) populations, although these identities are defined by molecular markers and/or transcriptomic signatures, and their functional equivalence to *in vivo* prospermatogonia remains uncertain [52, 53]. During extended *in vitro* differentiation of hPSC-derived germ cells, populations resembling prospermatogonia have been observed. These cells typically exhibit reduced expression of early PGC markers together with increased expression of genes associated with later germline development and display transcriptional similarity to fetal germ cells identified in human developmental datasets [54]. Representative examples include the *in vitro* specification of prospermatogonia-like cells from hPSC-derived hPGCLCs reported by Hwang et al. [53, 55] or strategy published by Murase et al. [52]. These findings represent a significant step toward reconstructing early stages of human spermatogenic development *in vitro*, although the developmental competence of PSC-derived prospermatogonia-like cells and their ability to give rise to functional spermatogonial stem cells remain to be established. Efforts to generate spermatogonial stem cell-like (SSC-like) populations from hPSCs have also been reported. Several differentiation systems have produced cell populations expressing markers characteristic of undifferentiated spermatogonia. For instance, Zhao et al. described differentiation protocol capable of generating hPSC-derived PLZF-positive spermatogonium-like cells (SLCs) that likely represent a heterogeneous spermatogonial population [56]. Earlier work also demonstrated that hPSCs can acquire spermatogonial-like molecular markers signatures during *in vitro* differentiation [57]. More recent studies have further explored derivation and expansion of human SSC-like cells (hSSLCs) populations from hPSCs, reporting germ cells expressing not only canonical spermatogonial markers but also exhibiting gene expression profiles similar to SSCs isolated from human testes [58]. Subsequent studies have further refined differentiation strategies and reported additional systems supporting the generation of hSSLCs under defined culture conditions [59, 60]. Together, although PSC-derived SSC-like cells represent a promising model for studying early spermatogonial development, their functional equivalence to human SSCs *in vivo*—particularly their

capacity for long-term self-renewal and full spermatogenesis—has not yet been convincingly demonstrated. Additionally, several studies have reported the generation of cells expressing meiotic markers or displaying haploid-like DNA content following differentiation of hPSCs [56, 57, 61, 62]; however, the identity and functionality of these cells remain highly debated [43, 63].

In summary, recent advances have enabled the derivation of human germ cell–like populations resembling also the later developmental stages; however, their functional competence and broader reproducibility remain to be established. Further application and independent validation of these emerging differentiation systems will be important for assessing their robustness and developmental relevance. Major challenges remain in advancing these models toward meiotic and post-meiotic stages, including establishing accurate meiotic recombination and faithful epigenetic reprogramming, processes that remain difficult to achieve in hPSC-derived models [52, 64]. To date, the faithful recapitulation of meiotic and post-meiotic stages of human spermatogenesis - including meiosis and spermiogenesis - has not yet been robustly and reproducibly achieved in stem-cell-based *in vitro* systems. These limitations highlight the importance of molecular regulators and microenvironmental interactions that are yet unknown but likely required to support further human germ cell maturation. Additionally, stem-cell-based germline differentiation has progressed further in rodent systems, where pluripotent stem cells can be directed through multiple stages of male germ-cell development, including meiosis and post-meiotic differentiation under appropriate experimental conditions, typically in the presence of supportive gonadal somatic environments [65, 66]. At the same time, key regulatory mechanisms governing human spermatogenesis remain incompletely understood, highlighting the need to identify human-specific molecular regulators required for the generation of mature male gametes *in vitro*.

From a practical perspective, the choice of culture conditions represents an important consideration when implementing or further developing stem-cell-based germline differentiation protocols. Approaches relying on feeder layers or animal-derived components may introduce experimental variability, whereas more defined culture systems may facilitate protocol standardization and reproducibility across laboratories.

Nevertheless, human stem-cell-based models remain valuable experimental systems for dissecting molecular pathways regulating human germline development and studying germline biology *in vitro*. Importantly, these approaches may provide a renewable source of germ cell–like populations, potentially reducing reliance on limited human testicular tissue. Increasing evidence suggests also that successful advancement of germ cell differentiation requires not only appropriate developmental signaling pathways [44, 45], but also a supportive microenvironment that recapitulates key structural and cellular features of the testicular niche [67]. Consequently, recent efforts have increasingly focused also on developing 3D culture systems designed to better reproduce the architecture and multicellular organization of the seminiferous epithelium. These can be also stratified based on complexity, ranging from conventional 3D systems, through organoids, to advanced testicular models discussed in later sections.

Conventional 3D culture systems

Conventional three-dimensional (3D) culture systems represent an intermediate level of complexity between traditional two-dimensional (2D) monolayer cultures and more advanced *in vitro* models. In the testis, germ cells develop within the seminiferous tubules in close association with Sertoli cells and other somatic cell types that together form a highly organized microenvironment in a three-dimensional way. This spatially structured niche plays a key role in supporting germ cell development but is largely absent in conventional monolayer cultures. Three-dimensional culture systems therefore aim to recapitulate aspects of this environment by enabling cell growth within a volumetric context that facilitates cell–cell and cell–matrix interactions.

Conventional 3D models can be broadly divided into two methodological strategies: scaffold-based culture systems and scaffold-free culture systems. These approaches differ mainly in how the 3D architecture is generated, either by providing an external structural scaffold or by allowing cells to self-assemble into multicellular aggregates. Compared with 2D cultures, 3D systems more closely resemble physiological tissue architecture and often promote improved cellular communication, differentiation potential, and gene expression patterns.

Scaffold-based 3D culture systems use materials that provide structural support for cell attachment, growth, and spatial organization *in vitro*. These scaffolds may be composed of natural extracellular matrix–derived biomaterials, synthetic polymers, or hybrid materials that combine biological and synthetic components to mimic the mechanical and biochemical properties of native tissue. It should be noted that the classification of scaffold biomaterials is not always strict, as many materials share overlapping properties and may fall into more than one category. For example, extracellular matrix–derived materials such as collagen or laminin are naturally occurring polymers, while many hydrogels (composed of hydrophilic polymers [68]), for example agarose-based hydrogels used in 3D culture systems, may incorporate extracellular matrix (ECM)-derived biomaterials or synthetic polymers. In addition, hybrid scaffolds may combine multiple biomaterial classes. In the context of human spermatogenesis, scaffold-based systems have primarily been used to culture mixed populations of human testicular cells or spermatogonial stem cells to support their survival. Several studies using human testicular cells have investigated whether matrix-supported environments can improve the maintenance and expansion of SSCs. For example, studies using 3D decellularized human testicular ECM scaffold have reported improved survival and maintenance of human SSCs compared with conventional culture conditions [69, 70]. Biologically derived scaffolds obtained from decellularized testicular ECM have attracted considerable attention, as they preserve key components of the native testicular niche and may promote the survival and spatial organization of human testicular cells *in vitro* [71]. In another study, Jabari et al. co-cultured human SSCs and Sertoli cells, isolated from testicular biopsies, in soft agar–based 3D systems supplemented with laminin and growth factors [39]. Under these conditions, germ cells were able to form colonies and maintain markers associated with

undifferentiated spermatogonia, results indicated that a semi-solid scaffold environment supported SSCs more effectively than standard 2D culture. Subsequent study of this group reported the formation of morphologically spermatozoa-like cells, as confirmed by expression of specific markers, following long-term culture of human SSCs co-cultured with Sertoli cells in a hybrid hydrogel of agarose and laminin [72]. Another example is the study by Sun et al., who reported the 3D-induced culture system, supported by matrigel matrix, reported to promote the differentiation of isolated human SSCs (co-cultured with Sertoli cells) into haploid spermatid-like cells [73]. The resulting cells were characterized not only by the expression of stage-specific markers, but also through transcriptomic analyses and DNA methylation profiling in comparison with *in vivo* round spermatids. However, it remains unclear whether the observed haploid cells arose *de novo* or originated from pre-existing germ cells in the culture, and independent validation of these findings is still needed [12].

Despite these advances, scaffold-based systems generally provide only a partial approximation of the native testicular environment. While matrix-supported cultures can enhance cell viability and facilitate interactions between germ cells and somatic cells, they typically do not reproduce the complex spatial organization and developmental coordination characteristic of the seminiferous epithelium *in vivo*. Consequently, these platforms have been used mainly to study early spermatogonial biology, stem cell maintenance, and germ cell–niche interactions rather than to achieve complete human spermatogenesis *in vitro*.

Scaffold-free 3D culture systems rely on the intrinsic ability of cells to self-assemble into multicellular aggregates without the use of exogenous biomaterial matrices. In the context of human spermatogenesis research, such systems are typically generated by culturing dissociated testicular cells under non-adherent conditions, allowing cells to establish direct cell–cell interactions. Within conventional scaffold-free 3D culture systems, two main experimental approaches can be distinguished for the purpose of this review: spheroid cultures and testicular cell reassembly models. Spheroids generally represent relatively simple multicellular aggregates that arise through spontaneous cell–cell adhesion under scaffold-free culture conditions. Such structures can be generated using various aggregation techniques, including hanging-drop cultures [74], ultra-low attachment plates, and micro-well systems. While spheroids improve cell–cell communication compared with monolayer cultures, they typically lack the sophisticated spatial organization and functional compartmentalization characteristic of native tissue. Scaffold-free aggregation of dissociated human testicular cells can lead to the formation of multicellular aggregates that facilitate interactions between germ cells and testicular somatic cells. For example, Baert et al. [75] demonstrated that primary human testicular cells can self-assemble into spheroid structures. These early spheroid-like structures subsequently reorganized into more complex assemblies containing both germ and somatic cell populations. In some experimental systems, such spheroids may represent an early stage of cellular self-organization during the establishment of more complex 3D testicular models. An interesting example of spheroid-like structures are embryoid bodies (EBs), which are a specialized type of spheroid formed by aggregation of

pluripotent stem cells under non-adherent conditions. In studies of human germ cell development, EB formation is commonly used as an intermediate step to generate primordial germ cell-like cells (PGCLCs) *in vitro* using a stem-cell based model [44].

Alongside spheroids, testicular cell reassembly models might represent another category of conventional scaffold-free 3D systems. These multicellular constructs are generated by reaggregating dissociated testicular cells, allowing partial reconstruction of cellular interactions present in the testicular microenvironment. A representative example of a testicular cell reassembly system was reported by Mincheva et al. [76], who demonstrated that dispersed adult human testicular cells can reorganize *in vitro* into cord-like structures through dynamic interactions between Sertoli and peritubular cells. This model illustrated the capacity of human testicular somatic cells to reassemble and form structures partially resembling testicular tissue organization.

Before discussing more advanced *in vitro* models of human spermatogenesis, it is important to clarify the terminology used to describe different types of 3D cell assemblies. In the context of testicular research, the terms spheroids, reassembled testicular structures, and organoids are sometimes used interchangeably, although they refer to models with different levels of structural organization. In this review, spheroids are defined as relatively simple three-dimensional cell aggregates formed through the aggregation of cells under non-adherent culture conditions. Testicular cell reassembly models refer to multicellular structures generated by reaggregation of dissociated testicular cells that partially recreate cellular interactions of the testicular microenvironment. Testicular organoids are considered more advanced 3D systems that exhibit a higher degree of spatial organization and more closely reproduce key structural and functional features of native testicular tissue.

Although conventional scaffold-based and scaffold-free 3D culture systems have substantially improved the physiological relevance of *in vitro* spermatogenesis models, their capacity to reproduce the structural complexity of the human testis remains limited. In most currently available conventional 3D systems, only selected aspects of the testicular microenvironment are recapitulated, and the precise spatial organization of germ cells within the seminiferous epithelium as well as the characteristic tubular architecture of seminiferous tubules are not fully reproduced. Consequently, these platforms have primarily been used to investigate early spermatogonial biology, stem cell maintenance, and germ cell–niche interactions rather than to achieve complete human spermatogenesis *in vitro*. To address these limitations, increasing attention has been directed toward testicular organoid systems, which aim to more faithfully reconstruct the cellular composition and spatial organization of the testicular niche. These models represent an important step toward creating advanced *in vitro* platforms that better mimic the structural and functional properties of human testicular tissue.

Testicular organoids and 3D organotypic testis culture systems

In an effort to address the limitations of conventional 3D culture systems, increasing attention has been directed toward models that better preserve the cellular composition as well as spatial organization of the human testicular tissue. In this context, two related experimental approaches have emerged – testicular organoids and organotypic testis culture systems. Although these models differ in their experimental design, they are often discussed together because both aim to recreate key aspects of the seminiferous microenvironment and enable more physiologically relevant interactions between germ cells and supporting somatic cell populations.

Organoids have been described as miniaturized and simplified *in vitro* model systems of organs by Hofer and Lutolf [77]. They can also be defined as more advanced 3D cellular models that arise through the guided self-organization of stem or tissue-derived cells and reproduce selected structural and functional characteristics of the organs *in vivo* [67, 78]. Consequently, testicular organoids (TOs) typically arise through the organization of dissociated testicular cells cultured under defined conditions that promote multicellular aggregation and the formation of tissue-like structures [67, 79].

Several studies have demonstrated the feasibility of generating testicular organoids from human testicular cells, although only a subset of these systems exhibit a higher degree of spatial organization consistent with more advanced classification perspective. Notably, many reported “testicular organoids” appear to resemble reaggregated multicellular structures rather than fully organized organoids, reflecting the difficulty of reproducing the complex spatial organization of cells within the seminiferous tubules of the human testis *in vivo*. For example, study of Baert et al. [75], also mentioned in the context of spheroid-like systems, showed that primary human testicular cells can self-organize into multicellular structures containing germ cells together with key somatic cell types, including Sertoli and Leydig cells. These structures formed partially organized arrangements that recapitulated selected aspects of the native testicular microenvironment and were therefore described as testicular organoids (TOs). In an effort to promote *de novo* formation of testicular architecture, Pendergraft and colleagues [80] reported the generation of human TOs with a comparable level of advancement. These TOs were derived from primary testicular cell cultures and consisted of spermatogonial stem cells together with Sertoli, Leydig, and peritubular cells. In addition, this system was proposed as a platform for assessing gonadotoxic effects of chemotherapeutic agents. Similar to the structures described by Baert et al. [75], these models lacked testis-specific topography but were able to exhibit steroidogenic activity, as evidenced by testosterone production. The above-described TOs primarily supported the maintenance of spermatogonial populations, whereas no clear evidence of meiotic progression was reported. This indicates that, although these models partially recapitulate the cellular composition and functional interactions of the human testicular niche, they remain limited in their ability to support complete spermatogenic differentiation *in vitro*.

In contrast to testicular organoids, organotypic testis culture systems are based on the *in vitro* maintenance of intact or minimally disrupted fragments of testicular tissue, thereby preserving key aspects of the native cellular composition and architecture. While organoid models reconstruct features of the testicular niche through guided cellular self-assembly, organotypic cultures rely on the intrinsic organization of the tissue itself. Organotypic culture of human testicular tissue aims to preserve native architecture and cellular composition under *ex vivo* conditions. Such systems are often based on air–liquid interface (ALI) conditions, which allow tissue fragments to be maintained at the interface between air and culture medium while preserving seminiferous tubule structure and cell–cell interactions. Studies using cultured human testicular tissue fragments have demonstrated that seminiferous tubule structure, together with key somatic cell populations such as Sertoli and Leydig cells, can be maintained for extended periods under optimized culture conditions. For example, de Michele et al. [81] showed that organotypic culture supports the survival and maintenance of human prepubertal testicular tissue, preserving germ cells and somatic cell compartments *in vitro*. Similarly, Medrano et al. [82] demonstrated that human prepubertal testicular tissue cultured under organotypic conditions retains structural integrity and supports the survival of early germ cell populations. More broadly, organotypic testis culture systems have been applied to both prepubertal and adult human tissue to investigate germ cell behavior and responses to experimental or toxicological stimuli. However, despite preservation of tissue architecture, progression beyond early stages of germ cell development remains limited. In addition, variability between donor samples and restricted availability of human testicular tissue represent important challenges for the reproducibility and broader application of these models.

Despite methodological differences between testicular organoids and organotypic testis culture systems, both approaches provide valuable platforms for studying human spermatogenesis *in vitro* because they allow germ cells to interact with multiple somatic cell types within a more structured microenvironment than conventional cell aggregates. However, neither of these systems have so far supported the complete generation of functional human spermatozoa *in vitro*. While these approaches have improved conventional *in vitro* models, current organoid and organotypic culture systems still face several limitations. The spatial organization of seminiferous tubules is often only partially reproduced in reconstructed systems, and long-term maintenance of complete spermatogenic progression remains difficult to achieve. In addition, diffusion-limited transport of oxygen and nutrients can restrict the size and longevity of these 3D structures under static culture conditions. Consequently, recent efforts have focused on integrating tissue engineering strategies with dynamic culture platforms that allow improved control of the cellular microenvironment. These developments have led to the emergence of microfluidic and testis-on-a-chip systems, which aim to provide controlled perfusion and more precise regulation of the biochemical and physical conditions required for human germ cell development *in vitro*.

Microfluidic and Testis-on-a-Chip Systems - bioengineered platforms

To overcome limitations inherent to existing *in vitro* models of human spermatogenesis, microfluidic and testis-on-a-chip (ToC) platforms have been developed as advanced systems incorporating bioengineered features that enable dynamic control of the testicular microenvironment. In contrast to static culture systems, these approaches are designed to incorporate controlled fluid flow and microscale architecture, thereby improving the delivery of nutrients, oxygen, and soluble factors such as hormones or drugs, as well as facilitating the removal of metabolic waste [10]. By enabling precise regulation of biochemical gradients and mechanical conditions, microfluidic platforms offer the potential to more closely mimic the dynamic *in vivo* environment of the human testis. In this context, testis-on-a-chip systems represent a specialized application of microfluidic technology designed to recapitulate key aspects of testicular tissue organization and function within an integrated microfluidic platform. The testis-on-a-chip systems can be combined with other materials, for example extracellular matrix components or hydrogels, to support 3D organization and more defined cell–cell interactions.

In general, these approaches have been explored for the culture of testicular tissue fragments, as well as 3D culture models and isolated testicular cells. Although still limited in number, available studies suggest that controlled perfusion conditions can support tissue viability and contribute to the preservation of key cell populations under *in vitro* conditions. For example, Sharma et al. [83] described a microfluidic testis-on-a-chip platform enabling *ex vivo* culture of seminiferous tubules derived from human and non-human primate testes under continuous perfusion. This system supported tissue integrity and viability, highlighting the potential of dynamic culture conditions for maintaining human testicular tissue *ex vivo*. A recent example of a human tissue-based microfluidic platform was reported by Shen et al. [84], who developed a microfluidic chip for the culture of human testicular tissue fragments. This system integrates a perfusable microchannel network with a dedicated tissue chamber, enabling continuous medium flow and improved control of the local microenvironment. Under these conditions, the cultured tissue maintained structural integrity, including preservation of seminiferous tubule morphology, as well as the viability of both somatic and germ cell populations. In addition, the platform was proposed as a tool for pharmacological and toxicological studies, highlighting the potential of microfluidic approaches for investigating human testicular function under controlled *in vitro* conditions. Microfluidic systems have also been adapted for cell-based approaches, including both isolated testicular cells and three-dimensional multicellular models derived from human testicular cells. For instance, Li et al. [85] developed a microfluidic testicular organ-on-a-chip composed of human spermatogonial stem cells co-cultured with Sertoli and Leydig cells under standardized perfusion conditions. The system supported the formation of a spermatogenic epithelial structure and maintained fundamental cellular functions and interactions within a dynamic microenvironment. Also Park et al. [86] developed a human cell-based testis-on-a-chip model incorporating Sertoli and Leydig cells as well as various human testicular tissue constituent cells, which enabled the study of intercellular

communication and functional interactions within a controlled microenvironment. However, while current testis-on-a-chip models enable the study of germ cell behavior and cell–cell interactions, evidence for progression beyond early stages of germ cell development remains limited.

In the context of human models these approaches remain at an early stage of development, with limited number of studies demonstrating their application using exclusively human cells or tissues. The growing interest in microfluidic approaches for modeling human spermatogenesis is reflected in recent reviews highlighting their potential to recreate dynamic aspects of the testicular microenvironment and to improve the physiological relevance of such *in vitro* systems [87, 88]. Despite their potential, these advanced systems still face several challenges, including limited availability of human testicular tissue, variability between donor samples, and the difficulty of reconstructing the complex architecture of seminiferous tubules. Moreover, while dynamic perfusion improves environmental control, it does not yet overcome the intrinsic biological and structural barriers that limit complete spermatogenic progression *in vitro*. Nevertheless, these platforms represent an important technological advancement, bridging the gap between conventional three-dimensional cultures and more physiologically relevant models of human testicular function. Future efforts integrating microfluidic technologies with organoid and organotypic culture systems may further enhance the ability to recreate the human testicular niche and support more advanced stages of germ cell development *in vitro*.

Conclusions

Significant advances have been achieved in the development of experimental systems designed to study human spermatogenesis *in vitro*, ranging from simplified cell culture approaches to increasingly sophisticated 3D and bioengineered models. Despite these developments, the reliable generation of fully functional human male gametes *in vitro* has not yet been accomplished. Existing platforms provide important insights into germ cell biology and the regulatory role of the testicular microenvironment, supporting spermatogonial stem cell maintenance and, in some cases, partial progression through meiosis. However, robust functional validation remains limited. To assess whether germ cells produced *in vitro* truly resemble authentic human gametes, several biological parameters should be evaluated. According to the proposed “gold standard” criteria for evaluating *in vitro*-derived germ cells [89], these include morphological and phenotypic features, correct DNA and chromosome content, proper chromosomal pairing and recombination during meiosis, preservation of genomic integrity, appropriate imprinting patterns, and ultimately the capacity of the resulting haploid cells to support fertilization and early embryonic development. Although a number of experimental systems have reported the formation of haploid germ-cell-like cells derived from human spermatogonial stem cells or pluripotent stem cells, currently available models reproduce only selected aspects of spermatogenic development. Future progress in this field will therefore depend not only on improving the structural and biochemical fidelity of testicular models but also on the application of rigorous validation criteria to determine whether *in*

vitro-generated cells faithfully recapitulate the genetic, epigenetic, and functional properties of human male gametes.

For potential clinical applications, culture systems used for *in vitro* spermatogenesis will need to rely on fully defined and xeno-free media and biomaterials. The elimination of animal-derived components is important not only to ensure greater consistency and reproducibility between cultures but also to minimize the risk of transmitting animal-associated pathogens [12]. The development of standardized xeno-free culture components and conditions therefore represents an important step toward the clinical translation of human spermatogenesis models.

The development of advanced bioengineered platforms, such as microfluidic testis-on-a-chip systems, represents a promising direction for more faithfully replicating the complex microenvironment required for human spermatogenesis *in vitro*. By enabling precise control over biochemical and physical parameters, these approaches may help address some of the current limitations and move the field closer to the generation of sperm cells *in vitro*. However, despite these technological advances, our understanding of human spermatogenesis appears to rely largely on studies performed in simpler human *in vitro* cell culture models of the male germline. These models serve as accessible experimental systems that allow the investigation of cellular processes, the identification of key molecular regulators, and the exploration of mechanisms that are specific to human germ cell development and cannot be directly inferred from animal studies. In particular, uncovering the critical, and still incompletely defined, molecular players and pathways governing human spermatogenesis will require continued investigation using well-controlled and tractable model systems. Ultimately, the integration of knowledge gained from simpler models with the physiological relevance of advanced bioengineered platforms is likely to be essential for achieving complete human spermatogenesis *in vitro*.

The successful generation of human spermatozoa *in vitro* would represent a major scientific breakthrough, but would also raise profound ethical considerations and require the establishment of robust regulatory frameworks, particularly in the context of potential clinical application in assisted reproduction. Given that sperm quality directly influences the health and long-term outcomes of the offspring, rigorous evaluation of genetic and epigenetic integrity, as well as safety, will be essential. Addressing these challenges in parallel with continued technological and biological advances will be critical for the responsible translation of *in vitro* spermatogenesis into clinical practice.

Abbreviations

2D two-dimensional

3D three-dimensional

SSCs spermatogonial stem cells

BTB blood-testis barrier

ARs androgen receptors

FSH follicle-stimulating hormone

PTMs peritubular myoid cells
 LH luteinizing hormone
 hPSCs human pluripotent stem cells
 hESCs human embryonic stem cells
 hiPSCs human induced pluripotent stem cells
 IVG *in vitro* gametogenesis
 hPGCLCs human primordial germ cell-like cells
 PGCs primordial germ cells
 ECM extracellular matrix
 SLCs spermatogonium-like cells
 EBs embryoid bodies
 TOs testicular organoids
 ALI air-liquid interface
 ToC testis-on-a-chip

Data Availability

Not applicable.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization: N.R., A.J.B.; Writing – original draft: A.J.B.; Writing – review & editing: A.J.B., N.R.; Supervision: N.R.; Funding acquisition: N.R. All authors read and approved the manuscript.

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References

1. Griswold MD. Spermatogenesis: The Commitment to Meiosis. *Physiol Rev.* 2016;96:1–17. <https://doi.org/10.1152/physrev.00013.2015>.
2. McSwiggan HM, O'Doherty AM. Epigenetic reprogramming during spermatogenesis and male factor infertility. *Reproduction.* 2018;156:R9–21. <https://doi.org/10.1530/REP-18-0009>.
3. Guo J, Grow EJ, Mlcochova H, Maher GJ, Lindskog C, Nie X, et al. The adult human testis transcriptional cell atlas. *Cell Res.* 2018;28:1141–57. <https://doi.org/10.1038/S41422-018-0099-2>.

4. Hermann BP, Cheng K, Singh A, Roa-De La Cruz L, Mutoji KN, Chen IC, et al. The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. *Cell Rep.* 2018;25:1650-1667.e8. <https://doi.org/10.1016/J.CELREP.2018.10.026>.
5. Soumillon M, Necsulea A, Weier M, Brawand D, Zhang X, Gu H, et al. Cellular Source and Mechanisms of High Transcriptome Complexity in the Mammalian Testis. *Cell Rep.* 2013;3:2179–90. <https://doi.org/10.1016/J.CELREP.2013.05.031>.
6. Tharmalingam MD, Jorgensen A, Mitchell RT. Experimental models of testicular development and function using human tissue and cells. *Mol Cell Endocrinol.* 2018;468:95–110. <https://doi.org/10.1016/j.mce.2017.12.011>.
7. Siebert-Kuss LM, Krenz H, Tekath T, Wöste M, Di Persio S, Terwort N, et al. Transcriptome analyses in infertile men reveal germ cell-specific expression and splicing patterns. *Life Sci Alliance.* 2022;6:e202201633. <https://doi.org/10.26508/LSA.202201633>.
8. Amann RP. The cycle of the seminiferous epithelium in humans: A need to revisit? *J Androl.* 2008;29:469–87. <https://doi.org/https://doi.org/10.2164/jandrol.107.004655>.
9. Eisenberg ML, Esteves SC, Lamb DJ, Hotaling JM, Giwercman A, Hwang K, et al. Male infertility. *Nat Rev Dis Prim.* 2023;9:49. <https://doi.org/10.1038/s41572-023-00459-w>.
10. Kaltsas A, Kyrgiagini M-A, Markou E, Koumenis A, Mamuris Z, Dimitriadis F, et al. Artificial Gametogenesis and In Vitro Spermatogenesis: Emerging Strategies for the Treatment of Male Infertility. *Int J Mol Sci.* 2025;26. <https://doi.org/10.3390/IJMS26157383>.
11. Guo J, Nie X, Giebler M, Mlcochova H, Wang Y, Grow EJ, et al. The Dynamic Transcriptional Cell Atlas of Testis Development during Human Puberty. *Cell Stem Cell.* 2020;26:262-276.e4. <https://doi.org/10.1016/J.STEM.2019.12.005>.
12. Robinson M, Sparanese S, Witherspoon L, Flannigan R. Human in vitro spermatogenesis as a regenerative therapy — where do we stand? *Nat Rev Urol.* 2023;20:461–79. <https://doi.org/10.1038/s41585-023-00723-4>.
13. Mruk DD, Cheng CY. The Mammalian Blood-Testis Barrier: Its Biology and Regulation. *Endocr Rev.* 2015;36:564–91. <https://doi.org/10.1210/ER.2014-1101>.
14. Wanjari UR, Gopalakrishnan AV. Blood-testis barrier: a review on regulators in maintaining cell junction integrity between Sertoli cells. *Cell Tissue Res.* 2024;396:157–75. <https://doi.org/10.1007/S00441-024-03894-7>.
15. Teves ME, Roldan ERS. Sperm bauplan and function and underlying processes of sperm formation and selection. *Physiol Rev.* 2021;102:7. <https://doi.org/10.1152/PHYSREV.00009.2020>.
16. Hermo L, Pelletier RM, Cyr DG, Smith CE. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 2: changes in spermatid organelles associated with development of spermatozoa. *Microsc Res Tech.* 2010;73:279–319. <https://doi.org/10.1002/JEMT.20787>.
17. Skinner MK, editor. *Encyclopedia of Reproduction - 2nd Edition.* Elsevier; 2018.
18. Hofmann MC, McBeath E. Sertoli Cell-Germ Cell Interactions Within the Niche: Paracrine and

- Juxtacrine Molecular Communications. *Front Endocrinol (Lausanne)*. 2022;13:897062. <https://doi.org/10.3389/FENDO.2022.897062>.
19. Han S, Zhao L, Luo J, Shi C, Yao C, Ji Z, et al. Identification and isolation of human testicular peritubular myoid cells and Leydig cells by a combination of ITGA9 and NGFR. *Reprod Biol Endocrinol*. 2025;23:82. <https://doi.org/10.1186/s12958-025-01389-w>.
 20. Mayerhofer A. Peritubular cells of the human testis: prostaglandin E2 and more. *Andrology*. 2020;8:898–902. <https://doi.org/10.1111/andr.12669>.
 21. Mayerhofer A. Human testicular peritubular cells: more than meets the eye. *Reproduction*. 2013;145:R107–16. <https://doi.org/10.1530/REP-12-0497>.
 22. Adamczewska D, Słowikowska-Hilczner J, Walczak-Jędrzejowska R. The Fate of Leydig Cells in Men with Spermatogenic Failure. *Life (Basel, Switzerland)*. 2022;12. <https://doi.org/10.3390/LIFE12040570>.
 23. Ge R, Chen G, Hardy MP. The role of the Leydig cell in spermatogenic function. *Adv Exp Med Biol*. 2008;636:255–69. https://doi.org/10.1007/978-0-387-09597-4_14.
 24. De Jong J, Stoop H, Gillis AJM, Hersmus R, Van Gurp RJHLM, Van De Geijn GJM, et al. Further characterization of the first seminoma cell line TCam-2. *Genes Chromosomes Cancer*. 2008;47:185–96. <https://doi.org/10.1002/GCC.20520>.
 25. Looijenga LHJ, Stoop H, Biermann K. Testicular cancer: Biology and biomarkers. *Virchows Arch*. 2014;464:301–13. <https://doi.org/10.1007/S00428-013-1522-1/FIGURES/7>.
 26. De Windt S, Nekounazar Azad N, Wyns C. Human Testicular Tissue Digestion, Testicular Cell Selection, and Downstream Characterization for Reproductive Purposes: A Scoping Review. *Int J Mol Sci* 2025, Vol 26,. 2025;26. <https://doi.org/10.3390/ijms262010150>.
 27. Kwaspen L, Kanbar M, Wyns C, Kwaspen L, Kanbar M, Wyns C. Mapping the Development of Human Spermatogenesis Using Transcriptomics-Based Data: A Scoping Review. *Int J Mol Sci* 2024, Vol 25,. 2024;25. <https://doi.org/10.3390/IJMS25136925>.
 28. Sadri-Ardekani H, Mizrak SC, Van Daalen SKM, Korver CM, Roepers-Gajadien HL, Koruji M, et al. Propagation of Human Spermatogonial Stem Cells In Vitro. *JAMA*. 2009;302:2127–34. <https://doi.org/10.1001/jama.2009.1689>.
 29. Sadeghian-Nodoushan F, Borzouie Z, Hekmatimoghaddam S. Human Spermatogonial Stem Cell Culture In Vitro: A Review of New Materials and Recent Advances. *Cell J*. 2025;27. <https://doi.org/10.22074/cellj.2025.2034670.1616>.
 30. Sahare MG, Suyatno, Imai H. Recent advances of in vitro culture systems for spermatogonial stem cells in mammals. *Reprod Med Biol*. 2018;17:134. <https://doi.org/10.1002/rmb2.12087>.
 31. Goharbakhsh L, Mohazzab A, Salehkhoul S, Heidari M, Zarnani AH, Parivar K, et al. Isolation and Culture of Human Spermatogonial Stem Cells Derived from Testis Biopsy. *Avicenna J Med Biotechnol*. 2013;5:54.
 32. Barszcz A, Tutak K, Zyprych-Walczak J, Dassi E, Ilaslan E, Dąbrowska M, et al. Multi-omic

- screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis. *bioRxiv*. 2025. <https://doi.org/10.1101/2025.09.23.677621>.
33. Barszcz A, Tutak K, Malcher A, Dąbrowska M, Zyprych-Walczak J, Dassi E, et al. The RNA helicase DDX53 (CAGE) contributes to RNA metabolism in a human germ cell model. *bioRxiv*. 2026. <https://doi.org/10.64898/2026.02.03.703580>.
 34. Hau RK, Miller SR, Wright SH, Cherrington NJ. Generation of a hTERT-Immortalized Human Sertoli Cell Model to Study Transporter Dynamics at the Blood-Testis Barrier. *Pharm* 2020, Vol 12,. 2020;12:1–21. <https://doi.org/10.3390/pharmaceutics12111005>.
 35. Gaur M, Ramathal C, Pera RAR, Turek PJ, John CM. Isolation of human testicular cells and co-culture with embryonic stem cells. *Reproduction*. 2018;155:153–66. <https://doi.org/10.1530/REP-17-0346>.
 36. Guo Y, Hai Y, Yao C, Chen Z, Hou J, Li Z, et al. Long-term culture and significant expansion of human Sertoli cells whilst maintaining stable global phenotype and AKT and SMAD1/5 activation. *Cell Commun Signal*. 2015;13:20-. <https://doi.org/10.1186/s12964-015-0101-2>.
 37. Richer G, Baert Y, Goossens E. In-vitro spermatogenesis through testis modelling: Toward the generation of testicular organoids. *Andrology*. 2020;8:879–91. <https://doi.org/10.1111/andr.12741>.
 38. Sousa M, Cremades N, Alves C, Silva J, Barros A. Developmental potential of human spermatogenic cells co-cultured with Sertoli cells. *Hum Reprod*. 2002;17:161–72. <https://doi.org/10.1093/humrep/17.1.161>.
 39. Jabari A, Sadighi Gilani MA, Koruji M, Gholami K, Mohsenzadeh M, rastegar T, et al. Three-dimensional co-culture of human spermatogonial stem cells with Sertoli cells in soft agar culture system supplemented by growth factors and Laminin. *Acta Histochem*. 2020;122:151572. <https://doi.org/10.1016/j.acthis.2020.151572>.
 40. Medrano J V., Rombaut C, Simon C, Pellicer A, Goossens E. Human spermatogonial stem cells display limited proliferation in vitro under mouse spermatogonial stem cell culture conditions. *Fertil Steril*. 2016;106:1539-1549.e8. <https://doi.org/10.1016/j.fertnstert.2016.07.1065>.
 41. Damyanova KB, Nixon B, Johnston SD, Gambini A, Benitez PP, Lord T. Spermatogonial stem cell technologies: applications from human medicine to wildlife conservation. *Biol Reprod*. 2024;111:757–79. <https://doi.org/10.1093/biolre/ioae109>.
 42. Saitou M, Hayashi K. Mammalian in vitro gametogenesis. *Science* (80-). 2021;374. <https://doi.org/10.1126/science.aaz6830>.
 43. Kurek M, Albalushi H, Hovatta O, Stukenborg JB. Human Pluripotent Stem Cells in Reproductive Science-a Comparison of Protocols Used to Generate and Define Male Germ Cells from Pluripotent Stem Cells. *Int J Mol Sci*. 2020;21. <https://doi.org/10.3390/ijms21031028>.
 44. Irie N, Weinberger L, Tang WWC, Kobayashi T, Viukov S, Manor YS, et al. SOX17 is a critical specifier of human primordial germ cell fate. *Cell*. 2015;160:253–68.

- <https://doi.org/10.1016/j.cell.2014.12.013>.
45. Sasaki K, Yokobayashi S, Nakamura T, Okamoto I, Yabuta Y, Kurimoto K, et al. Robust In Vitro Induction of Human Germ Cell Fate from Pluripotent Stem Cells. *Cell Stem Cell*. 2015;17:178–94. <https://doi.org/10.1016/j.stem.2015.06.014>.
 46. Kobayashi M, Kobayashi M, Odajima J, Shioda K, Hwang YS, Sasaki K, et al. Expanding homogeneous culture of human primordial germ cell-like cells maintaining germline features without serum or feeder layers. *Stem Cell Reports*. 2022;17:507–21. <https://doi.org/10.1016/j.stemcr.2022.01.012>.
 47. Murase Y, Yabuta Y, Ohta H, Yamashiro C, Nakamura T, Yamamoto T, et al. Long-term expansion with germline potential of human primordial germ cell-like cells in vitro. *EMBO J*. 2020;39. <https://doi.org/10.15252/embj.2020104929>.
 48. Irie N, Surani M. Efficient Induction and Isolation of Human Primordial Germ Cell-Like Cells from Competent Human Pluripotent Stem Cells. *Methods Mol Biol*. 2017;1463:217–26. https://doi.org/10.1007/978-1-4939-4017-2_16.
 49. Vijayakumar S, Sala R, Kang G, Chen A, Pablo MA, Adebayo AI, et al. Monolayer platform to generate and purify primordial germ-like cells in vitro provides insights into human germline specification. *Nat Commun*. 2023;14:5690-. <https://doi.org/10.1038/s41467-023-41302-w>.
 50. Esfahani SN, Zheng Y, Arabpour A, Irizarry AMR, Kobayashi N, Xue X, et al. Derivation of human primordial germ cell-like cells in an embryonic-like culture. *Nat Commun* 2024 151. 2024;15:167-. <https://doi.org/10.1038/s41467-023-43871-2>.
 51. Vaz Santos M, De Bruin IJ, Dartée N, Lakshmipathi M, Hamer G, Mulder CL, et al. Generating human primordial germ cell-like cells from pluripotent stem cells: a scoping review of in vitro methods. *Hum Reprod Open*. 2025;2025:hoaf056. <https://doi.org/10.1093/hropen/hoaf056>.
 52. Murase Y, Yokogawa R, Yabuta Y, Nagano M, Katou Y, Mizuyama M, et al. In vitro reconstitution of epigenetic reprogramming in the human germ line. *Nature*. 2024;631:170–8. <https://doi.org/10.1038/s41586-024-07526-6>.
 53. Hwang YS, Suzuki S, Seita Y, Ito J, Sakata Y, Aso H, et al. Reconstitution of prospermatogonial specification in vitro from human induced pluripotent stem cells. *Nat Commun*. 2020;11:5656. <https://doi.org/10.1038/s41467-020-19350-3>.
 54. Sohni A, Tan K, Song HW, Burow D, de Rooij DG, Laurent L, et al. The Neonatal and Adult Human Testis Defined at the Single-Cell Level. *Cell Rep*. 2019;26:1501-1517.e4. <https://doi.org/10.1016/j.celrep.2019.01.045>.
 55. Seita Y, Hwang YS, Sasaki K. Reconstitution of Human Prospermatogonial Development from Human-Induced Pluripotent Stem Cells. *Methods Mol Biol*. 2023;2656:145–59. https://doi.org/10.1007/978-1-0716-3139-3_8.
 56. Zhao Y, Ye S, Liang D, Wang P, Fu J, Ma Q, et al. In Vitro Modeling of Human Germ Cell Development Using Pluripotent Stem Cells. *Stem Cell Reports*. 2018;10:509–23.

- <https://doi.org/10.1016/J.STEMCR.2018.01.001>.
57. Easley CA, Phillips BT, McGuire MM, Barringer JM, Valli H, Hermann BP, et al. Direct differentiation of human pluripotent stem cells into haploid spermatogenic cells. *Cell Rep*. 2012;2:440–6. <https://doi.org/10.1016/J.CELREP.2012.07.015>.
 58. Xu H, Yang M, Tian R, Wang Y, Liu L, Zhu Z, et al. Derivation and propagation of spermatogonial stem cells from human pluripotent cells. *Stem Cell Res Ther*. 2020;11:408. <https://doi.org/10.1186/s13287-020-01896-0>.
 59. Wang X, Qu M, Li Z, Long Y, Hong K, Li H. Valproic acid promotes the in vitro differentiation of human pluripotent stem cells into spermatogonial stem cell-like cells. *Stem Cell Res Ther*. 2021;12:553. <https://doi.org/10.1186/s13287-021-02621-1>.
 60. Khampang S, Lorthongpanich C, Laowtammathron C, Klaihmon P, Meesa S, Suksomboon W, et al. The dynamic expression of YAP is essential for the development of male germ cells derived from human embryonic stem cells. *Sci Rep*. 2024;14:15732. <https://doi.org/10.1038/s41598-024-66852-x>.
 61. Eguizabal C, Montserrat N, Vassena R, Barragan M, Garreta E, Garcia-Quevedo L, et al. Complete Meiosis from Human Induced Pluripotent Stem Cells. *Stem Cells*. 2011;29:1186–95. <https://doi.org/10.1002/stem.672>.
 62. Pierson Smela M, Adams J, Ma C, Breimann L, Widocki U, Shioda T, et al. Induction of Meiosis from Human Pluripotent Stem Cells. *bioRxiv Prepr Serv Biol*. 2024. <https://doi.org/10.1101/2024.05.31.596483>.
 63. Fang F, Li Z, Zhao Q, Li H, Xiong C. Human induced pluripotent stem cells and male infertility: An overview of current progress and perspectives. *Human Reproduction*. 2018;33:188–95. <https://doi.org/10.1093/humrep/dex369>.
 64. Lee SM, Surani MA. Epigenetic reprogramming in mouse and human primordial germ cells. *Exp Mol Med*. 2024;56:2578. <https://doi.org/10.1038/s12276-024-01359-z>.
 65. Ishikura Y, Ohta H, Sato T, Murase Y, Yabuta Y, Kojima Y, et al. In vitro reconstitution of the whole male germ-cell development from mouse pluripotent stem cells. *Cell Stem Cell*. 2021;28:2167–2179.e9. <https://doi.org/10.1016/j.stem.2021.08.005>.
 66. Zhou Q, Wang M, Yuan Y, Wang X, Fu R, Wan H, et al. Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells in Vitro. *Cell Stem Cell*. 2016;18:330–40. <https://doi.org/10.1016/j.stem.2016.01.017>.
 67. Alves-Lopes JP, Stukenborg JB. Testicular organoids: a new model to study the testicular microenvironment in vitro? 2018;24:176–91. <https://doi.org/10.1093/humupd/dmx036>.
 68. El-Sherbiny IM, Yacoub MH. Hydrogel scaffolds for tissue engineering: Progress and challenges. *Glob Cardiol Sci Pract*. 2013;2013:316. <https://doi.org/10.5339/gcsp.2013.38>.
 69. Salem M, Feizollahi N, Jabari A, Golmohammadi MG, Shirinsokhan A, Ghanami Gashti N, et al. Differentiation of human spermatogonial stem cells using a human decellularized testicular scaffold supplemented by platelet-rich plasma. *Artif Organs*. 2023;47:840–53.

<https://doi.org/10.1111/aor.14505>.

70. Naeemi S, Eidi A, Khanbabaee R, Sadri-Ardekani H, Kajbafzadeh AM. Differentiation and proliferation of spermatogonial stem cells using a three-dimensional decellularized testicular scaffold: a new method to study the testicular microenvironment in vitro. *Int Urol Nephrol* 2021 538. 2021;53:1543–50. <https://doi.org/10.1007/s11255-021-02877-9>.
71. Murdock MH, David S, Swinehart IT, Reing JE, Tran K, Gassei K, et al. Human Testis Extracellular Matrix Enhances Human Spermatogonial Stem Cell Survival In Vitro. *Tissue Eng Part A*. 2019;25:663. <https://doi.org/10.1089/ten.tea.2018.0147>.
72. Jabari A, Gholami K, Khadivi F, Koruji M, Amidi F, Gilani MAS, et al. In vitro complete differentiation of human spermatogonial stem cells to morphologic spermatozoa using a hybrid hydrogel of agarose and laminin. *Int J Biol Macromol*. 2023;235:123801. <https://doi.org/10.1016/j.ijbiomac.2023.123801>.
73. Sun M, Yuan Q, Niu M, Wang H, Wen L, Yao C, et al. Efficient generation of functional haploid spermatids from human germline stem cells by three-dimensional-induced system. *Cell Death Differ* 2018 254. 2018;25:749–66. <https://doi.org/10.1038/s41418-017-0015-1>.
74. Oliver E, Stukenborg JB. Rebuilding the human testis in vitro. *Andrology*. 2020;8:825–34. <https://doi.org/10.1111/andr.12710>.
75. Baert Y, De Kock J, Alves-Lopes JP, Söder O, Stukenborg JB, Goossens E. Primary Human Testicular Cells Self-Organize into Organoids with Testicular Properties. *Stem Cell Reports*. 2017;8:30–8. <https://doi.org/10.1016/j.stemcr.2016.11.012>.
76. Mincheva M, Sandhowe-Klaverkamp R, Wistuba J, Redmann K, Stukenborg JB, Kliesch S, et al. Reassembly of adult human testicular cells: can testis cord-like structures be created in vitro? *Mol Hum Reprod*. 2018;24:55–63. <https://doi.org/10.1093/molehr/gax063>.
77. Hofer M, Lutolf MP. Engineering organoids. *Nat Rev Mater*. 2021;6:402. <https://doi.org/10.1038/s41578-021-00279-y>.
78. Lancaster MA, Knoblich JA. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* (80-). 2014;345. <https://doi.org/10.1126/science.1247125>.
79. Zhao Z, Chen X, Dowbaj AM, Sljukic A, Bratlie K, Lin L, et al. Organoids. *Nat Rev Methods Prim* 2022 21. 2022;2:94-. <https://doi.org/10.1038/s43586-022-00174-y>.
80. Pendergraft SS, Sadri-Ardekani H, Atala A, Bishop CE. Three-dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity in vitro. *Biol Reprod*. 2017;96:720–32. <https://doi.org/10.1095/BIOLREPROD.116.143446>.
81. De Michele F, Poels J, Giudice MG, De Smedt F, Ambroise J, Vermeulen M, et al. In vitro formation of the blood–testis barrier during long-term organotypic culture of human prepubertal tissue: comparison with a large cohort of pre/peripubertal boys. *Mol Hum Reprod*. 2018;24:271–82. <https://doi.org/10.1093/MOLEHR/GAY012>.
82. Medrano J V., Vilanova-Pérez T, Fornés-Ferrer V, Navarro-Gomezlechón A, Martínez-Triguero

- ML, García S, et al. Influence of temperature, serum, and gonadotropin supplementation in short- and long-term organotypic culture of human immature testicular tissue. *Fertil Steril*. 2018;110:1045-1057.e3. <https://doi.org/10.1016/j.fertnstert.2018.07.018>.
83. Sharma S, Venzac B, Burgers T, Schlatt S, Le Gac S. Testis-on-chip platform to study ex vivo primate spermatogenesis and endocrine dynamics. *Organs-on-a-Chip*. 2022;4:100023. <https://doi.org/10.1016/J.OOC.2022.100023>.
84. Shen J, Wang X, Yang C, Ren G, Wang L, Piao S, et al. Development and evaluation of a microfluidic human testicular tissue chip: a novel in vitro platform for reproductive biology and pharmacology studies. *Lab Chip*. 2025;25:577–89. <https://doi.org/10.1039/D4LC00780H>.
85. Li Y, Cheng H, Lv LY, Liu Y, Xie Y, Chen J, et al. A testicular microfluidic organ-on-a-chip for mimicking spermatogenic epithelium. *Biomed Mater*. 2025;20. <https://doi.org/10.1088/1748-605X/ADD9DB>.
86. Park SR, Kook MG, Kim SR, Lee CM, Lee JW, Park JK, et al. Development of a novel testis-on-a-chip that demonstrates reciprocal crosstalk between Sertoli and Leydig cells in testicular tissue. *Exp Mol Med* 2024 567. 2024;56:1591–605. <https://doi.org/10.1038/s12276-024-01258-3>.
87. Schneider BM, Hamurcu HI, Salzbrunn A, Kopylow K von. Microfluidic systems in testicular in vitro culture: a powerful model tool for spermatogenesis and reprotoxicity studies. *Asian J Androl*. 2025;27:659. <https://doi.org/10.4103/AJA20254>.
88. Shuchat S, Yossifon G, Huleihel M. Perfusion in Organ-on-Chip Models and Its Applicability to the Replication of Spermatogenesis In Vitro. *Int J Mol Sci*. 2022;23. <https://doi.org/10.3390/IJMS23105402>.
89. Handel MA, Eppig JJ, Schimenti JC. Applying “gold standards” to in-vitro-derived germ cells. *Cell*. 2014;157:1257–61. <https://doi.org/10.1016/j.cell.2014.05.019>.

4.2. Artykuł 2: „Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis”

Wstęp: Niepłodność męska stanowi jeden z globalnych problemów zdrowotnych, a wiele mechanizmów, które leżą u podstaw zaburzeń rozwoju linii płciowej oraz spermatogenezy człowieka pozostaje nadal nieznanymi. Przeprowadzone w Instytucie Genetyki Człowieka PAN i opublikowane wcześniej badania genetyczne niepłodnych pacjentów z azoospermią nieobturacyjną (NOA) pomogły zidentyfikować gen *RBMXL3* (ang. *RNA-binding motif X-linked-like-3*) jako potencjalnie związany z niepowodzeniem procesu spermatogenezy. Gen *RBMXL3* jest genem specyficznym dla naczelnymi, zlokalizowanym na chromosomie X, a dostępne dane literaturowe są bardzo ograniczone i obejmują jedynie nieliczne doniesienia. Chociaż ekspresję *RBMXL3* wykrywano dotąd głównie w męskich gonadach, jego rola w powstaniu męskich gamet nie była dotąd badana. Dostępne dane literaturowe wskazują, że białka z rodziny RBMX odgrywają istotną rolę w potranskrypcyjnej regulacji ekspresji genów, w tym w splicingu mRNA. Przykładem jest białko RBMXL2, które odpowiada za kontrolę splicingu podczas spermatogenezy u myszy, a inaktywacja genu *Rbmxl2* prowadzi do niepłodności u samców. W publikacji stanowiącej część niniejszej rozprawy doktorskiej przedstawiono wyniki kompleksowych analiz transkryptomicznych (RNA-seq oraz eCLIP-seq) i proteomicznych (Co-IP-MS), mających na celu identyfikację sieci powiązań oraz molekularnych interaktorów RBMXL3 w ludzkim modelu komórek płciowych *in vitro*, konkretnie z wykorzystaniem ludzkiej linii komórkowej nowotworu jądra, TCam-2.

Wyniki i dyskusja: W celu zbadania białka RBMXL3 w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka przygotowano model komórkowy z przejściową nadekspresją tego białka z wykorzystaniem linii TCam-2. Przeprowadzono transfekcję komórek TCam-2 typu dzikiego (WT) wektorem kodującym białko RBMXL3 znakowane epitopem FLAG lub GFP znakowane epitopem FLAG (zastosowane jako kontrola do eksperymentów razem z WT). Ekspresję obu białek potwierdzono na poziomie RNA oraz białka, wykorzystując technikę RT-qPCR oraz Western Blot (WB). Dzięki nawiązaniu współpracy z prof. J. Alcornem (The University of Texas) było możliwe zastosowanie w badaniach specyficznego przeciwciała anti-RBMXL3 jako potwierdzenie wyników otrzymanych przy pomocy stosowanego równolegle przeciwciała anti-FLAG. W celu określenia lokalizacji RBMXL3 w

komórkach TCam-2 wykonano podwójne barwienie immunofluorescencyjne z zastosowaniem przeciwciała anty-FLAG oraz anty-RBMXL3. Za pomocą mikroskopii konfokalnej potwierdzono silną kolokalizację sygnału w jądrze komórkowym. Następnie przeprowadzono frakcjonowanie komórkowe połączone z izolacją białek oraz WB, co wykazało wyraźne wzbogacenie RBMXL3 we frakcji chromatynowej. Kolejnym etapem badań wykonanym w celu zidentyfikowania sekwencji DNA bezpośrednio oddziałujących z RBMXL3 było przeprowadzenie eksperymentu ChIP-seq (immunoprecypitacja chromatyny połączona z sekwencjonowaniem). Chociaż eksperyment ChIP-seq z użyciem FLAG nie wykazał powtarzalnego wzbogacenia DNA względem kontroli GFP, w publikacji nie wykluczono możliwości występowania oddziaływań o charakterze przejściowym lub pośrednim. Istotnym wynikiem niniejszej pracy było wykazanie (po raz pierwszy z wykorzystaniem specyficznego przeciwciała w barwieniu immunohistochemicznym) obecności białka RBMXL3 w męskiej gonadzie, z jego identyfikacją w spermatogoniach oraz spermatocytach. W dalszej części publikacji przeprowadzono sekwencjonowanie RNA (RNA-seq) wykorzystując komórki z nadekspresją RBMXL3-FLAG oraz GFP-FLAG (zmodyfikowanych przy użyciu wektora lentiwirusowego), a także WT. Analiza danych z sekwencjonowania transkryptomu wykazała zmiany w poziomie ekspresji 170 genów, w tym transkryptów kodujących białka, które uczestniczą w utrzymaniu integralności strukturalnej macierzy zewnątrzkomórkowej, podkreślając potencjalne znaczenie RBMXL3 dla procesów migracji dojrzewających komórek płciowych w kanalikach nasiennych. Istotnym wynikiem było również wykazanie potencjalnego wpływu RBMXL3 na procesy alternatywnego splicingu (AS). Analiza danych RNA-seq pozwoliła na zidentyfikowanie 916 zdarzeń AS w 470 transkryptach. Spośród genów o zmienionym profilu splicingu wyłoniono 14 genów (w tym *YY1*), które zostały już zakwalifikowane do terminu GO (ontologia genów) powiązanego z tworzeniem męskich gamet. W ramach tej samej puli transkryptów potencjalnie ulegających alternatywnemu splicingowi w obecności białka RBMXL3 zidentyfikowano również m.in. *FOS* oraz *HNRNPH1*, powiązane w literaturze z regulacją spermatogenezy i utrzymaniem komórek linii płciowej. Ponadto, przeprowadzono także analizę wzbogacenia terminów ontologii genowych (GO) oraz analizę szlaków biologicznych (Reactome) dla genów o zmienionym splicingu (DSGs). W oparciu o uzyskane wyniki wykazano powiązanie genów potencjalnie ulegających alternatywnemu splicingowi z uwagi na obecność RBMXL3 z procesami kluczowymi dla metabolizmu RNA oraz istotnymi w kontekście spermatogenezy. W następnej części

publikacji zaprezentowano wyniki dotyczące identyfikacji interaktorów białkowych RBMXL3 z wykorzystaniem ko-immunoprecypitacji sprzężonej ze spektrometrią mas (Co-IP-MS). Interakcje białkowe zbadane metodą Co-IP-MS pozwoliły na identyfikację 16 potencjalnych interaktorów białkowych RBMXL3. Wśród nich znalazły się białka związane z procesem splicingu oraz metabolizmu RNA, takie jak EIF4A3 oraz DDX39B, a kolejnym etapem była dalsza walidacja ich interakcji z białkiem RBMXL3. W tym celu wykonano podwójne barwienia immunofluorescencyjne, a przy użyciu mikroskopii konfokalnej wykazano miejscową kolokalizację sygnału RBMXL3 z tymi białkami, co może potwierdzać rzeczywistą interakcję. Ważną część publikacji stanowią wyniki eksperymentu eCLIP (wzmocniona metoda sieciowania i immunoprecypitacji połączona z sekwencjonowaniem), przeprowadzonego w celu identyfikacji RNA bezpośrednio oddziałujących z białkiem RBMXL3. Eksperyment przeprowadzony na komórkach TCam-2 z przejściową nadekspresją *RBMXL3-FLAG* (oraz *GFP-FLAG* jako kontrola) pozwolił zidentyfikować ponad 1200 transkryptów RNA wiązanych przez to białko. Większość miejsc wiązania (ponad 6900 pików) zlokalizowana była w obrębie intronów. Analiza motywów wykazała preferencję RBMXL3 dla konsensusowych sygnałów splicingowych, co wskazuje na udział tego białka w procesie alternatywnego splicingu. W publikacji przeprowadzono analizę funkcjonalną GO oraz Reactome, która wykazała, że transkrypty wiązane przez RBMXL3 uczestniczą w procesach kluczowych dla cyklu komórkowego, regulacji splicingu oraz utrzymania populacji komórek macierzystych. Wyniki te wskazują na potencjalną rolę RBMXL3 w regulacji procesów istotnych dla jakości gamet i różnicowania komórek linii płciowej. Wśród bezpośrednich celów zidentyfikowano m.in. transkrypty kodujące *EP300*, *FANCM*, *PREP*, a także *FOXJ3*, powiązane uprzednio w literaturze z procesem spermatogenezy. Ponadto, wyniki eCLIP-seq wykazały, że białko RBMXL3 wiąże własny transkrypt RNA, co może sugerować potencjalny mechanizm autoregulacyjny, typowy dla wielu białek wiążących RNA. W ostatniej części wyników zbadano wpływ białka RBMXL3 na retrotranspozycję elementów LINE-1 (L1). Eksperyment przeprowadzono, ponieważ analiza danych eCLIP wykazała, że RBMXL3 wiąże sekwencje należące do retrotranspozonów z rodziny L1 (LINE-1), chociaż analiza danych RNA-seq nie wykazała znaczącego wpływu na poziom ekspresji takich transkryptów względem obu kontroli (*GFP-FLAG* oraz *WT*). Eksperymenty dotyczące retrotranspozycji zostały wykonane przez prof. Zbigniewa Warkockiego (Instytut Chemii Bioorganicznej PAN), eksperta w dziedzinie biologii LINE-1, jako uzupełnienie badań nad białkiem RBMXL3. Test retrotranspozycji wykazał

silne hamowanie aktywności L1 przez białko RBMXL3, co podkreśla jego znaczenie dla utrzymania integralności genomu, szczególnie istotne w kontekście różnicowania komórek linii płciowej. Możliwy mechanizm działania może obejmować m.in. bezpośrednie oddziaływanie z transkryptami L1 oraz potencjalny wpływ na ich regulację potranskrypcyjną lub interakcje z białkami związanymi z procesem retrotranspozycji.

Wnioski: Uzyskane wyniki dostarczają pierwszych dowodów na rolę białka RBMXL3 jako regulatora metabolizmu RNA w komórkach męskiej linii płciowej człowieka. Przeprowadzone eksperymenty wykazały, że białko RBMXL3 wiąże liczne RNA w ludzkich komórkach oraz ma potencjalny wpływ na regulację ekspresji i alternatywny splicing genów kluczowych dla prawidłowego przebiegu spermatogenezy. W publikacji potwierdzono jądrową lokalizację RBMXL3 w komórkach oraz po raz pierwszy wykazano obecność tego białka w męskiej gonadzie wykorzystując do tego celu specyficzne przeciwciała. Wykazano także, że RBMXL3 istotnie hamuje retrotranspozycję elementów L1, co wskazuje na jego potencjalną funkcję w ochronie integralności genomu człowieka. Uzyskane wyniki sugerują, że warianty genetyczne zakłócające prawidłową funkcję RBMXL3 mogą być przyczyną niepłodności męskiej i rzucają nowe światło na skomplikowane mechanizmy regulacji genów u ludzi. Podsumowując, uzyskane dane sugerują, że RBMXL3 odgrywa istotną rolę w regulacji dynamiki transkryptomu męskich komórek płciowych u człowieka, jednak pełne zrozumienie jego znaczenia dla prawidłowego procesu spermatogenezy wymaga dalszych badań.

Mój wkład w powstanie publikacji:

- udział w opracowaniu koncepcji i przygotowaniu planu badań
- prowadzenie hodowli komórkowych, wykonywanie transfekcji i transdukcji
- optymalizacja metodologii oraz wykonanie i interpretacja eksperymentów z wykorzystaniem linii komórkowej TCam-2
- wykonanie większości izolacji RNA i białek oraz przeprowadzenie analiz RT-qPCR, Western Blot
- optymalizacja i wykonanie barwień immunofluorescencyjnych oraz Co-IP
- analiza bioinformatyczna danych NGS oraz opracowanie graficznej prezentacji wyników
- napisanie oryginalnej wersji publikacji, przygotowanie większości załączników oraz rycin
- przygotowywanie odpowiedzi dla recenzentów w procesie publikacyjnym
- przygotowanie ostatecznej wersji publikacji

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Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis

Agata J. Barszcz¹; Katarzyna Tutak^{1,2}; Joanna Zypych-Walczak³; Erik Dassi⁴; Erkut Ilaslan^{1,5}; Małgorzata Dąbrowska¹; Agnieszka Malcher¹; Marta Olszewska¹; Dominik Cysewski⁶; Agnieszka Nadel¹; Tomasz Kolanowski¹; Zofia E. Madeja⁷; Michał Hrab⁸; Alexander Yatsenko⁹; Maciej Kurpisz¹; Zbigniew Warkocki¹⁰; Natalia Rozwadowska^{1*}

¹ Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

² Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland

³ Department of Mathematical and Statistical Methods, Poznan University of Life Sciences, Poznan, Poland

⁴ Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

⁵ DNRF Center for Chromosome Stability, University of Copenhagen, DK-2200 Copenhagen, Denmark

⁶ Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

⁷ Department of Genetics and Animal Breeding, Faculty of Veterinary Medicine and Animal Sciences, Poznan University of Life Sciences, Poznan, Poland

⁸ Department of Urology, Poznan University of Medical Sciences, Poznan, Poland

⁹ Department of OBGYN and Reproductive Sciences, Magee-Womens Research Institute, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

25 ¹⁰ Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

26 *Corresponding author: natalia.rozwadowska@igcz.poznan.pl

27 Abstract

28 **Background:** RBMXL3 is a primate-specific gene localized on the X chromosome, which expression is
29 detectable mainly in the male gonad. So far, very little is known about the RBMXL3 protein function and
30 its molecular interactions. However, recent reports mention the *RBMXL3* gene in the context of human
31 spermatogenesis, cancer, and a breathing disorder that affects newborns. In this study, we investigate the
32 RBMXL3's molecular network on a genome-wide scale using the human seminoma cell line (TCam-2) as
33 a male germline *in vitro* model.

34 **Methods:** By using transcriptomic (RNA sequencing (RNA-seq) and enhanced crosslinking and
35 immunoprecipitation (eCLIP)) and proteomic (Co-immunoprecipitation coupled with Mass Spectrometry,
36 (Co-IP-MS)) approaches we show RBMXL3 importance in RNA metabolism. Additionally, Western Blot,
37 qRT-PCR, immunostaining, and confocal imaging were used in order to investigate the function of
38 RBMXL3. Finally, we used a plasmid-based L1 retrotransposition assay to demonstrate the suppressive
39 effect of RBMXL3 on human Long Interspersed Nuclear Element-1 (LINE-1, L1) retrotransposition.

40 **Results:** Our RNA-seq data show that RBMXL3 expression drives gene expression changes and influence
41 alternative splicing in human cells. Moreover, by performing eCLIP we provide a proof that RBMXL3 binds
42 to a wide range of RNA transcripts. Additionally, we confirmed the nuclear localization of RBMXL3 in
43 TCam-2 cells and its presence in spermatogonia and spermatocytes within the human testis. Finally, we
44 report for the first time that RBMXL3 restricts human LINE-1 retrotransposition.

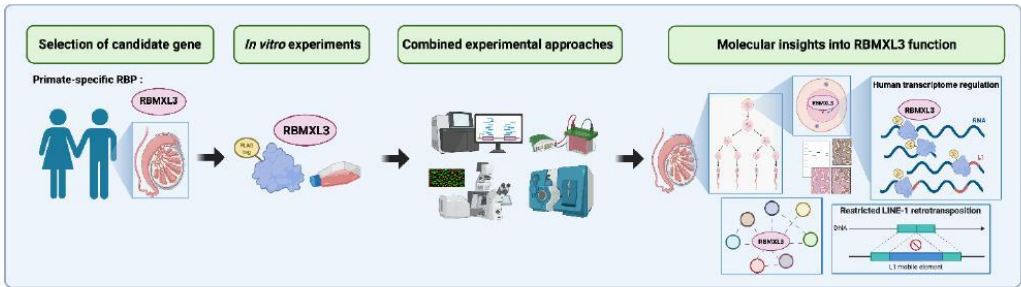
45 **Conclusions:** Our findings for the first time identify primate-specific RBMXL3 protein as a new upstream
46 regulator of RNA metabolism, characterized by broad RNA-binding activity in human TCam-2 cells.
47 Finally, we show that RBMXL3 expression heavily reduces LINE-1 retrotransposition in human cells,
48 underlying RBMXL3 importance in maintaining genome integrity. Our data suggest that RBMXL3 may

49 contribute to the regulation of transcriptome dynamics in male germ cells, while its broader functional
50 implications remain to be determined.

51 **Keywords**

52 RNA-binding proteins, RNA metabolism, splicing, retrotransposition, spermatogenesis, infertility

53 **Graphical abstract**



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56 **Background**

57 Infertility has become a global health issue that affects millions of couples worldwide [1–4]. Since
58 approximately half of infertility cases are associated with male factor it is necessary to explore the
59 mechanisms underlying spermatozoa differentiation. A plethora of factors must cooperate to ensure
60 successful male gametes differentiation during spermatogenesis, and the complexity of this process makes
61 it error-prone. Unfortunately, our knowledge about the genetic regulation of human germ cell development
62 and spermatogenesis is fragmented. High-throughput sequencing technologies, including whole-exome
63 sequencing (WES) and whole-genome sequencing (WGS) studies, pave the way for seeking new candidate
64 genes that may be responsible for some fertility disorders. Recent genome-wide genetic screening of non-
65 obstructive azoospermia (NOA) patients identified novel candidate genes, possibly associated with
66 spermatogenesis failure [5]. Among the potential NOA-associated gene candidates, the *RBMXL3* (RNA-
67 binding motif X-linked-like-3) gene, coding for a primate-specific protein, was identified. *RBMXL3* is

68 located on Xq23 within the first intron sequence of *LRCH2*. The *RBMXL3* is a primate-specific, intronless
69 gene which is transcribed in the opposite direction to its host gene and encodes the RBMXL3 protein
70 consisting of 1067 amino acids (114.9 kDa). This protein shares 50 % identity with its ancestral protein
71 RBMX (RNA binding motif protein X-linked, also localized on X chromosome). *RBMXL3* originated, as
72 many other *RBMX*-like genes, from spliced *RBMX* mRNA transcript in the process called retrotransposition
73 [6–9]. The RBMX protein, previously called heterogeneous nuclear ribonucleoprotein G – hnRNP G, is
74 ubiquitously expressed in multiple tissues and contains an N-terminal RRM (RNA recognition motif) [6].
75 In addition to the RRM domain, the RBMXL3 protein contains a unique long C-terminal glycine-rich region
76 with not yet defined function [10]. A schematic comparison of RBMXL3 and related RBMX proteins has
77 been previously published by Liu et al [10].

78 Multiple lines of evidence support the involvement of the RBMX-family proteins in post-transcriptional
79 regulation of gene expression [11], including mRNA splicing [12]. RBMXL2 controls splicing patterns in
80 meiotic prophase during spermatogenesis, and *Rbmxl2* gene knock-out results in male mice sterility [13].
81 Also RBMX is involved in splicing and associates co-transcriptionally with RNA polymerase II (RNAPII)
82 in humans [14–16]. Importantly, DNA sequence variants in the *RBMX* gene have already been associated
83 with NOA in humans [17].

84 Alternative splicing regulation has been shown to be especially important in the context of developmental
85 processes and is particularly prevalent in the testis and the brain [18–20]. There is an evident relation
86 between the human brain dysfunctions and testis disorders [21, 22]. For example, the already mentioned
87 RBMX has been linked to Shashi X-linked intellectual disability syndrome characterized, among others,
88 with macroorchidism (abnormally large testes) [23, 24]. Complementarily, studies on a zebrafish *rbmx*
89 orthologue have shown that it is essential for normal brain development [25]. Furthermore, the RBMX
90 protein has been shown to contribute to sister chromatid cohesion whereby its knockdown causes abnormal
91 nuclear morphology [26, 27]. RBMX also contributes to the efficient DNA repair and ensures genome
92 stability during replication [28–30] which is essential during gametogenesis.

93 According to the Human Protein Atlas (HPA), *RBMXL3* is expressed mostly in spermatogonia and
 94 spermatocytes in the human testis. At a lower level, however, its expression has been also found in brain
 95 [31]. Mass spectrometry-based proteome-wide studies of healthy human tissues confirmed *RBMXL3*
 96 protein in the testis and lung [32]. However, wide-scale experiments on human tissues have shown
 97 discrepancies concerning the expression of *RBMXL3*, at the mRNA and protein level, indicating *RBMXL3*
 98 elusive nature [32–34]. Rare variants of *RBMXL3* have been associated not only with NOA [5] but also with
 99 *MYCN* non-amplified neuroblastoma, which is the most common extracranial solid tumor in infants and
 100 children [35]. Two other reports mention this gene in the context of metastatic colorectal cancer [36, 37].
 101 The only report that showed the first functional aspect of *RBMXL3* refers to its presence in the human lung
 102 cancer cell line (A549) wherein it was proposed to stabilize the transcript encoding pulmonary surfactant
 103 protein B [10].

104 Since modeling human spermatogenesis *in vitro* is limited, we decided to address *RBMXL3* functionality
 105 in male germ cells using the TCam-2 cell line, derived from human seminoma (germ cell tumor) [38–41].
 106 In terms of gene expression, these cells resemble human primordial germ cells [42, 43] which are embryonic
 107 precursors of male germ cell lineage and hence are a valuable tool for studying germline-specific processes.
 108 Here, we generated modified TCam-2 cell lines expressing either *RBMXL3*-FLAG or GFP-FLAG and used
 109 them to characterize transcriptomic and proteomic changes resulting from *RBMXL3* overexpression. By
 110 using RNA-seq and eCLIP, we show that *RBMXL3* is a promiscuous RNA-binding protein influencing
 111 both gene expression as well as splicing of multiple target RNAs. In addition, we identify *RBMXL3* protein
 112 interactors of which most are involved in post-transcriptional regulation of gene expression, including
 113 splicing. Finally, we observe *RBMXL3* binding to LINE-1 (L1, Long Interspersed Nuclear Element-1)
 114 retrotransposon RNAs and show reduced retrotransposition of L1 in an *in vitro* L1 retrotransposition assay.
 115 This study adds important new information on the functionality of primate-specific *RBMXL3* placing it as
 116 a possible regulator of human spermatogenesis and differentiation processes.

117 **Methods**

118 Cell line

119 The human testicular cancer cell line (TCam-2) was gifted from Prof. Sohei Kitazawa (Division of
120 Molecular Pathology, Kobe University, Japan) [38, 39]. TCam-2 cells were cultured at 37°C in a humidified
121 5% CO₂ incubator in Roswell Park Memorial Institute 1640 Medium (RPMI 1640 Medium, GlutaMAX™
122 Supplement Thermo Fisher Scientific, 61870044) supplemented with 10% heat-inactivated Fetal Bovine
123 Serum (Cytiva, HYCLSV30160.03) and 1% Antibiotic-Antimycotic solution (100X) (Thermo Fisher
124 Scientific, 15240062).

125 HEK293T cells (used in retrotransposition assay, originally acquired in Invitrogen) were derived from a
126 female. The 293T cells (ATCC Cat# CRL-3216, RRID:CVCL_0063) were obtained from the lab of Jose L.
127 Garcia-Perez (IGMM Edinburgh) [44] and authenticated by SNP genotyping by Eurofins Genomics
128 (Ebersberg Germany), the chromatograms are available online in the supplementary materials to Janecki et
129 al., 2024 [45]. The cells were cultured in monolayers in high glucose Dulbecco's modified Eagle's medium
130 (DMEM, Gibco; 41966052) supplemented with 9% (v/v) fetal bovine serum (Gibco; 10270106), 100 U/ml
131 penicillin and 100 µg/ml streptomycin (Merck; P4458) at 37°C in a humidified 5% CO₂ incubator. Cells
132 were passaged every 2-3 day.

133 Generation of TCam-2 cell lines modified by lentiviral transduction

134 Lentiviral particles were packed using 2nd (for RBMXL3 construct) and 3rd (for eGFP construct) generation
135 packaging system in HEK293T cells. In case of 2nd generation system 1×10^6 HEK293T cells were
136 transfected according to manufacturer's recommendations (Sigma-Aldrich, CAPHOS) based on calcium
137 phosphate method with the mixture of plasmids: psPAX (Addgene, #12260), MD2G (Addgene, #12259)
138 and pLV[Exp]-Puro-EF1A>hRBMXL3[NM_001145346.1]/3xFLAG (Vector Builder, Vector ID
139 VB180321-1238ccw) in ratio 1:1:2. In case of 3rd generation system following plasmids were used: pRSV-
140 Rev (Addgene, #12253), pMDLg/prRE (Addgene, #12251), pMSCV-VSV-G (Addgene, #14888),
141 pLV[Exp]-Puro-EF1A>EGFP/3xFLAG (Vector Builder, Vector ID VB180409-1201grs) in ratio 1:1:1:2.
142 After 48 and 72h post transfection pseudoviral particles were collected and filtered through 0.45 µm

(Millipore, SLHVR13SL). Viral aliquots were frozen and stored at -80 °C. Next 1×10^5 TCam-2 cells were incubated with the viral supernatant in the presence of polybrene (final concentration 4µg/ml, Sigma-Aldrich, TR-1003-G) for 48h. After incubation with the pseudoviral particles, selection with puromycin (final concentration 1 ug/ml, Thermo Fisher Scientific, A1113803) was initiated and continued for 4 days.

Transfection of TCam-2 cell line

TCam-2 cells were seeded the day before transfection using JetPrime transfection reagent (Polyplus, 101000046). For immunofluorescence staining experiments, cells were cultured on 15 mm in diameter round cover glasses in a 12-well culture plates, and transfection mixture was prepared using 0.8 µg of plasmid, 1.6 µl of reagent, and 75 µl of buffer, according to the manufacturer's protocol. Next, cells were transfected using 75 µl of transfection mixture per single well. For other experiments, cells were cultured on 150 mm cell culture dishes and transfection mixture was prepared using 31.45 µg of plasmid, 62.9 µl of reagent, and 1.31 ml of buffer, according to the manufacturer's protocol. Next, cells were transfected using 1.31 ml of transfection mixture per single dish. The complete medium was changed before the transfection procedure. Cells were cultured in a complete medium with transfection mixtures for 2 days prior to downstream experiments. Transfection efficiency was monitored in our study by fluorescence microscopy 48 h after transfection. using transfection control samples (TCam-2 cells expressing GFP-FLAG). Additionally, all samples were validated via WB and qPCR prior to downstream experiments.

Plasmids used: pLV[Exp]-Puro-EF1A>hRBMXL3[NM_001145346.1]/3xFLAG (Vector Builder, Vector ID VB180321-1238ccw), pLV[Exp]-Puro-EF1A>EGFP/3xFLAG (Vector Builder, Vector ID VB180409-1201grs).

qRT-PCR and statistical analysis

RNA was isolated with RNeasy Plus Mini Kit (Qiagen, 74136) or AllPrep Mini Kit (Qiagen, 80004) and residual DNA was removed by TURBO DNA-free™ kit (Thermo Fisher Scientific, AM1907) treatment according to the manufacturer's protocol. One µg of total RNA was reverse transcribed by SuperScript IV

Reverse Transcriptase kit (Thermo Fisher Scientific, 18090050) with the use of random primers (Promega, C1181). For quantitative polymerase chain reaction (qPCR) 10 ng of cDNA was mixed with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25778), 0.3 μ M of each primer (all primer sequences are listed in Supplemental Table S16) and assayed using CFX96 Touch qPCR System (Bio-Rad). All samples were normalized to ACTB and GAPDH according to the geNorm algorithm. Statistical analyses were done using GraphPad Prism (v8.0.1) and performed using ordinary one-way ANOVA non-parametric test with Tukey's multiple comparisons test. Data with statistical significance are as indicated, * p < 0.05, ** p < 0.01, *** p < 0.001.

RNA sequencing and bioinformatic analysis

The RNA-seq experiment was done in duplicate and performed using TCam-2 cells transduced with *RBMXL3-FLAG*, *GFP-FLAG* and non-transduced WT. Cells transduced with *GFP-FLAG* and non-transduced WT TCam-2 cells were used as negative controls. The lentiviral transduction was used to generate stable cell lines in order to ensure relatively sustained and uniform expression of proteins across the cell populations, thereby reducing biological variability and increasing the robustness of transcriptomic analyses. Total RNA was isolated from cells and treated with DNase as it was described above. Libraries were prepared using the TruSeq Stranded mRNA LT kit (Illumina), enabling sequencing of polyadenylated mRNAs. Samples were run on the Illumina NovaSeq6000 platform, 150 bp paired-end reads with at least 30 M reads per sample. Library preparation and sequencing were performed by Macrogen NGS Service (Korea).

RNA-seq data pre-processing was performed by removing low-quality reads ($Q < 30$) and sequencing adapters using Trim Galore (v0.6.10) (<https://github.com/FelixKrueger/TrimGalore>). Further information regarding each specific RNA-seq analysis is provided in the sections below.

Differential expression analysis of RNA-seq data

190 The pre-processed RNA-seq reads were mapped to the human reference genome (GRCh38/hg38, Gencode
191 v44) using STAR (v2.7.11a) [46] (--outSAMtype BAM SortedByCoordinate --quantMode GeneCounts).
192 Sorted BAM files were subsequently indexed using samtools (v1.19.2) [47]. Read counts were then
193 normalized by DESeq2 R package [48]. Lowly expressed genes (with less than 0.1 mean CPM in each
194 condition i.e. RBMXL3, GFP or WT) were excluded from further analysis. Next, we performed the
195 differential expression analysis comparing the 'RBMXL3 vs. GFP' and the 'RBMXL3 vs. WT' conditions
196 using DESeq2. The set of significant differentially expressed genes (DEGs) was determined based on
197 adjusted p-value (FDR) < 0.05 and $|\log_2FC| \geq 1$. As the final list of genes we intersected the significant
198 DEGs of the two comparisons ('RBMXL3 vs. GFP', 'RBMXL3 vs. WT'), and we retrieved only those
199 genes with the same trend (upregulation or downregulation) in both comparisons.

200 The Gene ontology and pathway enrichment analyses were performed with the topGO and clusterProfiler
201 [49] R packages, using a 0.05 threshold on Benjamini-Hochberg (BH) adjusted p-value.

202 **Differential splicing analysis of RNA-seq data**

203 To quantify transcripts expression (TPM) in each condition (RBMXL3, GFP and WT) we employed the
204 Salmon tool (v1.10.2) [50] and aligned the pre-processed RNA-seq reads to the reference transcriptome
205 (Gencode v44). The alternative splicing analysis was then performed using the SUPPA2 tool (v2.3) [51,
206 52]. To identify splicing events a differential splicing analysis was performed setting a threshold of 0.05 on
207 the adjusted p-value and a cutoff of 0.15 on the absolute value of the dPSI. In order to identify potentially
208 more specific splicing events, the final list of differentially spliced genes (DSGs) was prepared by doing an
209 intersection between the significant predicted splicing events from the two comparisons ('RBMXL3 vs.
210 GFP' and 'RBMXL3 vs. WT'). During the SUPPA analysis, the WT and GFP samples were set always as
211 Cond1 and RBMXL3 as Cond2. The Gene ontology and pathway enrichment analyses were performed with
212 the topGO and clusterProfiler [49] R packages, using a 0.05 threshold on BH adjusted p-value.

213 **L1s differential expression of RNA-seq data**

L1s' expression was quantified using the BAM files obtained from the alignment with STAR and the TETranscripts tool (v2.2.3) [53]. Counts were generated using the --mode multi option, and the repetitive elements were annotated using the file GRCh38_GENCODE_rmsk_TE.gtf (https://labshare.cshl.edu/shares/mhammelllab/www-data/TETranscripts/TE_GTF/). Differential expression analysis of repetitive elements was performed using the DESeq2 R package [48] for two comparisons ('RBMXL3 vs. WT' and 'RBMXL3 vs. GFP'). Specifically, only repetitive elements that belong to the L1 family were considered. Significant L1s were retrieved using a threshold of $|\log_2 FC| \geq 0.585$ and the adjusted p-value (FDR) ≤ 0.05 .

RBMXL3 eCLIP

To obtain high-level protein expression, two biological replicates of each sample, TCam-2 cells transfected with *RBMXL3-FLAG* and *GFP-FLAG* (as control), were used in eCLIP-seq performed by Eclipse BioInnovations (USA). Briefly, 48h after transfection of TCam-2 cells on multiple 150 mm cell culture dishes, the cells were crosslinked at 254-nm UV with an energy setting of 400 mJoules/cm² (Analytik Jena, CL-3000). Overall, the cells were lysed, RNase I treated, and protein-RNA complexes were immunoprecipitated with anti-FLAG antibody coupled to magnetic beads. Sequencing was performed as SE72 on the NextSeq platform (Illumina). The eCLIP experiment was performed by Eclipse BioInnovations' standard RBP-eCLIP pipeline as previously described [54]. Raw sequencing reads were trimmed of UMIs and adapters, filtered out of reads that mapped to repeat elements, aligned to the reference genome (hg38) with STAR, and filtered of PCR duplicates.

Clusters (regions within the IP sample that contain a pileup of reads) were identified using CLIPper (<https://github.com/YeoLab/clipper>) [55, 56]. Each cluster was normalized against the paired input sample. Final peaks for each sample were filtered for enrichment over the input ($|\log_2 FC| \geq 2$) and statistical significance (adjusted p-value ≤ 0.01). Replicated peaks were then identified by positionally intersecting peaks of each replicate of the same condition with the intersect command of bedtools[57], with the -s option. Aspecific peaks (i.e. those also found in GFP samples) were then filtered out from RBMXL3_FLAG peaks

239 by positional intersection in the same way. Peaks were annotated using the GenomeRanges [58] and
240 wavClusteR R packages [59], while Gene ontology (GO) and pathway enrichment analyses were performed
241 with the topGO and clusterProfiler packages [49], using a 0.05 threshold on BH adjusted p-value.

242 De novo motif analysis was conducted by using the HOMER findMotifsGenome command [60] and the -
243 len 4,5,6,8,10,12 -size given -rna parameters.

244 Reads mapping to repetitive elements were then used to compute the enrichment of repetitive elements in
245 eCLIP reads with respect to input samples, obtaining fold-enrichment and entropy values as a measure of
246 confidence.

247 **Co-immunoprecipitation coupled with mass spectrometry (Co-IP-MS)**

248 3×10^6 *RBMXL3-FLAG* transfected or wild-type (WT, as negative control) TCam-2 cells were lysed with
249 1ml of RIPA buffer (Sigma-Aldrich, R0278) in the presence of protease inhibitors (Thermo Fisher
250 Scientific, 87786) at 4°C for 30 min, on rotator. Next, lysate was clarified by centrifugation: 4°C, 10 min,
251 11,000 x g. One ml of total protein lysates was incubated with 40µl of anti-FLAG M2 magnetic beads
252 (Sigma-Aldrich, M8823) at 4°C for 3 h with gentle rotation. After IP reaction, beads were washed three
253 times with ice-cold TBS washing buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4) and one time with DPBS
254 (Thermo Fisher Scientific, 14190169). IP reactions were performed in three technical replicates. If samples
255 were sent to mass spectrometry (MS), beads were snap frozen with liquid nitrogen and subsequently
256 proceeded to MS analysis. MS analysis was performed in the Mass Spectrometry Laboratory, IBB PAS.
257 Dried beads were suspended in 50µl of 100mM ABC. Proteins were reduced and alkylated with
258 TCEP/MMTS, digested overnight with trypsin (Promega, V5111) as described previously[61]. Peptide
259 digest was purified with HLB 96-well plates (Waters, WAT058951), vacuum dry then dissolved in 98%
260 water (J.T.Baker 4218-03)/ 2% MeCN (J.T. Baker HPLC JT9012)/ 0.1%TFA (Supelco, 80457) and
261 analyzed by an online LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using a
262 Nano-Acquity (Waters) UPLC system and QExactive Orbitrap mass spectrometer (Thermo Fisher

Scientific), by applying peptides to precolumn (nanoACQUITY UPLC Trapping Column Waters) using water containing 0.1% formic acid as a mobile phase and then transferred to a nano-column (nanoACQUITY UPLC BEH C18 Column (75 μ m inner diameter; 250 mm long; Waters, 186007482)) using an acetonitrile gradient (5–35% AcN in 180 min) in the presence of 0.1% formic acid with the flow rate of 250 nl/min. Three washing runs ensuring lack of cross-contamination from previous samples preceded each analysis. The column outlet was directly coupled to the ion source of the spectrometer working in the regime of data-dependent acquisition MS to MS/MS switch. Peptides were eluted directly to the ion source of the mass spectrometer. Data were acquired in the m/z range of 300–2000. Data were searched using MaxQuant [62] version 1.5.3.0 against a reference human protein sequence database (79 052 entries) downloaded from UniProt. Platform search parameters: a match between runs (match time window, 0,7 min; alignment time, 20 min); enzyme, trypsin/p specific; max missed, 2; minimal peptide length, 7 aa; variable modification, methionine, and proline oxidation; fixed, cysteine alkylation (Methylthio); main search peptide tolerance, 4.5 ppm; protein FDR 1%. Finally, sixteen proteins identified in each RBMXL3 replicate but not present in negative controls were classified as potential RBMXL3 protein partners. Gene ontology analysis among identified proteins was performed in Cytoscape version 3.9.1 using ClueGO plugin version 2.5.9, 4% enrichment cutoff was used meaning that the number of genes should represent at least 4% of total genes in a given process. STRING analysis of protein-protein interaction (PPI) networks was performed using StringApp version 1.7.1, a 0.4 cutoff score was used which means interactions below this value were filtered out.

Immunofluorescence of TCam-2 cells

After two washes in DPBS (Thermo Fisher Scientific, 14190169) TCam-2 cells were fixed with 4% paraformaldehyde (Boster Bio, AR1068) at 4 °C for 15 min. Next, cells were washed 3 times with DPBS, permeabilized with 0.55% Triton X-100 in DPBS for 30 mins at RT and blocked for 1h at RT in blocking solution (10% of suitable serum derived from the host animal species of the secondary antibody, diluted in 0.55% Triton-X100 in DPBS). Cells were incubated overnight at 4 °C with primary antibodies diluted in

the blocking solution. After washing 3 times with DPBS, the cells were incubated with secondary antibodies diluted in 0.55% Triton X-100 in DPBS for 1 h at RT in the dark. After washing 3 times with DPBS, cells were mounted in Fluoroshield with DAPI histology mounting medium (Sigma-Aldrich, F6057).

The following primary antibodies were used: mouse anti-FLAG 1:500 (Sigma-Aldrich, F1804), rabbit anti-DYKDDDDK 1:2000 (GenScript, A00170), rabbit anti-RBMXL3 in serum 1:500 (gift from Prof J. Alcorn [10]), mouse anti-EIF4A3 1:700 (Santa Cruz, sc-365549), rabbit anti-DDX39B 1:50 (Sigma-Aldrich, HPA058450), rabbit anti-DDX39B 1:400 (Proteintech, 14798-1-AP).

The following secondary antibodies were used: goat anti-mouse Alexa Fluor 594 1:500 (Thermo Fisher Scientific, A-11005), goat anti-rabbit Alexa Fluor 488 1:1000 (Abcam, ab150077), donkey anti-mouse Alexa Fluor 488 1:1000 (Thermo Fisher Scientific, A-21202), goat anti-rabbit Alexa Fluor 594 1:500 (Thermo Fisher Scientific, A-11012).

All images were obtained with Leica DMI8 confocal microscope with Leica STELLARIS STED system equipped with Leica HyD photodetectors (Leica Microsystems). We utilized HC PL APO CS2 objective with magnification of 100 and numerical aperture of 1.4 (Leica Microsystems). Data were collected and processed with Leica Application Suite X (LAS X) software (v4.4.0.24861).

Western blot

TCam-2 cells were lysed on a rotator for 30 min in RIPA buffer (Sigma-Aldrich, R0278) supplemented with protease inhibitors (Thermo Fisher Scientific, 87786), followed by three rounds of sonication at 4°C [20 s on, 30 s off] using Bioruptor Pico (Diagenode, Belgium). Next, protein lysates were centrifuged at 15,000 x g for 10 min at 4°C and supernatants were mixed with 4X Laemmli Sample Buffer (Bio-Rad, 1610747) with 340 mM DTT (Thermo Fisher, R0861) and denatured for 10 min at 95°C. Samples were then separated on the 8% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, 1620097). Membranes were blocked with 5% non-fat milk in TBS buffer with 0.1% Tween-20 (TBST) and proceeded to overnight incubation at 4°C with primary antibodies diluted in the blocking solution. Washing steps

312 (3x10min. with TBST) were followed by horseradish peroxidase (HRP)-conjugated secondary antibodies
313 incubation for 1h at RT. Blots were washed twice with T for 10 min and two times in TBS for 10 min. The
314 chemiluminescent signal was detected using the Clarity Western ECL Substrate (Bio-Rad, 1705061) and
315 developed by the ChemiDoc MP Imaging System (Bio-Rad).

316 Primary antibodies used: mouse anti-FLAG 1:1000 (Sigma Aldrich, F1804), mouse anti-ACTB 1:15 000
317 (Santa Cruz Biotechnology, sc-81178), mouse anti-GAPDH 1:10 000 (Santa Cruz, sc-365062), rabbit anti-
318 RBMXL3 in serum 1:2000 (a kind gift from Prof J. Alcorn[10]), rabbit anti-RBMXL2 1:400 (Sigma-
319 Aldrich, HPA051842).

320 Secondary antibodies used: HRP-conjugated anti-mouse 1:10 000 (Santa Cruz Biotechnology, sc-516102),
321 HRP-conjugated anti-rabbit 1:5000 (Abcam, ab6721).

322 Protein fractionation was performed according to the previously described method [63]. Primary antibodies
323 used for Western Blot after protein fractionation experiment were as follows: mouse anti-TUBB 1:5000
324 (Abcam, ab131205), mouse anti-SNRNP70 1:5000 (Santa Cruz, sc-390899), rabbit anti-H3C1 1:5000
325 (Abcam, ab18521).

326 **Immunohistochemistry of testis tissue samples**

327 For the immunohistochemistry staining, commercial human adult testis paraffin sections were used (Zyagen,
328 HP-401). Slides were incubated in 60°C for one hour. Immunohistochemistry (IHC) experiment was
329 performed using specific RBMXL3 antibody in serum (a kind gift from Prof J. Alcorn [10]). To determine
330 the appropriate antibody dilution, eliminate false positive results, and reduce the background staining, a
331 series of positive control reactions were performed using slides with normal testis cells present in section
332 derived from patient with seminoma. The immunohistochemistry procedure was performed using
333 EnVisionFlex+ visualization system kit (Dako, Agilent Technologies, K8002). Slides were then dewaxed
334 and epitopes were unmasked in PT-Link device (Dako, Agilent Technologies) with the use of EnVisionFlex
335 Target Retrieval Solution Low pH (Dako, Agilent Technologies, K8005). After that, the slides were

336 incubated with primary antibody (dilution 1:2000; incubation 20 min at RT). The antigen-antibody complex
337 was detected using EnVision FLEX+ Rabbit LINKER (Dako, Agilent Technologies, K8009) for 20 min at
338 RT and localized using 3-3'diaminobenzidine (DAB) as chromogen. In the end, the tissue sections were
339 counterstained with hematoxylin, dehydrated in ethanol, incubated in a series of xylenes, and cover-slipped
340 using mounting medium (Dako, Agilent Technologies). The protein expression was evaluated with the use
341 of light microscope (Leica DMI8).

342 **LINE-1 retrotransposition assay**

343 The assays were performed as described [64]. Briefly, 293T cells were seeded at 150,000 per well into 12-
344 well plates (Greiner Bio-one) in 1ml DMEM high-glucose medium (Invitrogen) without antibiotics. Next
345 day the cells were transfected with 750ng 99_PUR_RPS_EGFP and 200ng of either RBMXL3 encoding
346 plasmid (pLV[Exp]-Puro-EF1A>hRBMXL3[NM_001145346.1]/3xFLAG (Vector Builder, Vector ID
347 VB180321-1238ccw)) or pKK_TEV_MBP (pZW165) [65, 66] using 2,5 µl Lipofectamine2000 (Thermo
348 Fisher Scientific) in a total of 100 µl OPTI-MEM (Thermo Fisher Scientific). Next day the medium was
349 exchanged for one containing penicillin/streptomycin (Sigma) and 1µg/ml puromycin (Invivogen) to select
350 cells which received the 99_PUR_RPS_EGFP plasmid. After 2 days medium was exchanged. After a total
351 time of 4 days of selection the cells were detached by treatment with trypsin and analyzed by flow cytometry
352 using Attune NxT flow cytometer (A24861; Thermo Fisher Scientific). The cells in which retrotransposition
353 had occurred (0,01-2,2% of the entire population depending on the test condition) expressed EGFP and were
354 identified by excitation with a blue laser ($\lambda=488\text{nm}$) and detection using a bandpass filter (BL1
355 $\lambda=530/30\text{nm}$). Other cells did not express EGFP. The live cell population was set by gating FSC and SSC
356 parameters (gate 1), followed by subgating single cells ('singlets') using the FSC-A (area) versus FSC-H
357 (peak height) parameters. Control non-transfected (NT) cells were analyzed to set reference background
358 EGFP fluorescence (gating <0,005% of the NT cells). A control transfection with EGFP (pKK_TEV_EGFP,
359 pZW171) and mCherry (pKK_TEV_mCherry, pZW174) [65] encoding plasmids (125ng each per well) and
360 the RBMXL3-FLAG and MBP encoding plasmids were done to test whether RBMXL3-FLAG expression

361 influences EGFP and/or mCherry expression. mCherry was analyzed by excitation with a yellow laser (λ
362 =561nm) and detection with the band pass filter (YL2 λ =620/15nm). No cross-talk with EGFP is observed.

363 Pools of cells were sampled and used to analyze expression of the RBMXL3-FLAG and MBP proteins. The
364 cell lysates were prepared as described [66] and separated onto Novex 4-20% gradient Tris-glycine gels
365 (Thermo Fisher Scientific), wet-transferred on Protran nitrocellulose membrane (Amersham, 0,45 μ m) in a
366 buffer supplemented with methanol to 20% [66]. Membranes were stained with ponceau S solution, cut into
367 3 pieces (upper for staining RBMXL3-FLAG, middle for Lamin B, and lower for MBP or EGFP), blocked
368 in 5% low-fat milk in TBST20 and probed with primary antibodies against either FLAG (M2, Merck,
369 F3165), Lamin B (ProteinTech, 12987-1-AP), MBP (NEB, 8032S), or EGFP (control transfection to assess
370 the impact of RBMXL3-FLAG expression on EGFP production; antibodies sc-9996 from Santa Cruz) in
371 1:1000 (FLAG), and 1:4000 (other Abs) dilutions in 5% skimmed milk in TBST20 for 16hr at 6°C.
372 Secondary HRP-coupled antibodies (from goat) were against rabbit Abs (111-035-144) or mouse Abs (115-
373 035-146) (both Jackson Immunoscience (0,8mg/ml)), used in 1:20,000 dilutions in milk for 2hr at RT.
374 Signals were obtained by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad) and
375 photographed using Uvitec Q9 Alliance CCD camera.

376 **Results**

377 **RBMXL3 is a nuclear protein enriched in the chromatin fraction**

378 Due to limited information about RBMXL3 function and lack of available models with native *RBMXL3*
379 expression, we generated a cellular model with transient overexpression of this protein using human
380 seminoma cell line. We transfected TCam-2 wild-type (WT) cells with vector encoding either *RBMXL3*
381 tagged with FLAG or *GFP* tagged with FLAG (used as a control). The expression of *RBMXL3-FLAG* was
382 confirmed on both RNA and protein levels (Fig. 1A,B). To confirm that the signal observed with the anti-
383 FLAG antibody is a fusion with RBMXL3 we used antibodies specific to: (i) RBMXL3 (kindly gifted by
384 Prof. J. Alcorn [10]); and (ii) RBMXL2, which according to the Human Protein Atlas (HPA) detects not

385 only RBMXL2 but also the RBMXL3 protein (Fig. 1C; Supplemental Fig. S1). RBMXL3-FLAG band
386 appeared at ~140 kDa and larger than expected (~115 kDa plus ~3 kDa 3xFLAG_tag). This is in line with
387 a previous study and may result from posttranslational modifications [10]. To investigate RBMXL3
388 subcellular localization, the cells overexpressing it were co-stained for RBMXL3 and FLAG. Confocal
389 microscopy showed strong signal co-localization in cell nucleus (Fig. 1D).

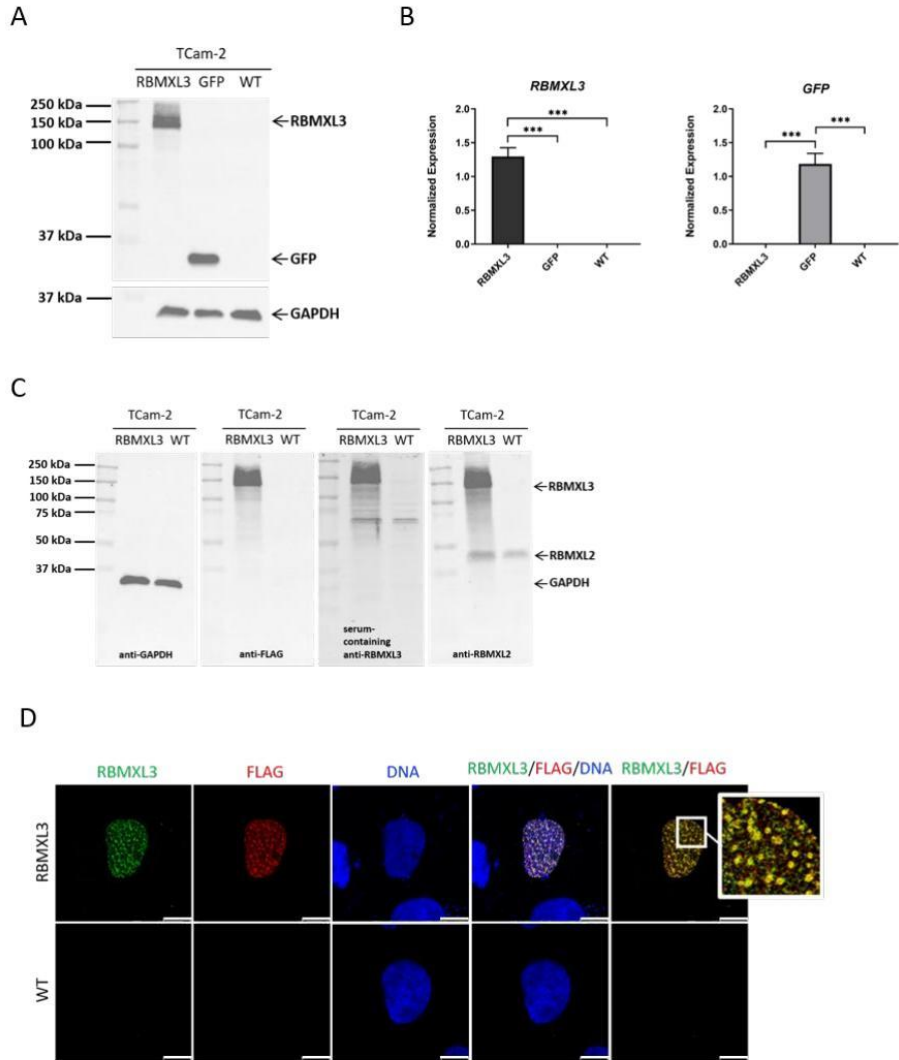
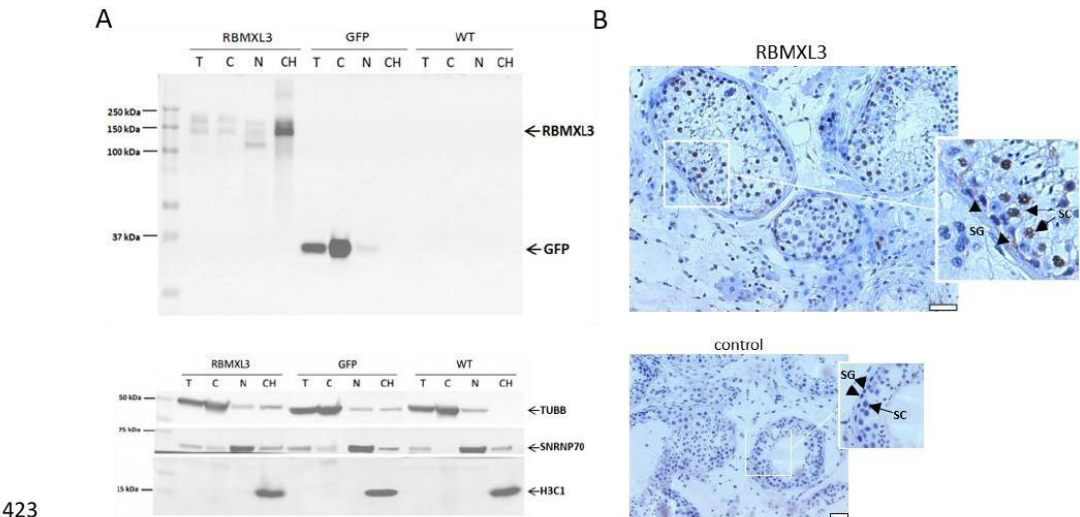


Figure 1. Transient overexpression of *RBMXL3* in TCam-2 cells. (A) Western Blot of transfected (RBMXL3, GFP) and wild-type (WT) TCam-2 cells using anti-FLAG antibody which detects the RBMXL3 and GFP, tagged with a FLAG tag. GAPDH served as a loading control. (B) Expression of *RBMXL3-FLAG* and *GFP-FLAG* in TCam-2 cells on mRNA level, assayed 48h after transfection. Normalized to *GAPDH* and *ACTB*. Ordinary one-way ANOVA non-parametric test with Tukey's multiple comparisons test was used for statistics. Error bars represent standard deviation (SD) with $n = 3$, *** $p < 0.001$. (C) Western Blot of TCam-2 cells detecting RBMXL3 protein with the use of anti-FLAG, anti-RBMXL3, and anti-RBMXL2 antibodies. GAPDH served as a loading control. The additional band appearing between 75 and 50 kDa was derived from serum which was present in one of two batches of RBMXL3 specific antibody used (Supplemental Fig. S1). (D) Co-immunofluorescence staining using anti-FLAG and specific anti-RBMXL3 antibodies in the wild-type (WT) and *RBMXL3*-expressing TCam-2 cells. The zoom window illustrates signal colocalization of RBMXL3 and FLAG. Scale bar 11.6 μm .

To further confirm RBMXL3 subcellular localization, TCam-2 cells overexpressing the protein of interest were subjected to cell fractionation. Visible enrichment of FLAG-tagged RBMXL3 was detected in the chromatin fraction (Fig. 2A). This result encouraged us to investigate whether RBMXL3 binds DNA by performing chromatin immunoprecipitation sequencing (ChIP-seq). However, the FLAG ChIP-seq did not detect reproducible DNA enrichment relative to the GFP control (unpublished data). This outcome may be influenced by technical constraints, as well as the possibility that RBMXL3 associates with chromatin indirectly or transiently, arguing against stable direct DNA binding.

Finally, to confront our observations in TCam-2 cells with *in vivo* RBMXL3 distribution in human testis we performed immunohistochemical staining for RBMXL3 using tissue section of a healthy adult human testis (Fig. 2B). The result was in line with transcriptomic data from the Human Protein Atlas according to which the highest *RBMXL3* mRNA level is detected in spermatogonia and spermatocytes. It is also worth noting that the localization of RBMXL3 protein, although nuclear for spermatocytes, seems to be rather

416 cytoplasmic in the case of spermatogonia. Importantly, previous data from the HPA did not provide exact
417 RBMXL3 protein staining due to the use of an RBMXL2 antibody (with positive signal in spermatogonia,
418 spermatocytes, and spermatids), as the antibody against RBMXL3 is not yet commercially available. To our
419 knowledge, this is the first demonstration of immunohistochemistry-based localization of RBMXL3 using
420 an RBMXL3-specific antibody. In summary, the presence of RBMXL3 in the human testis supports its
421 potential role in the spermatogenesis process, during which precise alternative splicing regulation and
422 genome integrity maintenance are crucial and remain the major challenges.



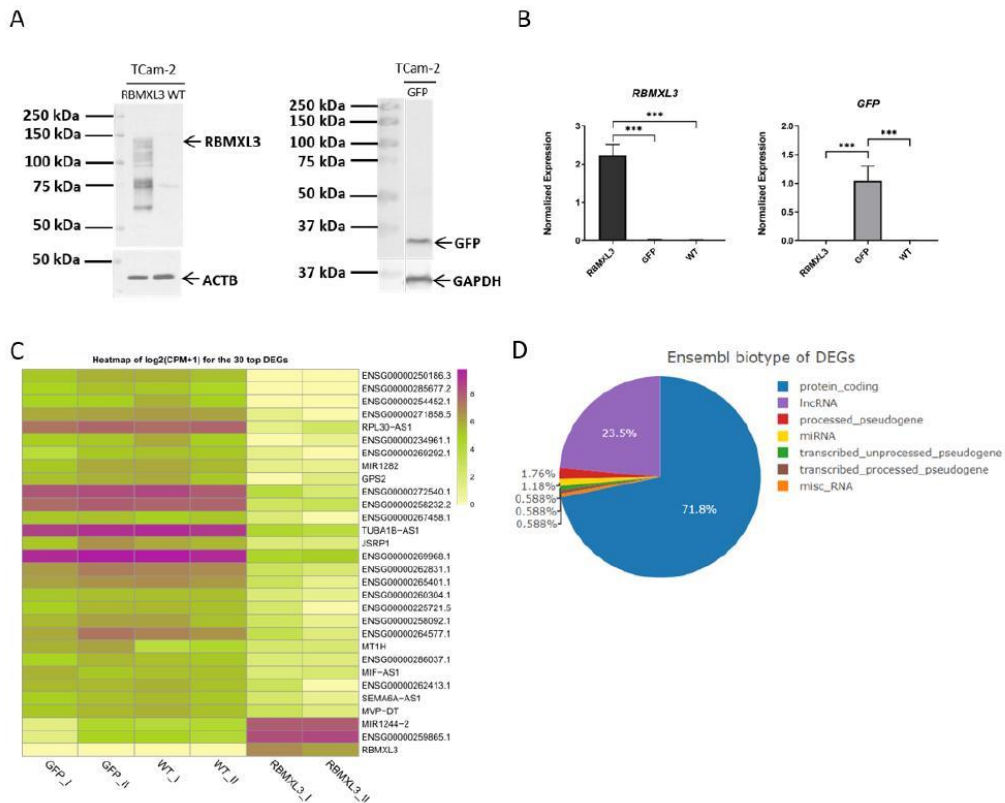
424 Figure 2. RBMXL3 enrichment in the chromatin fraction of TCam-2 cells. (A) Western Blot analysis using
425 anti-FLAG antibody performed on total (T), cytoplasmic (C), nuclear (N), and chromatin (CH) protein
426 fractions obtained from TCam-2 cells (overexpressing RBMXL3 and two controls: GFP and WT). The
427 subcellular fractions were evaluated using the following markers: tubulin beta class I (TUBB) as a
428 cytoplasmic marker, small nuclear ribonucleoprotein U1 subunit 70 (SNRNP70) as a nuclear marker, and
429 H3 clustered histone 1 (H3C1) as a chromatin marker. In each fraction, there is visible enrichment of the
430 respective marker. (B) Immunohistochemistry of RBMXL3 using specific RBMXL3 antibody in the normal

431 adult human testis. Negative control is presented below. Scale bar 50 μ m. SG, spermatogonia (arrowheads);
432 SC, spermatocytes (arrows).

433

434 **RBMXL3 influences gene expression, mRNA splicing, and interacts with protein factors involved in**
435 **RNA metabolism**

436 To investigate how RBMXL3 protein affects the human transcriptome we first performed RNA-sequencing
437 using RNA from TCam-2 cells expressing either *RBMXL3* or *GFP* (both modified with lentivirus) as well
438 as from WT cells (Fig. 3A,B; Supplemental Table S1; Supplemental Fig. S2A). Since both the GFP and WT
439 TCam-2 cells were used as controls we focused on genes that were the differentially expressed genes
440 (DEGs) in both comparisons i.e. ‘RBMXL3 vs. GFP’ and ‘RBMXL3 vs. WT’ (see Materials and Methods
441 section for more details). Using the cutoff $|\log_2FC| \geq 1$ and $FDR < 0.05$ we identified 96 up-regulated and
442 74 down-regulated genes after *RBMXL3* overexpression (Fig. 3C; Supplemental Table S2, Supplemental
443 Fig. S2B,C). The transcriptome analysis detected both protein-coding and long non-coding RNA (lncRNA)
444 genes to be differentially expressed (Fig. 3D). Among DEGs there were transcripts coding for proteins that
445 contribute to the structural integrity of the extracellular matrix (e.g. COL3A1, COL7A1, COL9A3, FBN2,
446 LUM, THBS1, TNXB) which might be important in the context of differentiating germ cell migration during
447 spermatogenesis. Interestingly, after lentiviral transduction we observed differences in the RBMXL3 band
448 pattern on WB compared with that observed after transient transfection (Fig. 1A and 3A). Although the
449 underlying mechanism is difficult to determine, this effect may result from enhanced protein degradation in
450 the stable cell line. The lower-molecular-weight bands likely represent degradation products and are
451 detectable only in cells with long-term *RBMXL3* expression and prolonged RBMXL3 activity.



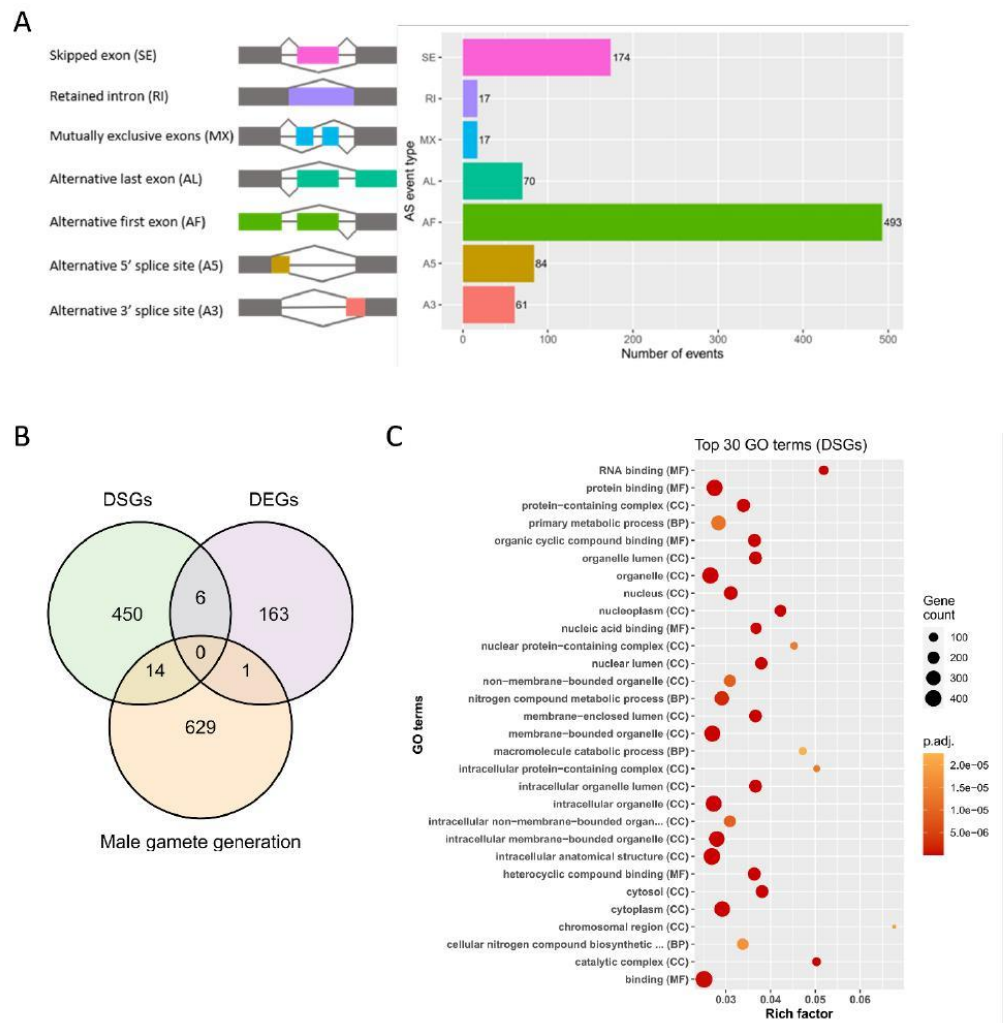
452

453 Figure 3. RBMXL3 drives transcriptome changes in human cells. (A) Western Blot of TCam-2 cells with
454 stable expression of *RBMXL3-FLAG* and *GFP-FLAG* as well as wild type (WT) using anti-FLAG antibody.
455 GAPDH and ACTB were used as loading controls. (B) The expression level of *RBMXL3-FLAG* and *GFP-FLAG*
456 *FLAG* in stable TCam-2 cell lines investigated at RNA level by RT-qPCR. Normalization was done to
457 *GAPDH* and *ACTB*. Ordinary one-way ANOVA non-parametric test with Tukey's multiple comparisons
458 test was used for statistics. Error bars represent standard deviation (SD) with n = 3, ***p < 0.001. (C)
459 Heatmap of log2(CPM+1) for the top 30 differentially expressed genes (DEGs) based on RNA-seq analysis
460 of TCam-2 cells, either WT or overexpressing *RBMXL3-FLAG* or *GFP-FLAG* ($|\log_2FC| \geq 1$, FDR < 0.05).
461 The top 30 DEGs presented on the heatmap were selected based on the highest $|\log_2FC|$ and the lowest FDR

462 values derived from the ‘RBMXL3 vs. GFP’ comparison (Supplemental Table S1,2). (D) The Ensembl
463 biotype of DEGs based on RNA-seq analysis of the TCam-2 cells.

464

465 Given the spermatogenesis-specific role of RBMXL2 in splicing regulation in mice [13] and the sequence
466 similarity between human RBMXL2 and RBMXL3 (85% and 56% at the DNA and protein levels,
467 respectively), we tested whether RBMXL3 can also play a role in this process. We analyzed our RNA-seq
468 data using SUPPA2 software to identify differential alternative splicing (AS) events. We focused on
469 significant predicted splicing events present in both comparisons (‘RBMXL3 vs. GFP’ and ‘RBMXL3 vs.
470 WT’, see Materials and Methods section for more details) and identified 916 splicing events in 470
471 transcripts ($p \leq 0.05$ and $|\Delta PSI| \geq 0.15$) upon expression of *RBMXL3-FLAG* (Supplemental Table S3,4). The
472 majority of AS events were alternative first exon (493) and exon skipping (174) (Fig. 4A). Next, to extract
473 the transcripts associated with human male gamete generation we utilized the genes list annotated to the GO
474 term (GO:0048232; male gamete generation) and compared them with differentially spliced genes (DSGs)
475 and DEGs. This approach led us to identify 14 genes: *DLD*, *YY1*, *CCNY*, *ATN1*, *GORASP2*, *GAMT*, *SKIL*,
476 *WDR33*, *SMAD4*, *GJA1*, *CEP57*, *BCL2L1*, *DHX36* and *LRRK2* that potentially underwent AS due to
477 *RBMXL3* expression (Fig. 4B; Supplemental Table S5). Gene ontology (GO) [67, 68] enrichment analysis
478 of DSGs showed that they are associated with terms such as mitotic cell cycle process, chromosome
479 organization, spindle, but also with regulation of RNA splicing, spliceosomal complex, transcription factor
480 binding, ncRNA metabolic process and translation, to name a few (Fig. 4C, for the complete list of GO
481 terms see Supplemental Table S6). To further explore the RBMXL3 function we also performed Reactome
482 pathway analysis of DSGs which underlined terms like Nonsense-Mediated Decay (NMD), mRNA splicing,
483 translation, and M phase, among others (Supplemental Fig. S3, for the complete list of significant Reactome
484 terms see Supplemental Table S6). This data indicates that RBMXL3 might play a role in the alternative
485 splicing of key genes, important for spermatogenesis and beyond.



486

487 Figure 4. Characterization of differentially spliced genes (DSGs) and alternative splicing (AS) events upon
488 *RBML3* expression. (A) SUPPA2 analysis of RNA-seq data from TCam-2 cells identified 916 differential
489 alternative splicing events of different types ($p \leq 0.05$ and $|dPSI| \geq 0.15$) (Supplemental Table S4). (B) Venn
490 diagram illustrating the total number of DSGs and DEGs based on RNA-seq data in comparison to the list
491 of genes associated with human male gamete generation (Supplemental Table S5). The male gamete
492 generation genes list annotated to GO:0048232 was retrieved from AMIGO2 (accession date 2024-02-

02)[115]. Intersection was prepared using Ensembl IDs. (C) Gene ontology (GO) enrichment analysis of differentially spliced genes (DSGs) due to overexpression of the RBMXL3 protein in TCam-2 cells (adjusted p-value < 0.05). For the complete list of GO terms see Supplemental Table S6. The abbreviations in the brackets represent the three GO categories: biological process (BP), molecular function (MF), and cellular component (CC). The rich factor represents the ratio of the number of genes from the input list associated with a given GO term (significant) to the total number of genes annotated to that term (annotated).

To deepen our understanding of the molecular protein interaction network of RBMXL3 we performed RBMXL3-FLAG co-immunoprecipitation followed by mass spectrometry analysis (Co-IP-MS) to identify RBMXL3-interacting proteins (Fig. 5A). This approach allowed us to select 16 potential interactors (Supplemental Table S7). Among them were known splicing factors (SFs) such as EIF4A3, a core component of the splicing-dependent multiprotein exon junction complex (EJC) and translation initiation factor and DDX39B, a protein involved in the processing and nuclear export of mRNA. Some of the potential interactors were also linked to processes such as rRNA biogenesis (e.g. NOLC1) and protein degradation (e.g. PSMD2). Furthermore, analysis performed with the STRING database indicated that most proteins identified by Co-IP-MS create a functional network involved in the regulation of gene expression (Fig. 5B; Supplemental Table S8). In addition, GO annotation performed on the Co-IP-MS detected proteins highlighted terms such as: negative regulation of RNA binding, U4 snRNP, U2-type prespliceosome assembly, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, translation release factor activity, codon specific and cytoplasmic translational termination (Fig. 5C; Supplemental Table S9). Moreover, according to the original STRING database network for RBMXL3, the majority of predicted interactors are small nuclear ribonucleoproteins (snRNPs), which are core components of the spliceosome (Supplemental Fig. S4A), further supporting the involvement of RBMXL3 in RNA splicing. However, it is worth noting that 7 out of 10 predicted interactors are shared between RBMXL3 and RBMXL2, which may arise also from sequence similarity between these proteins.

For further validation, two interactors (involved in splicing) were chosen. We used immunofluorescence co-staining and confocal imaging of the two selected proteins, EIF4A3 and DDX39B also known as UAP56 (Supplemental Fig. S4B,C,D). Although signal co-localization between RBMXL3 and both interactors was not ubiquitous, we observed several co-localization events. It is worth noting that two DDX39B antibodies (both polyclonal, coming from different manufacturers) had different molecular patterns of staining.

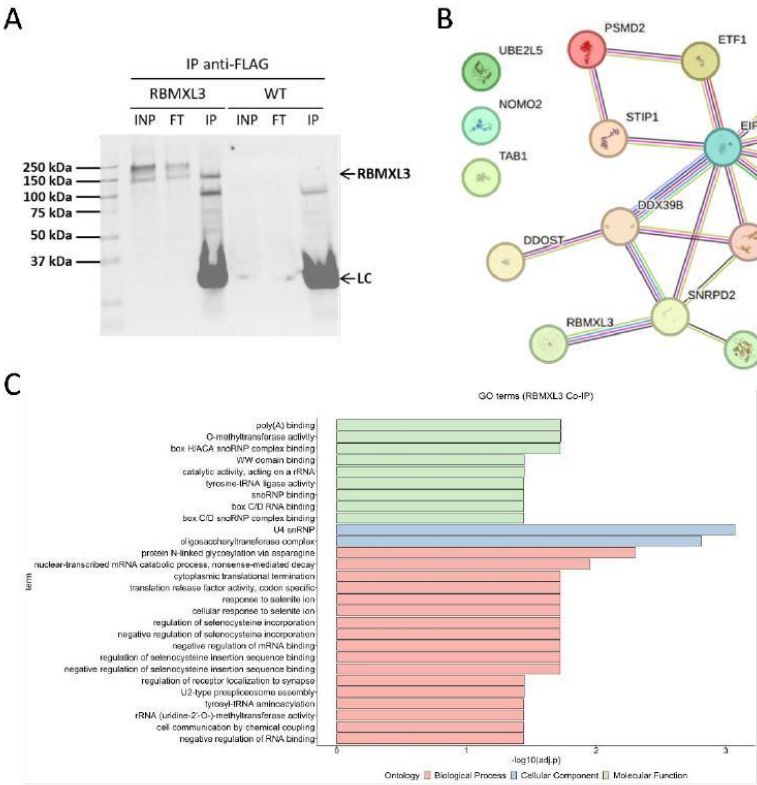


Figure 5. Identification of RBMXL3 protein interactors by Co-IP-MS. (A) Representative Western blot after immunoprecipitation using magnetic beads coated with anti-FLAG M2 antibody. RBMXL3-FLAG in the input (INP), flow-through (FT), and immunoprecipitated (IP) fractions was detected using FLAG antibody. LC - light chains of antibody. (B) Protein-protein interaction (PPI) network created among 16 identified potential protein interactors and RBMXL3 based on the STRING database (Supplemental Table S7,8). The

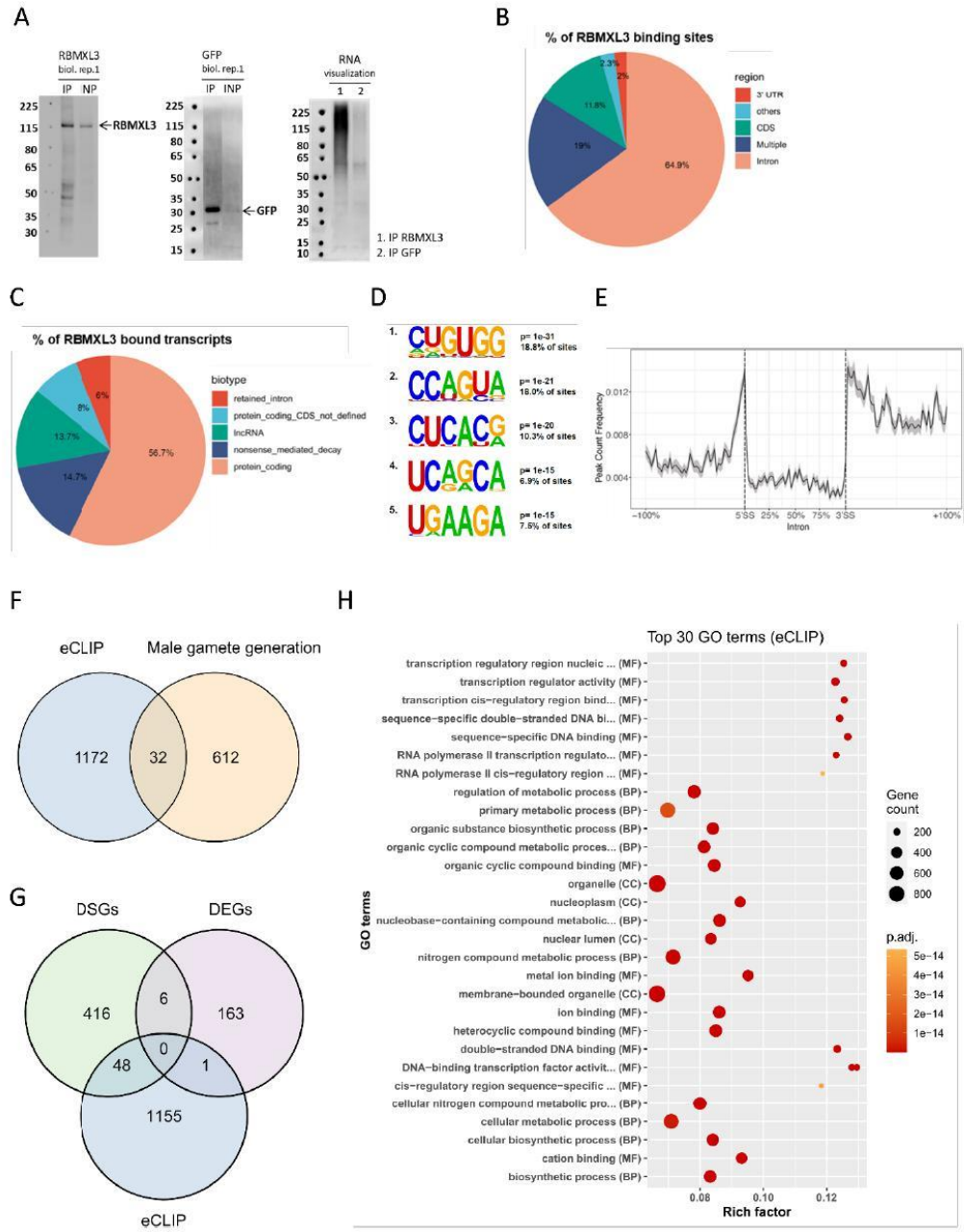
529 0.4 cutoff score was used to filter out interactions. (C) GO analysis of proteins identified by Co-IP-MS. GO
530 terms with adjusted p-value ≤ 0.05 were considered significant (Supplemental Table S9). The colors
531 represents the three GO categories (green: MF, blue: CC, red: BP).

532

533 **Identification of RBMXL3 targets by eCLIP**

534 In order to identify RNAs directly interacting with RBMXL3 we performed enhanced crosslinking and
535 immunoprecipitation (eCLIP) followed by high-throughput sequencing [54]. Using FLAG antibody, two
536 biological replicates of each sample, Tcam-2 cells transfected with *RBMXL3-FLAG* or *GFP-FLAG* (as
537 control) were subjected to eCLIP (Fig. 6A). For comprehensive analysis, we focused on binding sites that
538 intersected between the two 'RBMXL3' replicates and were not detected in any of the 'GFP' replicates. We
539 identified more than 1,200 annotated transcripts with over 6,900 RBMXL3-binding peaks (Supplemental
540 Table S10,11). As the RBMXL3-binding site position may be different within the same transcript (e.g.
541 *MSH3*) between the two replicates and thus do not meet the positional intersection condition we prepared
542 an extended list of transcripts intersected not only at the positional level but also at the transcripts level
543 (Supplemental Table S11). Distribution of enriched peaks in the RBMXL3-FLAG IP sample showed that
544 the majority of RBMXL3 binding sites are present within introns, suggesting the role of RBMXL3 in
545 splicing regulation (Fig. 6B). Moreover, eCLIP data suggest that RBMXL3 interacts with both protein-
546 coding and long non-coding RNAs (lncRNAs) (Fig. 6C). Enriched motif analysis revealed that the top two
547 most enriched represent the 5' and 3' splicing signal consensus (Fig. 6D). We have also analyzed the profile
548 of eCLIP peaks binding to the vicinity of the RNA splice sites and show that there is a visible enrichment
549 near splice sites, further supporting the role of RBMXL3 in splicing (Fig. 6E). Analysis of eCLIP peaks
550 coverage on an mRNA model showed a strong enrichment of RBMXL3 binding observed also around the
551 5'UTR region and start codon (Supplemental Fig. S5A). Then, we again employed the male gamete
552 generation genes list by comparing it with transcripts bound by RBMXL3 and identified 32 genes, including
553 *BRCA2*, *CLOCK*, *MSH6*, *TSGA10*, *ATRX*, *MLH3*, *KNL1*, *SETX*, *JAM3*, *NECTIN2*, *TCFL5*, *IGF2R*, *TDRD5*,

554 and *CDYL*, among others (Fig. 6F, Supplemental Table S12). Moreover, we investigated how many
 555 transcripts of the differentially spliced genes (DSGs) were directly bound by RBMXL3 based on eCLIP
 556 data and identified 48 transcripts that might be direct targets of RBMXL3 protein, including *HSPH1*, *CENPJ*,
 557 *HDAC2*, *FTX*, *TFDP2*, *PLEKHA5*, *CBFB*, *RIF1*, *E2F5*, *ZNF131*, *SP4*, and *CEP57*, among many others
 558 (Fig. 6G; Supplemental Table S13). In this group of genes, only one (*CEP57*) was assigned to the male
 559 gamete generation GO term. However, it is important to emphasize that many genes important for this
 560 process in humans still remain to be discovered and properly annotated. Functional characterization of
 561 identified RNAs using GO revealed that targets are involved in processes such as sister chromatid
 562 segregation, nuclear chromosome segregation, spindle organization, mitotic cell cycle process, and stem
 563 cell population maintenance (Fig. 6H, for the complete list of GO terms see Supplemental Table S14). These
 564 data indicate that RBMXL3 may play a crucial role in human spermatogenesis, particularly in germ cell
 565 differentiation, regulating the processes important for stem cells biology. Other significantly enriched GO
 566 terms among RBMXL3 RNA targets included the transcription regulator complex, regulation of RNA
 567 splicing, DNA-binding transcription factor activity, protein methylation, regulation of protein deacetylation
 568 and epigenetic regulation of gene expression. To further understand how the functions of RBMXL3-bound
 569 RNAs may relate to spermatogenesis we performed Reactome pathway analysis. This identified terms like
 570 centrosome maturation, separation of sister chromatids, and recruitment of mitotic centrosome proteins and
 571 complexes, pathways which are crucial to ensure gamete quality (Supplemental Fig. S5B, for the complete
 572 list of significant Reactome pathways see Supplemental Table S14). It is worth noting that among the
 573 detected RBMXL3 targets we found the RBMXL3 mRNA, suggesting that the RBMXL3 binds its own
 574 transcript, as several RNA-binding proteins (RBPs) do. Summarizing, by using eCLIP-seq we confirmed
 575 that RBMXL3 is a functional RBP and identified its RNA targets. Our data indicate that the primate-specific
 576 RBMXL3 protein exerts a significant impact on human cell transcriptome.



577

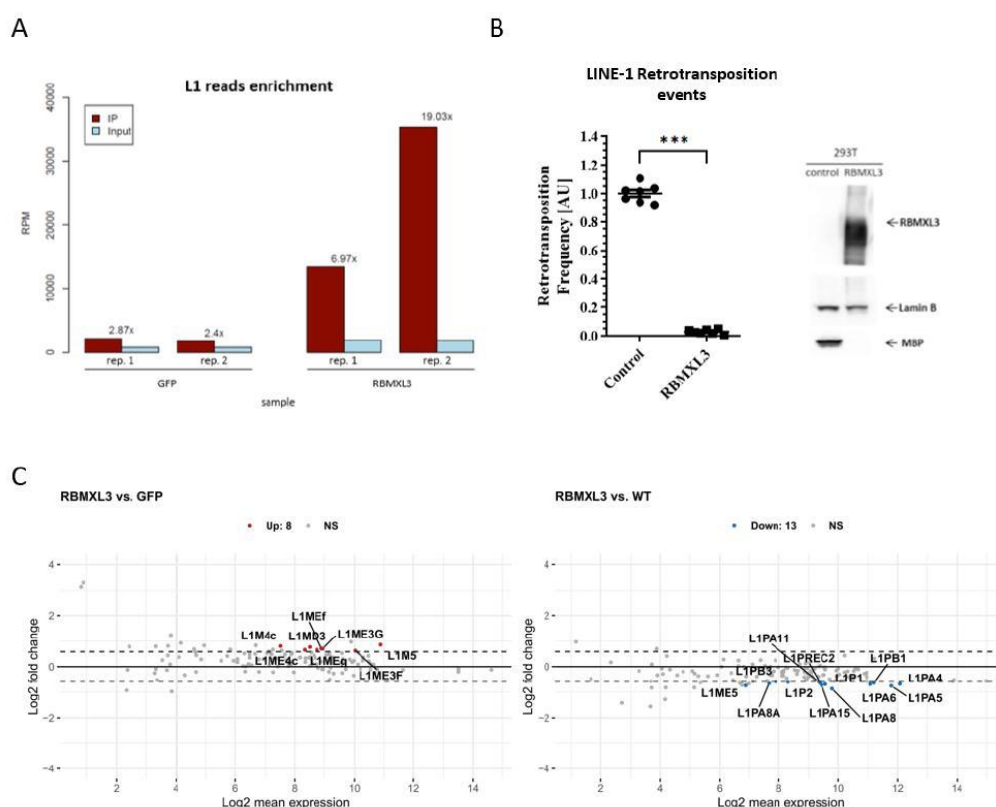
Figure 6. Global profiling of RNA target sites directly interacting with RBMXL3 by eCLIP. (A) Immunoblotting of RBMXL3-FLAG using anti-FLAG antibody (left) and visualization of immunoprecipitated RNA (right) in TCam-2 cells. (B) Distribution of the RBMXL3 binding sites in the different regions. All eCLIP peaks that map to a specific genomic location with multiple, different transcripts (thus possessing the same or different biotypes) are within the "Multiple" category. (C) The Ensembl biotype of the RBMXL3-bound transcripts in TCam-2 cells. (D) Significant binding motifs found in at least 5% of binding sites, sorted by p-value and identified by HOMER *de novo* motif analysis. (E) Visualization of the RBMXL3 RNA-binding sites with eCLIP peaks coverage in the vicinity of the RNA splice sites. (F) Venn diagram of transcripts bound by RBMXL3 and genes associated with human male gamete generation. The male gamete generation genes list annotated to GO:0048232 was retrieved from AMIGO2 (accession date 2024-02-02) [115] (Supplemental Table S12). Intersection was prepared using Ensembl IDs. (G) Venn diagram illustrating the total number of DSGs and DEGs based on RNA-seq data in comparison to the list of transcripts detected in RBMXL3 eCLIP thus directly bound by RBMXL3 (Supplemental Table S13). Intersection was prepared using Ensembl IDs. (H) GO analysis for the transcripts bound by RBMXL3 based on eCLIP data. For the complete list of significant GO terms see Supplemental Table S14 (adjusted p-value < 0.05). The rich factor represents the ratio of the number of genes from the input list associated with a given GO term (significant) to the total number of genes annotated to that term (annotated).

596

597 **The primate-specific RBMXL3 protein suppresses human LINE-1 retrotransposition**

598 We analyzed the eCLIP data in regards to repetitive elements and showed that RBMXL3 binds sequences
599 belonging to the L1 (Long Interspersed Nuclear Element-1 also known as LINE-1) family of
600 retrotransposons (Fig. 7A). This result encouraged us to investigate the effect of RBMXL3 on L1
601 retrotransposition by performing a plasmid-based L1 retrotransposition assay using HEK293T cells (see
602 Materials and Methods section for more details). We observed that the RBMXL3 protein heavily reduces

603 L1 retrotransposition (Fig. 7B) [66] in this assay, indicating that it may play a protective role against LINE-
604 1 mobilization, which is especially important in the context of germline and spermatogenesis [69]. To
605 confirm that this strong effect of RBMXL3 on L1 retrotransposition is a true positive result we performed
606 a series of control experiments. The lack of statistically significant changes in the percentage of transfected
607 cells indicated that RBMXL3 plasmid and expression do not significantly affect the transfection process or
608 generally reduce EGFP and mCherry protein production (Supplemental Fig. S6A,B). In fact, we detected
609 higher EGFP and mCherry intensities in the RBMXL3 expression condition which currently cannot be
610 explained. To complement this result, we also tested whether RBMXL3 affects endogenous L1 mRNA
611 levels. Based on our RNA-seq data, we analyzed L1s expression in Tcam-2 cells expressing *RBMXL3*,
612 using *GFP*-expressing and WT cells as controls (Fig. 7C; Supplemental Table S15). Our analysis showed
613 that some L1s are differentially expressed in sample with RBMXL3, but no subfamily displayed a similar
614 up- or down-regulation pattern between the two comparisons when using a threshold of $|\log_2FC| \geq 0.585$,
615 $FDR \leq 0.05$. We did not observe significant changes in the expression of endogenous L1HS class (also
616 called L1PA1 and Ta) [70–72] of retrotransposition competent L1s in humans (of which the L1RP element
617 used in the plasmid-based assay is a representative). Overall, our data provide evidence that RBMXL3
618 participates in the effective silencing of transposable elements (TEs), specifically L1, which is important
619 for safeguarding spermatogenesis.



620

621 Figure 7. RBMXL3 binds sequences belonging to L1 family and heavily reduces L1 retrotransposition. (A)

622 L1 reads enrichment in IP vs. Input sample based on repetitive elements analysis of the eCLIP data indicates

623 that RBMXL3 protein binds sequences belonging to the L1 family. (B) Effect of overexpression of *RBMXL3*

624 and *MBP* (as control) on L1 retrotransposition in HEK293T cells using L1 retrotransposition reporter assay.

625 The results of independent experiments were normalized relative to the control (MBP). *RBMXL3* expression

626 induces a ~15-20 fold reduction in L1 retrotransposition; each point represents a biological replicate,

627 *** $p < 0.001$ (left). Western blot analysis using anti-FLAG, anti-Lamin B and anti-MBP antibodies to detect

628 respectively: RBMXL3, Lamin B (loading control), and MBP protein (right).

629 (C) MA plots showing the L1s from RNA-seq analysis using TETranscripts package (Supplemental Table S15). Differentially up-

630 regulated L1s ($|FC| \geq 1.5$, $FDR \leq 0.05$) are labeled red, whereas differentially down-regulated L1 ($|FC| \leq -$
631 1.5 , $FDR \leq 0.05$) are labeled blue. Horizontal dashed lines indicate $\log_2(1.5)$ or $-\log_2(1.5)$.

632

633 Discussion

634 This study presents novel findings on the primate-specific *RBMXL3* and its critical role in RNA metabolism.
635 We confirmed the nuclear localization of *RBMXL3* protein in TCam-2 cell line and its presence in
636 spermatogonia and spermatocytes within the human testis. For the first time, we demonstrate that *RBMXL3*
637 acts as an upstream regulator of RNA metabolism by binding to a wide range of RNA molecules in human
638 TCam-2 cells. Our data reveal that *RBMXL3* drives transcriptomic changes by influencing both gene
639 expression as well as alternative splicing of multiple transcripts. By using LINE-1 retrotransposition reporter
640 assays we discovered that *RBMXL3* suppresses L1 retrotransposition in human cells. Although the TCam-
641 2 cell line serves as an important *in vitro* model of human germ cell biology [42, 73], it does not recapitulate
642 the complex differentiation program of germ cells within the human testis. Therefore, we interpreted the
643 TCam-2 data primarily as indicative in the context of human spermatogenesis and focused on gene/protein
644 targets that are expected to be present in germ cells where *RBMXL3* is expressed *in vivo*.

645 Alternative splicing regulation has been shown to be pivotal in the context of differentiation processes [74]
646 and is crucial for proper spermatogenesis [13, 42, 75–77]. We and others identified mutations in *RBMXL3*
647 gene found in non-obstructive azoospermia (NOA) patients with Sertoli cell-only syndrome (SCOS) [5] and
648 recently in patients with asthenozoospermia. By using modified TCam-2 cells as a model of human
649 primordial germ cells we demonstrate the role of *RBMXL3* in regulation of transcriptome. Our analyses led
650 to identification of potential *RBMXL3* target transcripts some of which might undergo AS depending on
651 *RBMXL3* presence. Among potential targets is *FOS* (also known as *c-FOS*), a protooncogene involved in
652 cell proliferation [78], which was highly enhanced upon *RBMXL3* overexpression. *FOS* protein is present
653 in the human testis and has been linked to spermatogenesis [79]. It is decreased in the testis of men with

NOA. Furthermore, among the genes detected as potentially differentially spliced in TCam-2 cells upon RBMXL3 overexpression, we found the *YY1* transcription factor. This gene has been shown to influence the heterochromatin in mouse spermatocytes which affects genome stability during male meiosis [80]. It has been also suggested that YY1 is a key factor for maintaining the viability of spermatogonia and the stemness of spermatogonial stem cells (SSCs) [81]. In patients with *RBMXL3* sequence defects, the precise control of YY1 during spermatogenesis might be disrupted, which in theory could lead to exhaustion of the spermatogonia pool and azoospermia. Another interesting gene found in the group of differentially spliced genes (DSGs) was *GJA1*, coding for the predominant testicular gap junction protein involved in the regulation of fate decisions in human SSCs [82]. Our data show also that RBMXL3 may influence AS of *HNRNPH1*, a known splicing factor. In mice, HNRNPH1 has been shown to be involved in the establishment of crosstalk between Sertoli cells (SCs) and germ cells, further, conditional knock-out of this gene in SCs resulted in mice infertility [83]. Another study confirmed the presence of HNRNPH1 protein in the human testis, detecting it mainly in the nucleus of spermatocytes and indicating its role in the suppression of spermatogenesis induced by heat exposure [84]. Other examples of RNA-binding proteins involved in spermatogenesis are RBMXL2 [13] and RBM46 [85], whose knockout causes male infertility in mice. RBMXL2 has been implicated in suppressing aberrant splice sites during mouse spermatogenesis to ensure proper sperm production [13]. Our data suggest that RBMXL3 might play an important role in spermatogenesis and that the variants in the *RBMXL3* gene may potentially disturb normal protein function, also influencing the pool of RBMXL3-regulated RNAs and lead to male infertility.

Studies concerning human spermatogenesis are extremely limited due to the lack of comprehensive *in vitro* models of spermatogenesis and the fact that many genes crucial for this process still remain to be discovered. Although the functions of the majority of specific isoforms are not known yet, precise regulation of gene expression and AS is necessary to ensure the successful completion of male gamete formation. Taking into consideration that SCOS (diagnosed by testicular biopsy in men with *RBMXL3* variants) is generally characterized by a lack of germ cells in the testis, based on our data, we conclude that RBMXL3 may play

679 a role in processes important for spermatogonial stem cells (SSCs) differentiation towards spermatozoa
680 and/or their self-renewal. In patients with disrupted RBMXL3 function, these processes might be affected,
681 leading to spermatogenic impairment.

682 In our eCLIP data we identified more than 1,200 transcripts with over 6,900 RBMXL3 binding sites. Among
683 the RBMXL3 RNA targets, we found multiple transcripts coding for proteins involved in DNA repair
684 pathways. Two of these, important in the context of spermatogenesis, are BRCA1 and BRCA2, which have
685 established roles in cancer but are also involved in meiotic sex chromosome inactivation during
686 spermatogenesis and meiotic homologous recombination [86, 87]. Another interesting examples could be
687 the *MSH2* and *MSH6* transcripts, coding for mismatch repair proteins and also expressed during
688 spermatogenesis. These proteins ensure the correction of replication errors predisposing to different
689 malignancies and participate in the genomic integrity and stability maintenance in male germline [88].
690 Among the transcripts bound by RBMXL3 we also found the *EP300* mRNA (bound within its coding
691 sequence). The protein product of this gene functions as a histone acetyltransferase that regulates
692 transcription via chromatin remodeling and is important in the context of human stem cells and
693 differentiation [89, 90]. Moreover, the knockdown of *EP300* reduced stem cell content in primary cultures
694 of undifferentiated spermatogonia from mice [91]. Another RBMXL3 RNA target is *PREP*, bound within
695 its intronic sequence. PREP is recognized as a multifunctional protein, which has been detected in human
696 testicular tissue [92] and numerous reports suggested its role in mouse, rat, and also human spermatogenesis
697 [92, 93]. Another potentially interesting RNA target in the context of NOA and SCOS might be *FANCM*, a
698 testis-enhanced gene that has already been related to such phenotype in human male patients [94]. Our data
699 indicate that RBMXL3 protein participates in upstream regulation of many key genes important for
700 spermatogenesis, where precise regulation of sophisticated gene expression patterns is necessary to produce
701 haploid spermatozoa. In the wide pool of RNAs bound by RBMXL3 (eCLIP data) we also found *FOXJ3* (a
702 transcription factor expressed in human testis). Studies of *Foxj3* in mice have shown that deletion of *Foxj3*
703 from spermatogonia caused SCOS in males and deletion from spermatocytes led to meiotic arrest (both

models exhibited complete sterility) [95]. The conclusion was that FOXJ3 is required for spermatogonia survival and participates in meiosis. According to our eCLIP data, RBMXL3 binds *FOXJ3* RNA within its intronic sequence, which may influence its post-transcriptional regulation as well as downstream modifications, specific for humans. In NOA patients with RBMXL3 impaired function such disruption potentially may lead to SCOS phenotype. Any DNA sequence variants found in RBMXL3 may potentially affect the wide repertoire of RBMXL3 targets and perturb the human male germ cell differentiation process, which still remains to be fully elucidated. Our eCLIP data also suggest that RBMXL3 may contribute to the epigenetic regulation of gene expression as it binds a wide range of RNAs coding for proteins involved in the regulation of histone modifications such as PRDM2, PRMT2, CREBBP, TET1, and TET2. This is important in the context of the spermatogenesis process which is accompanied by alterations in histone modifications in order to modulate gene expression changes during gamete differentiation [96, 97] and might be disrupted in patients with *RBMXL3* mutations. The RBMXL3 interplay with epigenetic pathways might also contribute to the silencing of transposable elements [98].

Although the overlap between RBMXL3-induced DSGs (RNA-seq) and RBMXL3-bound transcripts (eCLIP) is relatively low, we found proof for direct binding of RBMXL3 to almost 10% of potential DSGs. RBMXL3 may participate in splicing of these transcripts by interacting with other proteins, for example, known splicing factors (EIF4A3, DDX39B) that we identified in our Co-IP-MS experiment. Moreover, eCLIP peak coverage showed an enrichment of RBMXL3 binding around the 5' UTR regions and the start codon. This observation may be particularly relevant given the high abundance of alternative first exon (AF) events in our analysis. Such events are common in RNA-seq studies, particularly in complex transcriptomes, and often reflect alternative transcription start site and promoter usage [99, 100]. However, as RNA-seq results capture steady-state transcript levels and are influenced by post-transcriptional regulation, the underlying cause cannot be conclusively determined. Considering the protein-protein interaction of RBMXL3 with translation factor EIF4A3, it is also possible that RBMXL3 may modulate translation in primates. Additionally, our RNA-seq and eCLIP-seq data indicate that RBMXL3 affects not only protein-

729 coding transcripts but also lncRNAs and although their role in spermatogenesis remains largely unknown
730 [101] it is currently certain that they are important players in regulation of many crucial cellular processes,
731 stem cell maintenance and differentiation [102]. Finally, we acknowledge the small sample size used in this
732 study (two biological replicates per condition), which may limit the interpretation of the sequencing data.

733 Furthermore, a recent study examined the genomic profiles of *MYCN* non-amplified neuroblastoma and
734 revealed novel recurrently mutated genes, and among them was *RBMXL3*, mutated in 19% of samples [35].
735 As neuroblastoma is the most common extracranial solid tumour in infants and children [103], it is possible
736 that the *RBMXL3* mutations may affect cellular differentiation programs. This could relate to the *RBMXL3*
737 protein binding to *ZFHX3* mRNA, coding for a transcription factor (known also as a neuronal differentiation
738 marker) which has been shown to affect different types of cancers. *ZFHX3* might have an opposing influence
739 being associated with a suppressor or oncogenic activity, for example in prostate and breast cancer
740 separately [104]. *ZFHX3* has been also shown to affect the expression of some pluripotency factors and
741 stemness markers, including *MYC*, *TBX3*, *OCT4*, *NANOG*, and *SOX2* [104].

742 Finally, by using a well-established retrotransposition reporter assay, we show that *RBMXL3* expression
743 heavily reduces L1 retrotransposition in human cells. Since during spermatogenesis epigenetic
744 reprogramming occurs leading to erasure of epigenetic silencing marks [98, 105] L1s might regain their
745 transcriptional activities. This suggests that *RBMXL3* expressed specifically in spermatocytes and
746 spermatogonia at the time of L1 transcriptional activation might contribute to a yet unknown L1 regulatory
747 mechanism. This further supports the concept of *RBMXL3* importance in maintaining genome integrity.
748 Although the mechanism of this action remains unknown, we propose here a few possible scenarios. Firstly,
749 we identified significant enrichment of L1 with *RBMXL3* in our eCLIP data, demonstrating direct
750 interaction between *RBMXL3* and L1 transcripts and implying a post-transcriptional regulatory mechanism
751 [106]. Although we did not identify L1 retrotransposon proteins in our proteomic data, *RBMX* has been
752 identified among the proteins associated with the L1 ORF1 protein in human cells [107]. It is therefore
753 possible that *RBMXL3* interacts with L1-encoded proteins and/or manages their posttranslational

754 modifications restricting L1 mobilization. A similar mechanism has been shown, for example, for mouse
755 TEX19.1 in pluripotent mouse embryonic stem cells [44]. We also cannot exclude the possibility that
756 RBMXL3 is involved in piwi-interacting RNAs (piRNAs) biogenesis pathways as one of its possible
757 interactors (DDX39B/UAP56) participates in germline piRNAs production and transposon silencing in
758 *Drosophila* [108]. Involvement of RBMXL3 in splicing of *YY1* might affect retrotransposition as YY1 is a
759 known L1 regulatory factor [109]. In conclusion, the postulated RBMXL3 interplay with L1s seems to be a
760 worthwhile direction to follow in the context of germ cell development and beyond.

761 The discrepancies concerning RBMXL3 detection in transcriptome- and proteome-wide studies indicate the
762 need for a more in-depth analysis of RBMXL3 expression patterns. As RBMXL3 is a primate-specific gene
763 the sense of RBMXL3 research on non-primates animal models is questionable. It is also worth noting that
764 the RBMXL3 protein action might be cell cycle phase-specific, hence the experiments using the phase-
765 arrested human cells would be very beneficial. This might lead to the discovery of new protein interactors
766 and RNA targets. To further investigate RBMXL3 functions more in detail, we would suggest utilizing
767 human stem cell-based approaches and advanced human spermatogenesis models [110, 111] to capture its
768 natural molecular environments and possible transient existence. The extensive repertoire of RBMXL3
769 RNA targets identified in the eCLIP experiment, spanning a broad range of biological processes, suggests
770 that RBMXL3 expression may be more widespread than currently appreciated, yet remains challenging to
771 detect.

772 Finally, it is worth noting that RBMXL3 binds its own mRNA as demonstrated by our eCLIP data. Such
773 self-regulatory RBP interaction mode is common among many other RBPs emerging as pivotal post-
774 transcriptional regulators of gene expression [112–114]. In agreement with a possible regulatory feedback
775 loop of RBMXL3 repressing its own mRNA translation we encountered several technical difficulties with
776 maintaining RBMXL3 expression at the protein level, but not at the mRNA level in TCam-2 cells following
777 transduction. We hypothesize that the negative loop might exist between RBMXL3 protein and its RNA

778 which may be the reason why RBMXL3 has such an elusive nature across human tissues and its fine-tuning
779 physiological role.

780 **Conclusions**

781 Our study provides the first evidence that the primate-specific RBMXL3 protein acts as an upstream
782 regulator of RNA metabolism through extensive RNA-binding activity in human TCam-2 cells.
783 Furthermore, we found that RBMXL3 expression significantly suppresses L1 retrotransposition in human
784 cells, highlighting its importance in maintaining human genome integrity. Additionally, we confirmed the
785 nuclear localization of RBMXL3 in TCam-2 cells (*in vitro* male germline model) upon ectopic expression
786 and, for the first time, demonstrated the presence of the RBMXL3 protein in human testis. Collectively, our
787 data imply that RBMXL3 contributes to the control of transcriptome dynamics in human male germ cells,
788 with its full biological relevance yet to be explored.

789 **List of abbreviations**

790 **RNA-seq** RNA sequencing

791 **eCLIP** enhanced crosslinking and immunoprecipitation

792 **Co-IP-MS** Co-immunoprecipitation coupled with Mass Spectrometry

793 **LINE-1** L1, Long Interspersed Nuclear Element-1

794 **WES** Whole-exome sequencing

795 **WGS** Whole-Genome sequencing

796 **NOA** Non-obstructive azoospermia

797 **HPA** Human Protein Atlas

798 **ChIP** Chromatin Immunoprecipitation

799 **IHC** Immunohistochemistry

800 **DEGs** differentially expressed genes

801 **AS** alternative splicing

802 **DSGs** differentially spliced genes

803 **GO** Gene ontology

804 **RBPs** RNA-binding proteins

805 **TEs** transposable elements

806 **SCOS** Sertoli cell-only syndrome

807 **SSCs** spermatogonial stem cells

808 **Declarations**

809 **Ethics approval and consent to participate**

810 Not applicable.

811 **Consent for publication**

812 The paper has been read and approved by all authors. All authors approved the submission of this paper to
813 Cellular & Molecular Biology Letters for publication. All authors confirmed that neither the manuscript
814 submitted nor any part of it has been published or is being considered for publication elsewhere.

815 **Availability of data and materials**

816 The datasets generated and/or analyzed during the current study are available in the GEO repository,
817 GSE294696 (RNA-seq) and GSE308226 (eCLIP-seq). All data generated during this study are included in
818 this published article and are available in Zenodo: <https://doi.org/10.5281/zenodo.12788249>.

819 **Competing interests**

820 The authors declare no competing interests.

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826 **Authors' contributions**

827 **Conceptualization:** N.R.; **Methodology:** A.J.B., K.T., N.R.; **Investigation:** A.J.B., K.T., M.D., A.M.,
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829 M.O., A.Y. (selection of gene of interest based on previous studies), M.H.; **Writing – original draft:** A.J.B.;
830 **Writing – review & editing:** Z.W., E.D., K.T., E.I., A.J.B., J.Z.-W., A.N., T.K., A.Y., Z.E.M., N.R.;
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832 All authors read and approved the manuscript.

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841 **Supplemental Tables**

- 842 Supplemental Table S1 Normalized counts (CPM) based on RNA-seq of TCam-2 cells.
- 843 Supplemental Table S2 Differentially expressed genes (DEGs) based on RNA-seq of TCam-2 cells.
- 844 Supplemental Table S3 Normalized transcript-level values (TPM) for TCam-2 cells quantified with Salmon
- 845 from RNA-seq data.
- 846 Supplemental Table S4 Differentially spliced genes (DSGs) based on SUPPA2 analysis of RNA-seq data.
- 847 Supplemental Table S5 Genes overlap results for DSGs, DEGs, and genes involved in male gamete
- 848 generation.
- 849 Supplemental Table S6 Gene Ontology and pathway enrichment analysis of DSGs detected upon RBMXL3
- 850 overexpression.
- 851 Supplemental Table S7 Results of RBMXL3 co-immunoprecipitation followed by mass spectrometry.
- 852 Supplemental Table S8 RBMXL3 protein-protein interaction network analysis performed with the STRING
- 853 database.
- 854 Supplemental Table S9 Gene Ontology enrichment analysis based on proteins identified in RBMXL3 co-
- 855 immunoprecipitation experiment.
- 856 Supplemental Table S10 Detailed information on detected RBMXL3 eCLIP peaks.
- 857 Supplemental Table S11 List of transcripts bound by RBMXL3 identified by eCLIP.
- 858 Supplemental Table S12 Genes overlap results for RBMXL3 eCLIP targets and genes involved in male
- 859 gamete generation.
- 860 Supplemental Table S13 Genes overlap results for DSGs, DEGs, and RBMXL3 eCLIP targets.

861 Supplemental Table S14 Gene Ontology and pathway enrichment analysis of RBMXL3 eCLIP targets.

862 Supplemental Table S15 L1 differential expression in TCam-2 cells based on RNA-seq data.

863 Supplemental Table S16 List of primers.

864 **References**

865 1. Agarwal A, Majzoub A, Parekh N, Henkel R. A Schematic Overview of the Current Status of Male
866 Infertility Practice. *World J Mens Health*. 2020;38:308–22. <https://doi.org/10.5534/WJMH.190068>.

867 2. Lotti F, Maggi M. Sexual dysfunction and male infertility. *Nat Rev Urol*. 2018;15:287–307.
868 <https://doi.org/10.1038/NRUROL.2018.20>.

869 3. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and
870 treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod*. 2007;22:1506–12.
871 <https://doi.org/10.1093/HUMREP/DEM046>.

872 4. Krausz C, Riera-Escamilla A. Genetics of male infertility. *Nat Rev Urol*. 2018;15:369–84.
873 <https://doi.org/10.1038/s41585-018-0003-3>.

874 5. Malcher A, Stokowy T, Berman A, Olszewska M, Jedrzejczak P, Sielski D, et al. Whole-genome
875 sequencing identifies new candidate genes for nonobstructive azoospermia. *Andrology*. 2022;10:1605–24.
876 <https://doi.org/10.1111/ANDR.13269>.

877 6. Elliott DJ, Dalglish C, Hysenaj G, Ehrmann I. RBMX family proteins connect the fields of nuclear
878 RNA processing, disease and sex chromosome biology. *Int J Biochem Cell Biol*. 2019;108:1–6.
879 <https://doi.org/10.1016/j.biocel.2018.12.014>.

880 7. Lingenfelter PA, Delbridge ML, Thomas S, Hoekstra HE, Mitchell MJ, Marshall Graves JA, et al.
881 Expression and conservation of processed copies of the RBMX gene. *Mamm genome*. 2001;12:538–45.
882 <https://doi.org/10.1007/S00335001-0003-Z>.

- 883 8. Soulard M, Valle V Della, Siomi MC, Pinol-roma S, Codogno P, Bauvy C, et al. hnRNP G: sequence
884 and characterization of a glycosylated RNA-binding protein. *Nucleic Acids Res.* 1993;21:4210–7.
885 <https://doi.org/10.1093/NAR/21.18.4210>.
- 886 9. Elliott DJ, Venables JP, Newton CS, Lawson D, Boyle S, Eperon IC, et al. An evolutionarily conserved
887 germ cell-specific hnRNP is encoded by a retrotransposed gene. *Hum Mol Genet.* 2000;9:2117–24.
888 <https://doi.org/10.1093/HMG/9.14.2117>.
- 889 10. Liu L, Liu X, Bi W, Alcorn JL. A primate-specific RNA-binding protein (RBMXL3) is involved in
890 glucocorticoid regulation of human pulmonary surfactant protein B (SP-B) mRNA stability. *Am J Physiol*
891 *Lung Cell Mol Physiol.* 2021;320:L942–57. <https://doi.org/10.1152/AJPLUNG.00022.2020>.
- 892 11. Zuccotti P, Peroni D, Potrich V, Quattrone A, Dassi E. Hyperconserved Elements in Human 5'UTRs
893 Shape Essential Post-transcriptional Regulatory Networks. *Front Mol Biosci.* 2020;7.
894 <https://doi.org/10.3389/FMOLB.2020.00220/BIBTEX>.
- 895 12. Elliott DJ. The role of potential splicing factors including RBMY, RBMX, hnRNPG-T and STAR
896 proteins in spermatogenesis. *Int J Androl.* 2004;27:328–34. <https://doi.org/10.1111/J.1365->
897 [2605.2004.00496.X](https://doi.org/10.1111/J.1365-2605.2004.00496.X).
- 898 13. Ehrmann I, Crichton JH, Gazzara MR, James K, Liu Y, Grellscheid SN, et al. An ancient germ cell-
899 specific RNA-binding protein protects the germline from cryptic splice site poisoning. *Elife.*
900 2019;8:e39304. <https://doi.org/10.7554/eLife.39304>.
- 901 14. Zhou KI, Shi H, Lyu R, Wylder AC, Matuszek Ż, Pan JN, et al. Regulation of Co-transcriptional Pre-
902 mRNA Splicing by m6A through the Low-Complexity Protein hnRNPG. *Mol Cell.* 2019;76:70-81.e9.
903 <https://doi.org/10.1016/J.MOLCEL.2019.07.005>.
- 904 15. Heinrich B, Zhang Z, Raitskin O, Hiller M, Benderska N, Hartmann AM, et al. Heterogeneous nuclear
905 ribonucleoprotein G regulates splice site selection by binding to CC(A/C)-rich regions in pre-mRNA. *J*

- 906 Biol Chem. 2009;284:14303–15. <https://doi.org/10.1074/JBC.M901026200>.
- 907 16. Luzzi S, Hysenaj G, Siachisumo C, Cheung K, Gazzara M, James K, et al. RBMX enables productive
908 RNA processing of ultra-long exons important for genome stability. bioRxiv. 2020;:2020.10.09.333039.
909 <https://doi.org/10.1101/2020.10.09.333039>.
- 910 17. Tsujimura A, Fujita K, Komori K, Tanjapatkul P, Miyagawa Y, Takada S, et al. Associations of
911 homologous RNA-binding motif gene on the X chromosome (RBMX) and its like sequence on
912 chromosome 9 (RBMXL9) with non-obstructive azoospermia. Asian J Androl. 2006;8:213–8.
913 <https://doi.org/10.1111/J.1745-7262.2006.00107.X>.
- 914 18. Elliott DJ, Grellscheid SN. Alternative RNA splicing regulation in the testis. Reproduction.
915 2006;132:811–9. <https://doi.org/10.1530/REP-06-0147>.
- 916 19. Song H, Wang L, Chen D, Li F. The Function of Pre-mRNA Alternative Splicing in Mammal
917 Spermatogenesis. Int J Biol Sci. 2020;16:38–48. <https://doi.org/10.7150/IJBS.34422>.
- 918 20. Mazin P V., Khaitovich P, Cardoso-Moreira M, Kaessmann H. Alternative splicing during mammalian
919 organ development. Nat Genet. 2021;53:925–34. <https://doi.org/10.1038/S41588-021-00851-W>.
- 920 21. Matos B, Publicover SJ, Castro LFC, Esteves PJ, Fardilha M. Brain and testis: more alike than
921 previously thought? Open Biol. 2021;11:200322. <https://doi.org/10.1098/RSOB.200322>.
- 922 22. Naro C, Cesari E, Sette C. Splicing regulation in brain and testis: common themes for highly
923 specialized organs. Cell cycle. 2021;20:480–9. <https://doi.org/10.1080/15384101.2021.1889187>.
- 924 23. Shashi V, Xie P, Schoch K, Goldstein DB, Howard TD, Berry MN, et al. The RBMX gene as a
925 candidate for the Shashi X-linked intellectual disability syndrome. Clin Genet. 2015;88:386–90.
926 <https://doi.org/10.1111/CGE.12511>.
- 927 24. Cai T, Cinkornpumin JK, Yu Z, Villarreal OD, Pastor WA, Richard S. Deletion of RBMX RGG/RG

928 motif in Shashi-XLID syndrome leads to aberrant p53 activation and neuronal differentiation defects. *Cell*
929 *Rep.* 2021;36:109337. <https://doi.org/10.1016/j.celrep.2021.109337>.

930 25. Tsend-Ayush E, O'Sullivan LA, Grützner FS, Onnebo SMN, Lewis RS, Delbridge ML, et al. RBMX
931 gene is essential for brain development in zebrafish. *Dev Dyn.* 2005;234:682–8.
932 <https://doi.org/10.1002/DVDY.20432>.

933 26. Matsunaga S, Takata H, Morimoto A, Hayashihara K, Higashi T, Akatsuchi K, et al. RBMX: a
934 regulator for maintenance and centromeric protection of sister chromatid cohesion. *Cell Rep.* 2012;1:299–
935 308. <https://doi.org/10.1016/J.CELREP.2012.02.005>.

936 27. Cho Y, Ideue T, Nagayama M, Araki N, Tani T. RBMX is a component of the centromere noncoding
937 RNP complex involved in cohesion regulation. *Genes to Cells.* 2018;23:172–84.
938 <https://doi.org/10.1111/GTC.12562>.

939 28. Adamson B, Smogorzewska A, Sigoillot FD, King RW, Elledge SJ. A genome-wide homologous
940 recombination screen identifies the RNA-binding protein RBMX as a component of the DNA-damage
941 response. *Nat Cell Biol.* 2012;14:318–28. <https://doi.org/10.1038/NCB2426>.

942 29. Shin KH, Kim RHRH, Kang MK, Kim RHRH, Kim SG, Lim PK, et al. p53 promotes the fidelity of
943 DNA end-joining activity by, in part, enhancing the expression of heterogeneous nuclear
944 ribonucleoprotein G. *DNA Repair.* 2007;6:830–40. <https://doi.org/10.1016/J.DNAREP.2007.01.013>.

945 30. Zheng T, Zhou H, Li X, Peng D, Yang Y, Zeng Y, et al. RBMX is required for activation of ATR on
946 repetitive DNAs to maintain genome stability. *Cell Death Differ.* 2020;27:3162–76.
947 <https://doi.org/10.1038/S41418-020-0570-8>.

948 31. Brain tissue expression of RBMXL3 - Summary - The Human Protein Atlas.
949 <https://www.proteinatlas.org/ENSG00000175718-RBMXL3/brain>. Accessed 5 Oct 2025.

950 32. Wang D, Eraslan B, Wieland T, Hallström B, Hopf T, Zolg DP, et al. A deep proteome and

951 transcriptome abundance atlas of 29 healthy human tissues. *Mol Syst Biol.* 2019;15:e8503.
952 <https://doi.org/10.15252/msb.20188503>.

953 33. Kim MS, Pinto SM, Getnet D, Nirujogi RS, Manda SS, Chaerkady R, et al. A draft map of the human
954 proteome. *Nature.* 2014;509:575–81. <https://doi.org/10.1038/NATURE13302>.

955 34. Wilhelm M, Schlegl J, Hahne H, Gholami AM, Lieberenz M, Savitski MM, et al. Mass-spectrometry-
956 based draft of the human proteome. *Nature.* 2014;509:582–7. <https://doi.org/10.1038/nature13319>.

957 35. Lee E, Lee JW, Lee B, Park K, Shim J, Yoo KH, et al. Genomic profile of MYCN non-amplified
958 neuroblastoma and potential for immunotherapeutic strategies in neuroblastoma. *BMC Med Genomics.*
959 2020;13:171. <https://doi.org/10.1186/S12920-020-00819-5>.

960 36. Zheng P, Liang C, Ren L, Zhu D, Feng Q, Chang W, et al. Additional Biomarkers beyond RAS That
961 Impact the Efficacy of Cetuximab plus Chemotherapy in mCRC: A Retrospective Biomarker Analysis. *J*
962 *Oncol.* 2018;:5072987. <https://doi.org/10.1155/2018/5072987>.

963 37. Zheng P, Ren L, Feng Q, Zhu D, Chang W, He G, et al. Differences in clinical characteristics and
964 mutational pattern between synchronous and metachronous colorectal liver metastases. *Cancer Manag*
965 *Res.* 2018;10:2871–81. <https://doi.org/10.2147/CMAR.S161392>.

966 38. Mizuno Y, Gotoh A, Kamidono S, Kitazawa S. [Establishment and characterization of a new human
967 testicular germ cell tumor cell line (TCam-2)]. *Nihon Hinyokika Gakkai Zasshi Japanese J Urol.*
968 1993;84:1211–8. <https://doi.org/10.5980/JPNJUROL1989.84.1211>.

969 39. De Jong J, Stoop H, Gillis AJM, Hersmus R, Van Gurp RJHLM, Van De Geijn GJM, et al. Further
970 characterization of the first seminoma cell line TCam-2. *Genes Chromosomes Cancer.* 2008;47:185–96.
971 <https://doi.org/10.1002/GCC.20520>.

972 40. Oosterhuis JW, Looijenga LHJ. Testicular germ-cell tumours in a broader perspective. *Nat Rev*
973 *Cancer.* 2005;5:210–22. <https://doi.org/10.1038/nrc1568>.

- 974 41. Nettersheim D, Jostes S, Schneider S, Schorle H. Elucidating human male germ cell development by
975 studying germ cell cancer. *Reproduction*. 2016;152:R101–13. <https://doi.org/10.1530/REP-16-0114>.
- 976 42. Irie N, Weinberger L, Tang WWC, Kobayashi T, Viukov S, Manor YS, et al. SOX17 is a critical
977 specifier of human primordial germ cell fate. *Cell*. 2015;160:253–68.
978 <https://doi.org/10.1016/j.cell.2014.12.013>.
- 979 43. Smialek MJ, Ilaslan E, Sajek MP, Swiercz A, Janecki DM, Kusz-Zamelczyk K, et al. Characterization
980 of RNP Networks of PUM1 and PUM2 Post-Transcriptional Regulators in TCam-2 Cells, a Human Male
981 Germ Cell Model. *Cells*. 2020;9:984. <https://doi.org/10.3390/CELLS9040984>.
- 982 44. MacLennan M, García-Cañadas M, Reichmann J, Khazina E, Wagner G, Playfoot CJ, et al.
983 Mobilization of LINE-1 retrotransposons is restricted by Tex19.1 in mouse embryonic stem cells. *Elife*.
984 2017;6:e26152. <https://doi.org/10.7554/ELIFE.26152>.
- 985 45. Janecki DM, Sen R, Szóstak N, Kajdasz A, Kordys M, Plawgo K, et al. LINE-1 mRNA 3'
986 end dynamics shape its biology and retrotransposition potential. *Nucleic Acids Res*. 2024;52:3327.
987 <https://doi.org/10.1093/NAR/GKAD1251>.
- 988 46. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal
989 RNA-seq aligner. *Bioinformatics*. 2013;29:15–21. <https://doi.org/10.1093/BIOINFORMATICS/BTS635>.
- 990 47. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map
991 format and SAMtools. *Bioinformatics*. 2009;25:2078–9.
992 <https://doi.org/10.1093/BIOINFORMATICS/BTP352>.
- 993 48. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data
994 with DESeq2. *Genome Biol*. 2014;15:550. <https://doi.org/10.1186/s13059-014-0550-8>.
- 995 49. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: A universal enrichment tool for
996 interpreting omics data. *Innov*. 2021;2:100141. <https://doi.org/10.1016/J.XINN.2021.100141>.

997 50. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon: fast and bias-aware quantification of
998 transcript expression using dual-phase inference. *Nat Methods*. 2017;14:417–9.
999 <https://doi.org/10.1038/NMETH.4197>.

1000 51. Alamancos GP, Pagès A, Trincado JL, Bellora N, Eyra E. Leveraging transcript quantification for fast
1001 computation of alternative splicing profiles. *RNA*. 2015;21:1521–31.
1002 <https://doi.org/10.1261/RNA.051557.115>.

1003 52. Trincado JL, Entizne JC, Hysenaj G, Singh B, Skalic M, Elliott DJ, et al. SUPPA2: Fast, accurate, and
1004 uncertainty-aware differential splicing analysis across multiple conditions. *Genome Biol*. 2018;19:40.
1005 <https://doi.org/10.1186/s13059-018-1417-1>.

1006 53. Jin Y, Tam OH, Paniagua E, Hammell M. TETranscripts: a package for including transposable
1007 elements in differential expression analysis of RNA-seq datasets. *Bioinformatics*. 2015;31:3593–9.
1008 <https://doi.org/10.1093/BIOINFORMATICS/BTV422>.

1009 54. Van Nostrand EL, Pratt GA, Shishkin AA, Gelboin-Burkhart C, Fang MY, Sundararaman B, et al.
1010 Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP).
1011 *Nat Methods*. 2016;13:508–14. <https://doi.org/10.1038/nmeth.3810>.

1012 55. Yeo GW, Coufal NG, Liang TY, Peng GE, Fu XD, Gage FH. An RNA code for the FOX2 splicing
1013 regulator revealed by mapping RNA-protein interactions in stem cells. *Nat Struct Mol Biol*. 2009;16:130–
1014 7. <https://doi.org/10.1038/nsmb.1545>.

1015 56. Lovci MT, Ghanem D, Marr H, Arnold J, Gee S, Parra M, et al. Rbfox proteins regulate alternative
1016 mRNA splicing through evolutionarily conserved RNA bridges. *Nat Struct Mol Biol*. 2013;20:1434–42.
1017 <https://doi.org/10.1038/nsmb.2699>.

1018 57. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features.
1019 *Bioinformatics*. 2010;26:841–2. <https://doi.org/10.1093/BIOINFORMATICS/BTQ033>.

1020 58. Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, et al. Software for Computing
1021 and Annotating Genomic Ranges. *PLOS Comput Biol*. 2013;9:e1003118.
1022 <https://doi.org/10.1371/JOURNAL.PCBI.1003118>.

1023 59. Sievers C, Schlumpf T, Sawarkar R, Comoglio F, Paro R. Mixture models and wavelet transforms
1024 reveal high confidence RNA-protein interaction sites in MOV10 PAR-CLIP data. *Nucleic Acids Res*.
1025 2012;40:e160. <https://doi.org/10.1093/NAR/GKS697>.

1026 60. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-
1027 determining transcription factors prime cis-regulatory elements required for macrophage and B cell
1028 identities. *Mol Cell*. 2010;38:576–89. <https://doi.org/10.1016/J.MOLCEL.2010.05.004>.

1029 61. Kloska D, Kopacz A, Cysewski D, Aepfelbacher M, Dulak J, Jozkowicz A, et al. Nrf2 Sequesters
1030 Keap1 Preventing Podosome Disassembly: A Quintessential Duet Moonlights in Endothelium. *Antioxid*
1031 *Redox Signal*. 2019;30:1709–30. <https://doi.org/10.1089/ARS.2018.7505>.

1032 62. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass
1033 accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008;26:1367–72.
1034 <https://doi.org/10.1038/nbt.1511>.

1035 63. Winkle M, Tayari MM, Kok K, Duns G, Grot N, Kazimierska M, et al. The lncRNA KTN1-AS1 co-
1036 regulates a variety of Myc-target genes and enhances proliferation of Burkitt lymphoma cells. *Hum Mol*
1037 *Genet*. 2022;31:4193–206. <https://doi.org/10.1093/HMG/DDAC159>.

1038 64. Janecki DM, Sen R, Szóstak N, Kajdasz A, Kordyś M, Plawgo K, et al. LINE-1 mRNA 3' end
1039 dynamics shape its biology and retrotransposition potential. *Nucleic Acids Res*. 2024.
1040 <https://doi.org/10.1093/NAR/GKAD1251>.

1041 65. Szczesny RJ, Kowalska K, Klosowska-Kosicka K, Chlebowski A, Owczarek EP, Warkocki Z, et al.
1042 Versatile approach for functional analysis of human proteins and efficient stable cell line generation using

- 1043 FLP-mediated recombination system. PLoS One. 2018;13:e0194887.
- 1044 <https://doi.org/10.1371/JOURNAL.PONE.0194887>.
- 1045 66. Warkocki Z, Krawczyk PS, Adamska D, Bijata K, Garcia-Perez JL, Dziembowski A. Uridylation by
- 1046 TUT4/7 Restricts Retrotransposition of Human LINE-1s. Cell. 2018;174:1537-1548.e29.
- 1047 <https://doi.org/10.1016/j.cell.2018.07.022>.
- 1048 67. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene Ontology: tool for the
- 1049 unification of biology. Nat Genet. 2000;25:25–9. <https://doi.org/10.1038/75556>.
- 1050 68. Consortium TGO, Aleksander SA, Balhoff J, Carbon S, Cherry JM, Drabkin HJ, et al. The Gene
- 1051 Ontology knowledgebase in 2023. Genetics. 2023;224:iyad031.
- 1052 <https://doi.org/10.1093/GENETICS/IYAD031>.
- 1053 69. Kohlrausch FB, Berteli TS, Wang F, Navarro PA, Keefe DL. Control of LINE-1 Expression Maintains
- 1054 Genome Integrity in Germline and Early Embryo Development. Reprod Sci. 2022;29:328–40.
- 1055 <https://doi.org/10.1007/S43032-021-00461-1>.
- 1056 70. Boissinot S, Furano A V. The recent evolution of human L1 retrotransposons. Cytogenet Genome Res.
- 1057 2005;110:402–6. <https://doi.org/10.1159/000084972>.
- 1058 71. Boissinot S, Chevret P, Furano A V. L1 (LINE-1) Retrotransposon Evolution and Amplification in
- 1059 Recent Human History. Mol Biol Evol. 2000;17:915–28.
- 1060 <https://doi.org/10.1093/OXFORDJOURNALS.MOLBEV.A026372>.
- 1061 72. Goodier JL, Cheung LE, Kazazian HH. MOV10 RNA helicase is a potent inhibitor of
- 1062 retrotransposition in cells. PLOS Genet. 2012;8:e1002941.
- 1063 <https://doi.org/10.1371/JOURNAL.PGEN.1002941>.
- 1064 73. Looijenga LHJ, Stoop H, Biermann K. Testicular cancer: Biology and biomarkers. Virchows Arch.
- 1065 2014;464:301–13. <https://doi.org/10.1007/S00428-013-1522-1/FIGURES/7>.

- 1066 74. Baralle FE, Giudice J. Alternative splicing as a regulator of development and tissue identity. *Nat Rev*
1067 *Mol Cell Biol.* 2017;18:437–51. <https://doi.org/10.1038/NRM.2017.27>.
- 1068 75. Murase Y, Yokogawa R, Yabuta Y, Nagano M, Katou Y, Mizuyama M, et al. In vitro reconstitution of
1069 epigenetic reprogramming in the human germ line. *Nature.* 2024;631:170–8.
1070 <https://doi.org/10.1038/s41586-024-07526-6>.
- 1071 76. Hermann BP, Cheng K, Singh A, Roa-De La Cruz L, Mutoji KN, Chen IC, et al. The Mammalian
1072 Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. *Cell Rep.*
1073 2018;25:1650-1667.e8. <https://doi.org/10.1016/J.CELREP.2018.10.026/ATTACHMENT/40ED49CF->
1074 2212-4318-9D7D-46C85A4FAE79/MMC7.PDF.
- 1075 77. Sha QQ, Zheng W, Wu YW, Li S, Guo L, Zhang S, et al. Dynamics and clinical relevance of maternal
1076 mRNA clearance during the oocyte-to-embryo transition in humans. *Nat Commun.* 2020;11.
1077 <https://doi.org/10.1038/s41467-020-18680-6>.
- 1078 78. Güller M, Toualbi-Abed K, Legrand A, Michel L, Mauviel A, Bernuau D, et al. c-Fos overexpression
1079 increases the proliferation of human hepatocytes by stabilizing nuclear Cyclin D1. *World J Gastroenterol.*
1080 2008;14:6339–46. <https://doi.org/10.3748/WJG.14.6339>.
- 1081 79. Araújo FC, Oliveira CA, Reis AB, del Puerto HL, Martins AS, Reis FM. Expression of the proto-
1082 oncogene c-fos and the immunolocalization of c-fos, phosphorylated c-fos and estrogen receptor beta in
1083 the human testis. *Histol Histopathol.* 2009;24:1515–22. <https://doi.org/10.14670/HH-24.1515>.
- 1084 80. Wu S, Hu Y-C, Liu H, Shi Y. Loss of YY1 Impacts the Heterochromatic State and Meiotic Double-
1085 Strand Breaks during Mouse Spermatogenesis. *Mol Cell Biol.* 2009;29:6245–56.
1086 <https://doi.org/10.1128/MCB.00679-09>.
- 1087 81. Cheon Y-P, Choi D, Lee S-H, Chul †, Kim G. YY1 and CP2c in Unidirectional Spermatogenesis and
1088 Stemness. *Dev Reprod.* 2020;24:249–62. <https://doi.org/10.12717/DR.2020.24.4.249>.

- 1089 82. Chen W, Cui Y, Li C, He C, Du L, Liu W, et al. KLF2 controls proliferation and apoptosis of human
1090 spermatogonial stem cells via targeting GJA1. *iScience*. 2024;27:109024.
1091 <https://doi.org/10.1016/j.isci.2024.109024>.
- 1092 83. Feng S, Wen H, Liu K, Xiong M, Li J, Gui Y, et al. hnRNPH1 establishes Sertoli-germ cell crosstalk
1093 through cooperation with PTBP1 and AR, and is essential for male fertility in mice. *Development*.
1094 2023;150:dev201040. <https://doi.org/10.1242/DEV.201040/286819>.
- 1095 84. Zhu H, Cui Y, Xie J, Chen L, Chen X, Guo X, et al. Proteomic analysis of testis biopsies in men
1096 treated with transient scrotal hyperthermia reveals the potential targets for contraceptive development.
1097 *Proteomics*. 2010;10:3480–93. <https://doi.org/10.1002/PMIC.201000281>.
- 1098 85. Peart NJ, Johnson TA, Lee S, Sears MJ, Yang F, Quesnel-Vallieres M, et al. The germ cell-specific
1099 RNA binding protein RBM46 is essential for spermatogonial differentiation in mice. *PLOS Genet*.
1100 2022;18:e1010416. <https://doi.org/10.1371/JOURNAL.PGEN.1010416>.
- 1101 86. Alavattam KG, Kato Y, Sin HS, Maezawa S, Kowalski IJ, Zhang F, et al. Elucidation of the Fanconi
1102 Anemia Protein Network in Meiosis and Its Function in the Regulation of Histone Modifications. *Cell*
1103 *Rep*. 2016;17:1141–57. <https://doi.org/10.1016/j.celrep.2016.09.073>.
- 1104 87. Li Q, Engebrecht JA. BRCA1 and BRCA2 Tumor Suppressor Function in Meiosis. *Front Cell Dev*
1105 *Biol*. 2021;9:668309. <https://doi.org/10.3389/FCELL.2021.668309/BIBTEX>.
- 1106 88. Gunes S, Al-Sadaan M, Agarwal A. Spermatogenesis, DNA damage and DNA repair mechanisms in
1107 male infertility. *Reprod Biomed Online*. 2015;31:309–19. <https://doi.org/10.1016/j.rbmo.2015.06.010>.
- 1108 89. Jantine van Voorden A, Keijser R, Veenboer GJM, Lopes Cardozo SA, Diek D, Vlaardingerbroek JA,
1109 et al. EP300 facilitates human trophoblast stem cell differentiation. *Proc Natl Acad Sci U S A*.
1110 2023;120:e2217405120.
1111 https://doi.org/10.1073/PNAS.2217405120/SUPPL_FILE/PNAS.2217405120.SAPP.PDF.

- 1112 90. Xiao M, Wu CH, Meek G, Kelly B, Castillo DB, Young LEA, et al. PASK links cellular energy
1113 metabolism with a mitotic self-renewal network to establish differentiation competence. *Elife*.
1114 2023;12:e81717. <https://doi.org/10.7554/ELIFE.81717>.
- 1115 91. Lord T, Law NC, Oatley MJ, Miao D, Du G, Oatley JM. A novel high throughput screen to identify
1116 candidate molecular networks that regulate spermatogenic stem cell functions. *Biol Reprod*.
1117 2022;106:1175–90. <https://doi.org/10.1093/BIOLRE/IOAC048>.
- 1118 92. Venditti M, Fasano C, Minucci S, Serino I, Sinisi AA, Dale B, et al. DAAM1 and PREP are involved
1119 in human spermatogenesis. *Reprod Fertil Dev*. 2020;32:484–94. <https://doi.org/10.1071/RD19172>.
- 1120 93. Venditti M, Minucci S. Subcellular Localization of Prolyl Endopeptidase During the First Wave of Rat
1121 Spermatogenesis and in Rat and Human Sperm. *J Histochem Cytochem*. 2019;67:229–43.
1122 https://doi.org/10.1369/0022155418810064/ASSET/IMAGES/LARGE/10.1369_0022155418810064-
1123 [FIG9.JPEG](#).
- 1124 94. Kasak L, Laan M. Monogenic causes of non-obstructive azoospermia: challenges, established
1125 knowledge, limitations and perspectives. *Hum Genet*. 2021;140:135–54. <https://doi.org/10.1007/S00439->
1126 [020-02112-Y](#).
- 1127 95. Ni L, Xie H, Tan L. Multiple roles of FOXJ3 in spermatogenesis: A lesson from Foxj3 conditional
1128 knockout mouse models. *Mol Reprod Dev*. 2016;83:1060–9. <https://doi.org/10.1002/MRD.22750>.
- 1129 96. Wang L, Xu Z, Khawar MB, Liu C, Li W. The histone codes for meiosis. *Reproduction*.
1130 2017;154:R65–79. <https://doi.org/10.1530/REP-17-0153>.
- 1131 97. Odronec A, Olszewska M, Kurpisz M. Epigenetic markers in the embryonal germ cell development
1132 and spermatogenesis. *Basic Clin Androl*. 2023;33:6. <https://doi.org/10.1186/S12610-022-00179-3>.
- 1133 98. Yang F, Wang PJ. Multiple LINEs of retrotransposon silencing mechanisms in the mammalian
1134 germline. *Semin Cell Dev Biol*. 2016;59:118–25. <https://doi.org/10.1016/J.SEMCDB.2016.03.001>.

- 1135 99. Wang ET, Sandberg R, Luo S, Khrebtkova I, Zhang L, Mayr C, et al. Alternative isoform regulation
1136 in human tissue transcriptomes. *Nature*. 2008;456:470–6. <https://doi.org/10.1038/nature07509>.
- 1137 100. Reyes A, Huber W. Alternative start and termination sites of transcription drive most transcript
1138 isoform differences across human tissues. *Nucleic Acids Res*. 2018;46:582–92.
1139 <https://doi.org/10.1093/NAR/GKX1165>.
- 1140 101. Joshi M, Rajender S. Long non-coding RNAs (lncRNAs) in spermatogenesis and male infertility.
1141 *Reprod Biol Endocrinol*. 2020;18:103. <https://doi.org/10.1186/S12958-020-00660-6>.
- 1142 102. Mattick JS, Amaral PP, Carninci P, Carpenter S, Chang HY, Chen LL, et al. Long non-coding RNAs:
1143 definitions, functions, challenges and recommendations. *Nat Rev Mol Cell Biol*. 2023;24:430–47.
1144 <https://doi.org/10.1038/S41580-022-00566-8>.
- 1145 103. Dzieran J, Garcia AR, Westermarck UK, Henley AB, Sánchez EE, Träger C, et al. MYCN-amplified
1146 neuroblastoma maintains an aggressive and undifferentiated phenotype by deregulation of estrogen and
1147 NGF signaling. *Proc Natl Acad Sci U S A*. 2018;115:E1229–38.
1148 https://doi.org/10.1073/PNAS.1710901115/SUPPL_FILE/PNAS.1710901115.SD05.XLSX.
- 1149 104. Dong G, Ma G, Wu R, Liu J, Liu M, Gao A, et al. ZFHX3 Promotes the Proliferation and Tumor
1150 Growth of ER-Positive Breast Cancer Cells Likely by Enhancing Stem-Like Features and MYC and
1151 TBX3 Transcription. *Cancers (Basel)*. 2020;12:3415. <https://doi.org/10.3390/CANCERS12113415>.
- 1152 105. Bourc'his D, Bestor TH. Meiotic catastrophe and retrotransposon reactivation in male germ cells
1153 lacking Dnmt3L. *Nature*. 2004;431:96–9. <https://doi.org/10.1038/NATURE02886>.
- 1154 106. Warkocki Z. An update on post-transcriptional regulation of retrotransposons. *FEBS Letters*.
1155 2023;597:380–406. <https://doi.org/10.1002/1873-3468.14551>.
- 1156 107. Goodier JL, Cheung LE, Kazazian HH. Mapping the LINE1 ORF1 protein interactome reveals
1157 associated inhibitors of human retrotransposition. *Nucleic Acids Res*. 2013;41:7401–19.

- 1158 <https://doi.org/10.1093/NAR/GKT512>.
- 1159 108. Zhang F, Wang J, Xu J, Zhang Z, Koppetsch BS, Schultz N, et al. UAP56 couples piRNA clusters to
1160 the perinuclear transposon silencing machinery. *Cell*. 2012;151:871–84.
1161 <https://doi.org/10.1016/J.CELL.2012.09.040>.
- 1162 109. Sanchez-Luque FJ, Kempen MJHC, Gerdes P, Vargas-Landin DB, Richardson SR, Troskie RL, et al.
1163 LINE-1 Evasion of Epigenetic Repression in Humans. *Mol Cell*. 2019;75:590-604.e12.
1164 <https://doi.org/10.1016/J.MOLCEL.2019.05.024>.
- 1165 110. Nguyen AL V., Julian S, Weng N, Flannigan R. Advances in human In vitro spermatogenesis: A
1166 review. *Mol Aspects Med*. 2024;100. <https://doi.org/10.1016/j.mam.2024.101320>.
- 1167 111. Kaltsas A, Kyrgiagini M-A, Markou E, Koumenis A, Mamuris Z, Dimitriadis F, et al. Artificial
1168 Gametogenesis and In Vitro Spermatogenesis: Emerging Strategies for the Treatment of Male Infertility.
1169 *Int J Mol Sci*. 2025;26. <https://doi.org/10.3390/IJMS26157383>.
- 1170 112. Dassi E. Handshakes and fights: The regulatory interplay of RNA-binding proteins. *Front Mol*
1171 *Biosci*. 2017;4:67. <https://doi.org/10.3389/FMOLB.2017.00067/BIBTEX>.
- 1172 113. Quattrone A, Dassi E. The Architecture of the Human RNA-Binding Protein Regulatory Network.
1173 *iScience*. 2019;21:706–19. <https://doi.org/10.1016/J.ISCI.2019.10.058>.
- 1174 114. Pullmann RJ, Kim HH, Abdelmohsen K, Lal A, Martindale JL, Yang X, et al. Analysis of Turnover
1175 and Translation Regulatory RNA-Binding Protein Expression through Binding to Cognate mRNAs. *Mol*
1176 *Cell Biol*. 2007;27:6265–78. <https://doi.org/10.1128/MCB.00500-07>.
- 1177 115. Carbon S, Ireland A, Mungall CJ, Shu S, Marshall B, Lewis S, et al. AmiGO: online access to
1178 ontology and annotation data. *Bioinformatics*. 2009;25:288–9.
1179 <https://doi.org/10.1093/BIOINFORMATICS/BTN615>.

Supplementary Material

Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis

Agata J. Barszcz¹; Katarzyna Tutak^{1,2}; Joanna Zypych-Walczak³; Erik Dassi⁴; Erkut Ilaslan^{1,5}; Małgorzata Dąbrowska¹; Agnieszka Malcher¹; Marta Olszewska¹; Dominik Cysewski⁶; Agnieszka Nadel¹; Tomasz Kolanowski¹; Zofia E. Madeja⁷; Michał Hrab⁸; Alexander Yatsenko⁹; Maciej Kurpisz¹; Zbigniew Warkocki¹⁰; Natalia Rozwadowska^{1*}

¹ Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

² Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland

³ Department of Mathematical and Statistical Methods, Poznan University of Life Sciences, Poznan, Poland

⁴ Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

⁵ DNRF Center for Chromosome Stability, University of Copenhagen, DK-2200 Copenhagen, Denmark

⁶ Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

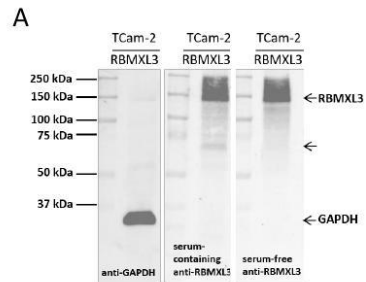
⁷ Department of Genetics and Animal Breeding, Faculty of Veterinary Medicine and Animal Sciences, Poznan University of Life Sciences, Poznan, Poland

⁸ Department of Urology, Poznan University of Medical Sciences, Poznan, Poland

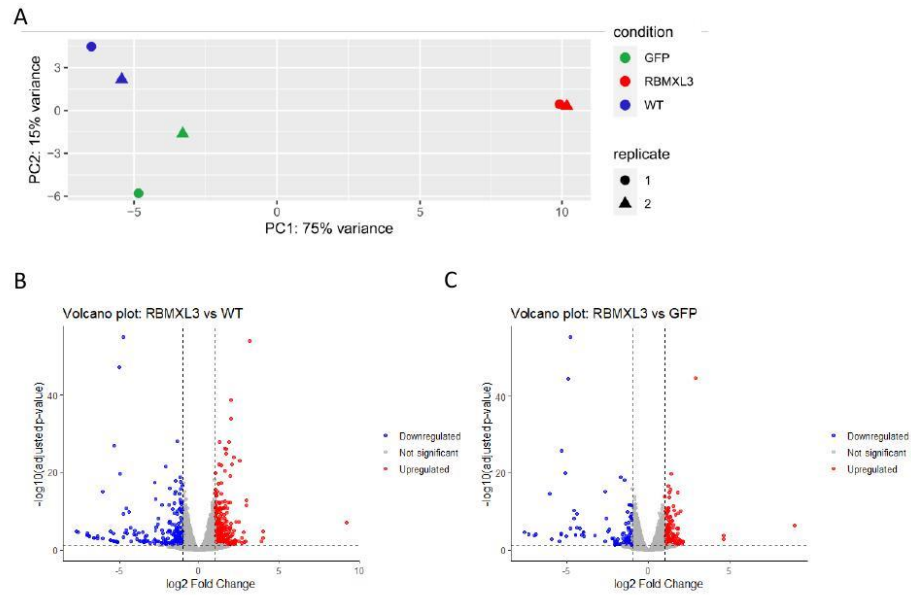
⁹ Department of OBGYN and Reproductive Sciences, Magee-Womens Research Institute, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

¹⁰ Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

*Corresponding author: natalia.rozwadowska@igcz.poznan.pl

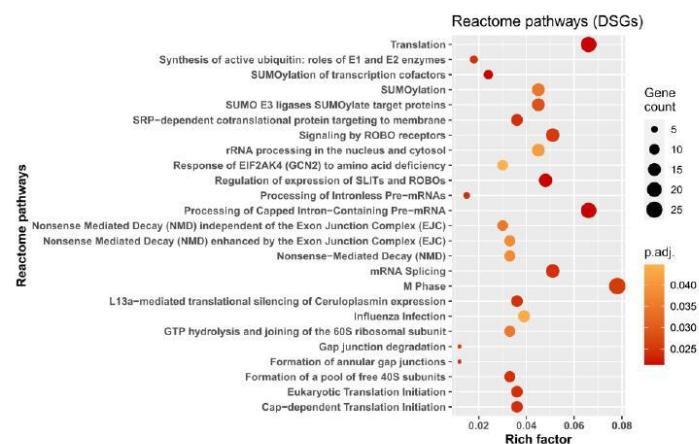


Supplemental Figure S1. RBMXL3 protein detection in TCam-2 cell lysates using RBMXL3-specific antibody. (A) Western Blot of RBMXL3 TCam-2 cells using serum-containing and serum-free RBMXL3 antibody. GAPDH served as a loading control. The additional band appearing between 75 and 50 kDa was derived from serum which was present in one of two batches of RBMXL3 specific antibody used.

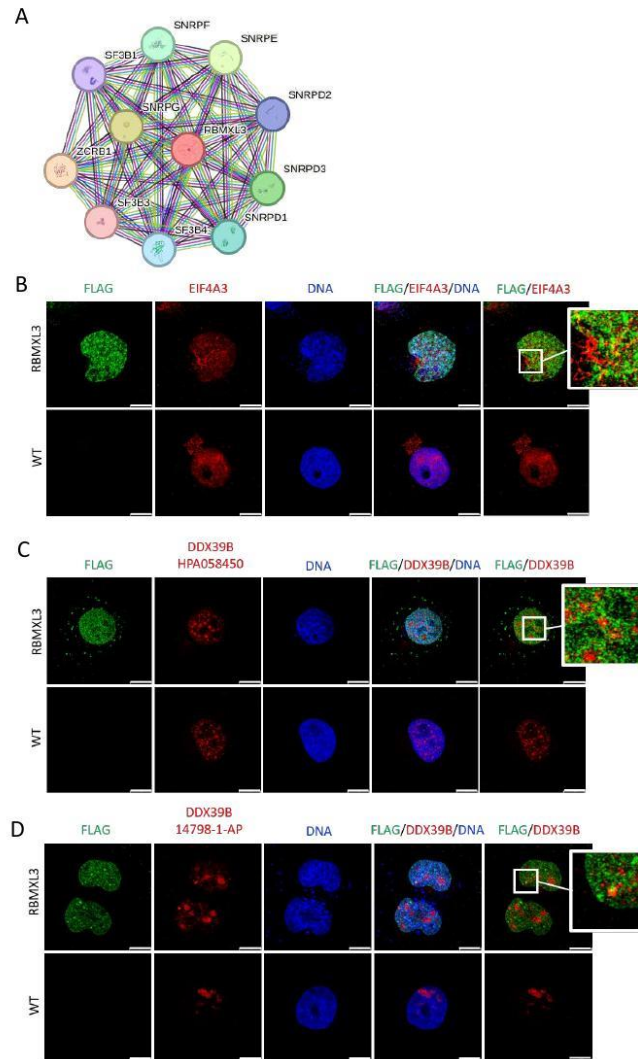


Supplemental Figure S2. Principal component analysis results of TCam-2 RNA-seq data and volcano plots. (A) Principal component analysis (PCA) of the TCam-2 RNA-seq samples. The two biological replicates corresponding to each condition (RBMXL3, GFP and WT) are presented with different shapes. The TCam-2 with RBMXL3 overexpression (highlighted in red) samples separate from the other control samples (GFP and WT, highlighted in green and blue) along principal component 1 (PC1). (B) Volcano plot of differentially expressed genes determined using DESeq2 analysis of RNA-seq data from TCam-2 cells with RBMXL3 overexpression and WT (Supplemental Table S2). Dashed lines represent twofold change in expression (vertical lines) and adjusted FDR < 0.05 cutoff (horizontal line). (C) Volcano plot of differentially expressed genes determined using DESeq2 analysis of RNA-seq data from TCam-2 cells with RBMXL3 and GFP overexpression (Supplemental Table S2). Dashed lines represent twofold change in expression (vertical lines) and adjusted FDR < 0.05 cutoff (horizontal line).

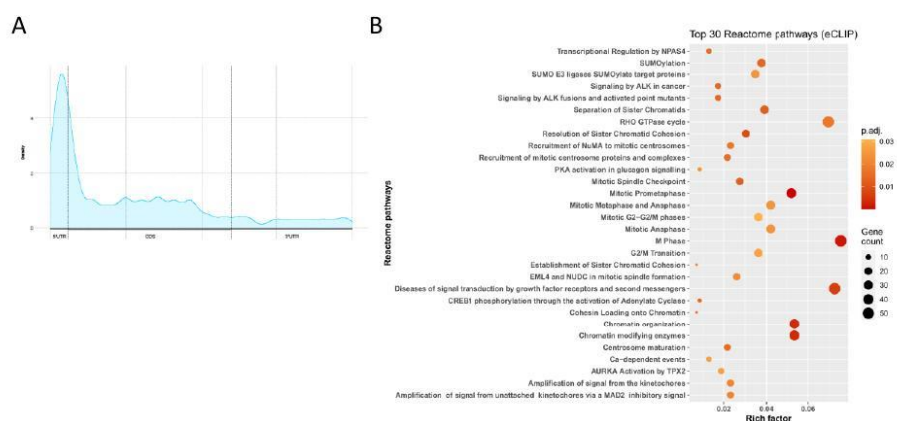
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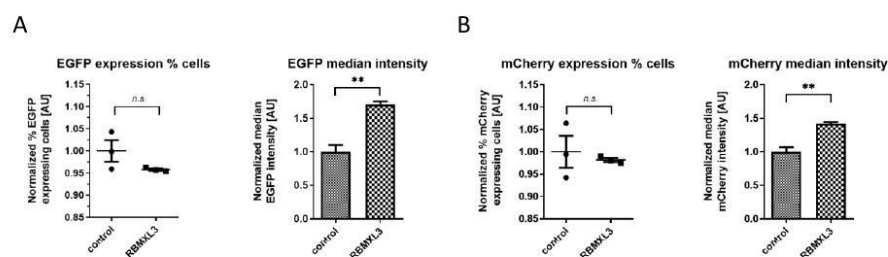
Supplemental Figure S3. Potential pathways regulated by DSGs detected upon *RBMXL3* overexpression and RNA-seq. (A) Reactome analysis for the DSGs based on the RNA-seq data. For the complete list of significant Reactome terms see Supplemental Table S6 (adjusted p-value < 0.05).



Supplemental Figure S4. STRING network for RBMXL3 and immunofluorescence co-staining of RBMXL3 and its potential protein interactors (EIF4A3 and DDX39B) identified by Co-IP-MS. (A) The STRING database network for RBMXL3 protein-protein interactions. (B) Confocal microscopy of TCam-2 cells expressing RBMXL3 and WT using anti-FLAG and anti-EIF4A3 antibodies. The zoom window illustrates signal colocalization of RBMXL3 and EIF4A3. Scale bar 11.6 μ m. (C) Confocal microscopy of TCam-2 cells expressing RBMXL3 and WT using anti-FLAG and anti-DDX39B (HPA058450) antibodies. The zoom window illustrates signal colocalization of RBMXL3 and DDX39B. Scale bar 11.6 μ m. (D) Confocal microscopy of TCam-2 cells expressing RBMXL3 and WT using anti-FLAG and anti-DDX39B (14798-1-AP) antibodies. The zoom window illustrates signal colocalization of RBMXL3 and DDX39B. Scale bar 11.6 μ m.



Supplemental Figure S5. Visualization of RBMXL3 binding and potential pathways regulated by RBMXL3. (A) Visualization of RBMXL3 RNA-binding sites with eCLIP peaks coverage alongside the standard mRNA model according to the MetaTX R package. (B) Reactome analysis for the transcripts bound by RBMXL3 based on eCLIP data. For the complete list of significant Reactome pathways see Supplemental Table S14 (adjusted p-value < 0.05).



Supplemental Figure S6. LINE-1 retrotransposition assay control experiments show that RBMXL3 does not generally reduce EGFP and mCherry protein production which support the role of RBMXL3 in restricting the L1 retrotransposition. (A) Effect of overexpression of RBMXL3 and MBP (as control) on EGFP expression and EGFP median intensity in HEK293T cells. The results of independent experiments were normalized relative to the control (MBP). Statistical significance was calculated using Student's unpaired t-test (n.s. not significant; ** $p < 0.01$; in comparison to MBP; each point = biological replicate). (B) Effect of overexpression of RBMXL3 and MBP (as control) on mCherry expression and mCherry median intensity in HEK293T cells. The results of independent experiments were normalized relative to the control (MBP). Statistical significance was calculated using Student's unpaired t-test (n.s. not significant, ** $p < 0.01$; in comparison to MBP; each point = biological replicate). See Materials and Methods section for more details.

4.3. Artykuł 3: „The RNA helicase DDX53 (CAGE) contributes to RNA metabolism in a human germ cell model”

Wstęp: DDX53 należy do rodziny helikaz RNA typu DEAD-box, określanych mianem kluczowych regulatorów metabolizmu RNA. Białka te uczestniczą m.in. w splicingu oraz regulacji transkryptomu, odgrywając istotną rolę w procesach rozwoju komórek linii płciowej. Jednym z mniej poznanych przedstawicieli tej rodziny jest białko DDX53 (znane również jako CAGE), kodowane przez gen zlokalizowany na chromosomie X. Białko to zalicza się do grupy antygenów nowotworowo-jądrowych (CTA). Mimo licznych doniesień o roli DDX53 w kontekście nowotworów, jego rola w związku z powstawaniem męskich komórek rozrodczych pozostawała dotąd zupełnie nieznana. Przesłanką do podjęcia niniejszych badań były analizy genetyczne przeprowadzone w Instytucie Genetyki Człowieka PAN, które zidentyfikowały warianty *DDX53* u mężczyzn dotkniętych niepłodnością. Dlatego celem publikacji stanowiącej część niniejszej rozprawy doktorskiej była identyfikacja sieci powiązań oraz molekularnych interaktorów białka DDX53 w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka. W badaniach wykorzystano ludzką linię komórkową nowotworu jądra (TCam-2). Pod względem ekspresji genów komórki te przypominają ludzkie pierwotne komórki płciowe, będące embrionalnymi prekursorami linii płciowej, co czyni je cennym modelem badawczym.

Wyniki i dyskusja: Opis wyników w publikacji rozpoczęto od przedstawienia wyników immunohistochemii, które potwierdziły obecność białka DDX53 w męskiej gonadzie. Następnie, w celu zbadania DDX53 wygenerowano model komórkowy TCam-2 z nadekspresją *DDX53-FLAG*, a także *GFP-FLAG* (użyty jako kontrola). Walidację uzyskanego modelu przeprowadzono z wykorzystaniem metod RT-qPCR, Western blot oraz podwójnego barwienia immunofluorescencyjnego z zastosowaniem przeciwciał anty-FLAG oraz anty-DDX53. W celu oceny wpływu nadekspresji białka DDX53 na transkryptom wykonano eksperyment RNA-seq z wykorzystaniem komórek TCam-2 z ekspresją *DDX53-FLAG*, *GFP-FLAG* oraz WT. Zidentyfikowano łącznie 135 genów o różnicowej ekspresji z uwagi na obecność białka DDX53, z których 3 geny (*SERPINF1*, *MZT1*, *FBN2*) wybrano do walidacji, a ich ekspresję potwierdzono metodą RT-qPCR. W kolejnej części publikacji przedstawiono wyniki analizy alternatywnego splicingu (AS). Analiza danych RNA-seq wykazała 1270 zdarzeń AS w 646 transkryptach. Porównanie listy genów potencjalnie ulegających alternatywnemu splicingowi w komórkach z

nadekspresją *DDX53-FLAG* z listą genów przypisanych do terminu GO powiązanego z tworzeniem męskich gamet pozwoliła wyłonić 24 geny, w tym gen *SEPTIN7* istotny dla spermatogenezy człowieka. W puli transkryptów o zróżnicowanym splicingu zidentyfikowano również m.in. *TFAM* oraz *SRSF1*, które zostały już opisane w literaturze jako istotne dla powstawania męskich komórek płciowych, podkreślając tym samym potencjalną rolę DDX53 w procesie spermatogenezy. Analiza funkcjonalna typu GO oraz Reactome wykazała powiązanie genów należących do tej grupy z procesami związanymi z metabolizmem RNA. W następnej części publikacji przedstawiono wyniki analizy dotyczące lokalizacji białka DDX53. W pierwszej kolejności, uwzględniając wcześniejsze doniesienia literaturowe wskazujące na pozytywne wyniki eksperymentów typu ChIP, komórki TCam-2 z nadekspresją DDX53-FLAG poddano analizie ChIP-seq. Przeprowadzony eksperyment ChIP-seq nie wykazał istotnego wzbogacenia sekwencji DNA, co może wynikać z różnic metodologicznych czy ograniczeń technicznych, jednocześnie nie wykluczając pośredniego lub przejściowego oddziaływania. Analiza lokalizacji subkomórkowej wykazała, że DDX53 występuje głównie w cytoplazmie komórek TCam-2, z niewielką frakcją jądrową (~11%), co potwierdzono metodami frakcjonowania i immunofluorescencji. Dodatkowo zaobserwowano obecność struktur przypominających granule germinalne. W publikacji podkreślono, że choć jest to interesująca obserwacja, jej jednoznaczne potwierdzenie wymaga dalszej i pogłębionej charakterystyki. W kolejnej części pracy opisano wyniki, dzięki którym zidentyfikowano RNA bezpośrednio oddziałujące z białkiem DDX53. W tym celu przeprowadzono eksperyment eCLIP-seq, a analiza wyników pozwoliła na identyfikację 181 transkryptów RNA wykrytych w obu replikatach TCam-2. Za pomocą analizy lokalizacji miejsc wiązania RNA przez DDX53 wykazano, że większość zlokalizowana jest w obrębie sekwencji kodujących (CDS), 3'UTR oraz intronów. Analiza motywów ujawniła wzbogacenie sekwencji typu CA. Przeprowadzono także analizę typu GO, która wskazała, że RNA wiązane przez białko DDX53 zaangażowane są m.in. w procesy metabolizmu RNA, transkrypcji oraz różnicowania komórek. Wśród zidentyfikowanych RNA wykryto między innymi transkrypty kodujące *TAF7*, *LIN28B* czy *NCBP2*, które w literaturze zostały już opisane jako istotne dla procesu spermatogenezy. Ponadto na podstawie danych eCLIP-seq wykazano wiązanie DDX53 do własnego transkryptu, co może sugerować istnienie mechanizmu autoregulacyjnego, typowego dla białek wiążących RNA. W celu identyfikacji sieci interakcji białkowych DDX53 przeprowadzono analizę Co-IP-MS, która pozwoliła zidentyfikować 35 potencjalnych

interaktorów zaangażowanych m.in. w regulację transkrypcji, procesy epigenetyczne oraz regulację transkryptów RNA. Przeprowadzone analizy wykazały funkcjonalne powiązania tych białek oraz ich udział w szlakach istotnych dla rozwoju komórek płciowych. Wybrane interakcje poddano dalszej walidacji metodą podwójnego barwienia immunofluorescencyjnego, wykazując kolokalizację DDX53 z białkami ACLY oraz THOC1 przy użyciu mikroskopii konfokalnej. Uzyskane wyniki wskazują na potencjalną interakcję DDX53 z tymi białkami w procesie spermatogenezy oraz możliwy udział DDX53 i jego interaktorów w strukturach przypominających granule germinalne. W publikacji podkreślono jednak, że jednoznaczna interpretacja tej obserwacji wymaga dalszych, ukierunkowanych badań.

Wnioski: Podsumowując, otrzymane wyniki wskazują, że DDX53 pełni funkcję białka wiążącego RNA oraz regulatora transkryptomu w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka. W publikacji wykazano jego obecność w ludzkiej tkance jądra oraz zdolność do wiązania szerokiego spektrum transkryptów. Przeprowadzone analizy RNA-seq, sugerują udział DDX53 w regulacji ekspresji genów oraz procesów alternatywnego splicingu istotnych dla gametogenezy. Lokalizacja DDX53 w cytoplazmatycznych kondensatach przypominających granule germinalne, w połączeniu z identyfikacją interaktorów białkowych, sugeruje jego potencjalnie specyficzną rolę w komórkach linii płciowej, co wymaga dalszej weryfikacji. Uzyskane w publikacji wyniki pozwalają na sformułowanie wniosku, że zaburzenia genetyczne w obrębie tego genu mogą prowadzić do niepowodzenia spermatogenezy u pacjentów, jednak dalsze badania są konieczne, aby poznać bardziej szczegółową rolę DDX53 podczas rozwoju komórek męskiej linii płciowej.

Mój wkład w powstanie publikacji:

- udział w opracowaniu koncepcji i przygotowaniu planu badań
- prowadzenie hodowli komórkowych, wykonywanie transfekcji i transdukcji
- optymalizacja metodologii oraz wykonanie i interpretacja eksperymentów z wykorzystaniem linii komórkowej TCam-2
- wykonanie większości izolacji RNA i białek oraz przeprowadzenie analiz RT-qPCR, Western Blot
- optymalizacja i wykonanie barwień immunofluorescencyjnych oraz Co-IP
- analiza bioinformatyczna danych NGS oraz opracowanie graficznej prezentacji wyników
- napisanie oryginalnej wersji publikacji, przygotowanie większości załączników oraz rycin
- przygotowywanie odpowiedzi dla recenzentów w procesie publikacyjnym
- przygotowanie ostatecznej wersji publikacji

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The RNA helicase DDX53 (CAGE) contributes to RNA metabolism in a human germ cell model

4 Agata J. Barszcz¹, Katarzyna Tutak^{1,2}, Agnieszka Malcher¹, Małgorzata Dąbrowska¹, Joanna Zyprych-
5 Walczak³, Erik Dassi⁴, Erkut Ilaslan^{1,5}, Marta Olszewska¹, Dominik Cysewski⁶, Michał Hrab⁷, Tomasz
6 Kolanowski¹, Alexander Yatsenko^{8,9,10}, Maciej Kurpisz¹ and Natalia Rozwadowska^{1*}

7 ¹ Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

8 ² Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznań,
9 Poland

³ Department of Mathematical and Statistical Methods, Poznan University of Life Sciences, Poznan, Poland

11 ⁴ Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

12 ⁵ DNRF Center for Chromosome Stability, University of Copenhagen, DK-2200 Copenhagen, Denmark

13 ⁶ Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

14 ⁷ Department of Urology, Poznan University of Medical Sciences, Poznan, Poland

15 ⁸ Department of Obstetrics, Gynecology, and Reproductive Sciences, Magee-Womens Research Institute, University of Pitts-
16 burgh School of Medicine, Pittsburgh, PA, United States

17 ⁹ Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States

18 10 Department of Genetics, University of Pittsburgh School of Public Health, Pittsburgh, PA, United States

19 * Correspondence: natalia.rozwadowska@igcz.poznan.pl; Tel.: +48 61 657 92 35

20 **Abstract**

DDX53 (DEAD-box helicase 53, known also as CAGE) is an intronless gene on the X chromosome, which expression shows strong testis specificity. It belongs to the group of cancer-testis (CT) antigens, with most studies to date focusing on its role in cancer, but the precise biological function of DDX53 remains unclear. Previous reports identifying rare *DDX53* variants in infertile men provided the rationale for investigating the role of DDX53 in the context of human spermatogenesis. By using the human seminoma cell line (TCam-2) as an *in vitro* male germline model, we aimed to investigate the function and molecular targets of DDX53 protein. Our eCLIP and RNA-seq data show that DDX53 protein directly interacts with numerous RNA molecules, drives transcriptome changes in human cells, and is involved in alternative splicing of RNA. Moreover, we identified potential DDX53 protein interactors

30 using Co-IP-MS approach. Subcellular localization analysis by confocal microscopy indicated a pre-
31 dominantly cytoplasmic distribution of DDX53, with partial nuclear presence in TCam-2 cells. We also
32 identified DDX53-positive structures that may correspond to germ granule-like assemblies, although
33 their precise nature remains to be determined. Additionally, we confirmed DDX53 presence in human
34 testis using a specific, commercially available anti-DDX53 antibody. Our data indicate that DDX53
35 protein acts as a regulator of RNA metabolism in human cells. Collectively, we show that DDX53 par-
36 ticipates in transcriptome regulation (including splicing) in male germ cells and exhibits transcriptome-
37 wide RNA interactions, but its wider biological role remains to be clarified.

38 **Keywords:** RNA binding; RNA metabolism; splicing; spermatogenesis; infertility

39 **1. Introduction**

40 The *DDX53* (DEAD-box helicase 53; known also as CAGE (cancer-associated gene protein, or can-
41 cer/testis antigen 26)) is an intronless gene, located in the Xp22.11 region, and rare variants within this
42 gene have been recently described as possibly associated with spermatogenesis failure based on whole
43 genome/exome sequencing (WGS/WES) genetic screening of infertile patients [1,2]. Published phylo-
44 genetic trees of *DDX53* demonstrated a high degree of sequence homology with most primate species
45 [3,4]. Interestingly, although it displays an evolutionary relationship with rodent species (such as the
46 European rabbit or guinea pig), it is not expressed in mice. Considering that species-specific differences
47 in the regulation and function of *DDX53* may exist, a comprehensive characterization of this protein and
48 the construction of a suitable research model remain challenging.

49 Importantly, the *DDX53* protein belongs to the group of cancer-testis antigens (CTAs), defined as pro-
50 teins that appear to be present only in germ cells and trophoblasts, but aberrantly expressed in various
51 cancer types [5,6]. Currently, CTAs are considered as potential therapeutic targets or novel biomarkers.
52 Although *DDX53* expression seems to be restricted to the testis and cancers [7–9], previous reports
53 indicate its expression also in the brain and normal colonic mucosa [4,10]. Studies that investigated the
54 role of *DDX53* in the context of cancer reported that it is associated with well known cancer hallmarks
55 [11] such as angiogenesis [12,13], proliferation [14–16], invasion [12,15,17] and metastasis [18–20]. It

has been shown that *DDX53* expression is regulated by hypomethylation of its promoter [21] and microRNAs (such as miR-200b, miR-22, and miR-30a) [12,22,23]. Moreover, *DDX53* possesses oncogenic potential and it has been suggested that hypomethylation of *DDX53* may be associated with tumorigenesis progression and may therefore serve as diagnostic marker in cancer [14,21]. *DDX53* has also attracted attention because its presence confers resistance to anti-cancer drugs [12,15–17,19,20,23–27]. A note of caution is warranted when interpreting existing data on *DDX53*. Our observations suggest that some antibodies commonly used to detect *DDX53* may show partial cross-reactivity with other DDX family members, including *DDX43* (unpublished data). In addition, certain antibodies previously applied have since been withdrawn, raising further questions about their specificity. These factors highlight the need for careful evaluation of antibody-based results and cautious interpretation of conclusions drawn from earlier studies.

According to the Human Protein Atlas (HPA) [28] *DDX53* is expressed predominantly in the human testis tissue, specifically, its presence on mRNA level has been detected in spermatogonia, spermatocytes, and early spermatids where it shows the highest expression.

The *DDX53* contains a DEAD [Asp-Glu-Ala-Asp] box domain which is known to contribute to the binding site of RNA molecules as well as ATP hydrolysis and has been resolved by X-ray crystallography [29]. This protein is thought to have an RNA helicase activity and proteins belonging to this family are known to have essential roles in RNA metabolism (including splicing) and have emerged as crucial players in various stages of germ cell development and not only [30–33]. Notably, the dynamic participation of DEAD-box RNA helicases in RNA metabolism shapes the transcriptomic landscape and thus may influence cell fate [34]. Members of the DEAD-box helicases, for example *DDX3Y*, *DDX25*, and *DDX4* (*VASA*), have well-documented and significant roles in germline generation, spermatogenesis, and male infertility [30].

Although great interest has been placed on CTAs in cancer research, their functions in germ cells of the testis remain poorly understood [35,36]. To our knowledge, this is the first report on the new candidate gene for non-obstructive azoospermia (NOA) [2], the *DDX53*, investigating its potential functions as well as protein and RNA interactors in the context of spermatogenesis. In this study, we examined the role of *DDX53* using the human seminoma cell line (TCam-2) which shares a similar gene expression

84 profile with human primordial germ cells (hPGCs, the precursor cells of the germline) [37] and could
85 serve as an *in vitro* male germline model. Our data provide an overview of DDX53-associated RNA
86 interactions in a human germ cell model, contributing to the current understanding of its potential in-
87 volvement in male gamete differentiation and offering a resource for future studies in cancer-related
88 contexts.

89 **2. Materials and Methods**

90 *2.1. Cell line*

91 All experiments were performed using the human testicular cancer cell line (TCam-2) which was gifted
92 from Prof. Sohei Kitazawa (Division of Molecular Pathology, Kobe University, Japan) [38,39]. The
93 TCam-2 cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640 Medium,
94 GlutaMAX™ Supplement Thermo Fisher Scientific, 61870044) supplemented with 10% heat-inacti-
95 vated Fetal Bovine Serum (Cytiva, HYCLSV30160.03) and 1% Antibiotic-Antimycotic solution (100X)
96 (Thermo Fisher Scientific, 15240062) at 37 °C and in a humidified 5% CO₂ incubator.

97 *2.2. TCam-2 cell line modification by lentiviral transduction*

98 The TCam-2 cells were modified using the lentiviral transduction (3rd generation packaging system)
99 according to the procedure described previously [40]. To generate lentiviruses, the following plasmids
100 were used: pRSV-Rev (Addgene, #12253), pMDLg/pRRE (Addgene, #12251), pMSCV-VSV-G
101 (Addgene, #14888), and pLV[Exp]-Puro-EF1A>EGFP/3xFLAG (Vector Builder, Vector ID
102 VB180409-1201grs) or pLV[Exp]-Puro-EF1A>hDDX53[NM_182699.3]/3xFLAG (Vector Builder,
103 Vector ID VB180321-1148yrw) in ratio 1:1:1:2. After 72h, post transfection pseudoviral particles were
104 collected and filtered through 0.45 µm (Millipore, SLHVR13SL). Viral aliquots were frozen and stored
105 at -80 °C. Next 1×10^5 TCam-2 cells were incubated with the viral supernatant in the presence of
106 polybrene (final concentration 4µg/ml, Sigma-Aldrich, TR-1003-G) for 48h. After incubation with the
107 pseudoviral particles, selection with puromycin (final concentration 1 µg/ml, Thermo Fisher Scientific,
108 A1113803) was initiated and continued for 4 days in order to obtain cells stably expressing the DDX53-
109 FLAG and GFP-FLAG (as control).

110 2.3. Transient transfection

111 The TCam-2 cells were transfected with plasmids listed below using jetPRIME transfection reagent
112 (Polyplus, 114-15). For immunofluorescence experiments, cells were seeded on 15 mm round glass
113 coverslips in 12-well plates and transfected using a mixture prepared according to the manufacturer's
114 protocol (0.8 µg plasmid, 1.6 µl reagent, 75 µl buffer; 75 µl per well). For other experiments, cells were
115 cultured in 150 mm dishes and transfected with a mixture prepared according to the same protocol (31.5
116 µg plasmid, 63 µl reagent, 1.3 ml buffer; 1.3 ml per dish) [40]. Next, the TCam-2 cells were cultured in
117 a complete medium with transfection mixtures for 2 days and then subjected to downstream experi-
118 ments. Plasmids used: (pLV[Exp]-Puro-EF1A>hDDX53[NM_182699.3]/3xFLAG (Vector Builder,
119 Vector ID VB180321-1148yrw), pLV[Exp]-Puro-EF1A>EGFP/3xFLAG (Vector Builder, Vector ID
120 VB180409-1201grs).

121 2.4. RT-qPCR and statistical analysis

122 Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, 74136) and DNA residues were removed
123 by TURBO DNA-free™ kit (Thermo Fisher Scientific, AM1907) treatment according to the manufac-
124 turer's protocol. Reverse transcription was conducted with SuperScript IV Reverse Transcriptase kit
125 (Thermo Fisher Scientific, 18090050) with the use of random primers (Promega, C1181) using 1 µg of
126 total RNA for each reaction. Ten ng of cDNA was used for qPCR, performed with PowerUp SYBR
127 Green Master Mix (Thermo Fisher Scientific, A25778) on CFX96 Touch qPCR System (Bio-Rad). All
128 samples were normalized to ACTB and GAPDH according to the geNorm algorithm. Statistical analyses
129 were performed in GraphPad Prism (v8.0.1) using ordinary one-way ANOVA non-parametric test with
130 Tukey's multiple comparisons test. All primer sequences are listed in Supplementary Table S14.

131 2.5. RNA sequencing and bioinformatic analysis

132 The human TCam-2 cells transduced with *DDX53-FLAG*, *GFP-FLAG* and non-transduced WT cells
133 were subjected to RNA sequencing (RNA-seq) experiment. The GFP-expressing and WT cells were
134 used as negative controls. Total RNA was isolated and DNase-treated according to the manufacturer's
135 instructions as it was described above. The cDNA libraries were prepared using TruSeq Stranded mRNA
136 LT kit (Illumina) and then the next-generation sequencing was performed on the Illumina NovaSeq6000

platform, 150 bp paired-end reads with at least 30 M reads per sample. Library preparation and sequencing were performed by Macrogen NGS Service (Korea). All RNA-seq experiments were done in duplicate.

RNA-seq data pre-processing was performed by removing low-quality reads ($Q < 30$) and sequencing adapters using Trim Galore (v0.6.10) (<https://github.com/FelixKrueger/TrimGalore>). Details of each specific RNA-seq analysis are provided in the following sections.

2.5.1. Differential expression analysis of RNA-seq data

The RNA-seq data were analyzed as we described previously [40]. The pre-processed RNA-seq reads were mapped to the human reference genome (GRCh38/hg38, Gencode v44) using STAR (v2.7.11a) [41] (`--outSAMtype BAM SortedByCoordinate --quantMode GeneCounts`). BAM files were sorted and indexed using samtools (v1.19.2) [42]. Read counts were then normalized by DESeq2 R package [43]. Lowly expressed genes (with less than 0.1 mean CPM in each condition i.e. DDX53, GFP or WT) were excluded from further analysis. The differential expression analysis was then performed to compare the ‘DDX53 vs. GFP’ and the ‘DDX53 vs. WT’ conditions using DESeq2. Significant differentially expressed genes (DEGs) were selected based on adjusted p-value ($FDR < 0.05$ and $|\log_2FC| \geq 1$). As the final list of genes we intersected the significant DEGs of the two comparisons (‘DDX53 vs. GFP’, ‘DDX53 vs. WT’), and we selected only those genes with the same trend (upregulation or downregulation) in both comparisons.

The Gene ontology (GO) and pathway enrichment analyses were performed with the topGO and clusterProfiler [44] R packages, using a 0.05 threshold on Benjamini-Hochberg (BH) adjusted p-value.

2.5.2. Differential splicing analysis of RNA-seq data

We employed the Salmon tool (v1.10.2) [45] to quantify transcript-level expression (TPM) in each condition (DDX53, GFP, and WT) and aligned the pre-processed RNA-seq reads to the reference transcriptome (Gencode v44). Next, we used the SUPPA2 tool (v2.3) [46,47] to perform a differential splicing analysis, setting a threshold of $p \leq 0.05$ and $|\Delta PSI| \geq 0.15$. To identify potentially more specific splicing events, the final list of differentially spliced genes (DSGs) was prepared by doing an intersection between the significant predicted splicing events from the two comparisons (‘DDX53 vs. GFP’ and

‘DDX53 vs. WT’). During the SUPPA analysis, the WT and GFP samples were set always as Cond1 and DDX53 as Cond2. The Gene ontology and pathway enrichment analyses were performed with the topGO and clusterProfiler [44] R packages, using a 0.05 threshold on BH adjusted p-value.

2.6. DDX53 eCLIP and bioinformatic analysis

Two days after transfection (using the pLV[Exp]-Puro-EF1A>hDDX53[NM_182699.3]/3xFLAG plasmid), TCam-2 cells on multiple 150 mm cell culture dishes were crosslinked at 254-nm UV with an energy setting of 400 mJoules/cm² (Analytik Jena, CL-3000). Two biological replicates of TCam-2 cells expressing the DDX53-FLAG were used in eCLIP-seq performed by Eclipse BioInnovations (USA). Briefly, the cells were lysed, RNase I treated, and protein-RNA complexes were immunoprecipitated with anti-FLAG antibody coupled to magnetic beads. Sequencing was performed as SE72 on the NextSeq platform (Illumina). The eCLIP experiment was performed according to Eclipse BioInnovations’ standard RBP-eCLIP pipeline as previously described [48]. Raw sequencing reads were trimmed of UMIs and adapters, filtered out of reads that mapped to repeat elements, aligned to the reference genome (hg38) with STAR (v2.7.11a), and filtered of PCR duplicates. Final peaks for each sample were filtered for enrichment over the input ($|\log_2FC| \geq 3$) and statistical significance (adjusted p-value ≤ 0.001). De novo motif analysis for DDX53 was performed with HOMER (v4.11).

2.7. Co-immunoprecipitation and mass spectrometry (Co-IP-MS)

3×10^6 of TCam-2 cells after transfection with plasmids coding for DDX53-FLAG or GFP-FLAG (used as a negative control) were crosslinked for 10 mins at RT using 0.3% formaldehyde (Thermo Fisher Scientific, 28906), then quenched with 125 mM glycine (BioShop, GLN001) in PBS for 5 min at RT and 5 mins on ice, and finally lysed with 1ml of RIPA buffer (Sigma-Aldrich, R0278) supplemented with protease inhibitors (Thermo Fisher Scientific, 87786) at 4°C for 30 min on rotator. Total protein lysates were centrifuged (4°C, 10 min, 11 000 x g) and 1 ml was incubated with 40µl of anti-FLAG M2 magnetic beads (Sigma-Aldrich, M8823) at 4°C for 3 h with gentle rotation. After IP reaction, beads were washed three times with ice-cold TBS washing buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4) and one time with DPBS (Thermo Fisher Scientific, 14190169). Three technical replicates of IP reactions were prepared. For mass spectrometry (MS), beads were snap frozen with liquid nitrogen and

processed as previously described [40]. MS analysis was carried out at the Mass Spectrometry Laboratory, IBB PAS. Dried beads were suspended in 50µl of 100mM ABC. Proteins were reduced and alkylated with TCEP/MMTS, followed by overnight digestion with trypsin (Promega, V5111) as described previously [49]. Peptide digest was purified with HLB 96-well plates (Waters, WAT058951), vacuum-dried, dissolved in 98% water (J.T.Baker 4218-03)/ 2% MeCN (J.T. Baker HPLC JT9012)/ 0.1%TFA (Supelco, 80457), and analyzed by an online LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using a Nano-Acquity (Waters) UPLC system and QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Peptides were first loaded onto a precolumn (nanoACQUITY UPLC Trapping Column Waters) using 0.1% formic acid in water as a mobile phase and then transferred to a nano-column (nanoACQUITY UPLC BEH C18 Column (75 µm inner diameter; 250 mm long; Waters, 186007482)) using an acetonitrile gradient (5–35% AcN in 180 min) in the presence of 0.1% formic acid with the flow rate of 250 nl/min. Each analysis was preceded by three washing runs to ensure there was no cross-contamination from previous samples. The column outlet was directly coupled to the ion source of the spectrometer working in the regime of data-dependent acquisition MS to MS/MS switch. Peptides were directly eluted into the ion source of the mass spectrometer. MS data were acquired in the m/z range of 300–2000. The resulting spectra were searched against the human UniProt reference proteome (79,052 entries) using MaxQuant (v1.5.3.0) [50]. The following parameters were applied: a match between runs (match time window, 0,7 min; alignment time, 20 min); enzyme, trypsin/p specific; max missed, 2; minimal peptide length, 7 aa; variable modification, methionine, and proline oxidation; fixed, cysteine alkylation (Methylthio); main search peptide tolerance, 4.5 ppm; protein FDR 1%. Finally, 36 proteins identified in each DDX53 replicate but not present in negative controls (GFP) were classified as potential DDX53 protein interactors. Gene ontology analysis among identified proteins was performed in Cytoscape (v3.9.1) using ClueGO plugin (v2.5.9), 4% enrichment cutoff was used meaning that the number of genes should represent at least 4% of total genes in a given process. STRING analysis of protein-protein interaction (PPI) networks was conducted using STRING website (v12.0), a 0.4 cutoff score was used which means interactions below this value were filtered out.

2.8. Immunofluorescence of TCam-2 cells

218 The TCam-2 cells were immunostained according to the procedure described previously [40]. Briefly,
219 cells were washed with DPBS (Thermo Fisher Scientific, 14190169), fixed with 4% paraformaldehyde
220 (Boster Bio, AR1068) at 4 °C for 15 min, washed again with DPBS, permeabilized with 0.55% Triton
221 X-100 in DPBS for 30 mins at RT and blocked for 1h at RT using blocking solution (10% of suitable
222 serum derived from the host animal species of the secondary antibody, diluted in 0.55% Triton-X100 in
223 DPBS). After overnight incubation at 4 °C with primary antibodies diluted in the blocking solution and
224 subsequent washing with DPBS, the cells were incubated with secondary antibodies diluted in 0.55%
225 Triton X-100 in DPBS for 1 h at RT in the dark. After washing, cells were mounted in Fluoroshield with
226 DAPI mounting medium (Sigma-Aldrich, F6057).

227 Primary antibodies used: rabbit DDX53 (CAGE) 1:100 (Sigma-Aldrich SAB1402037), mouse anti-
228 FLAG 1:500 (Sigma-Aldrich, F1804), rabbit anti-DYKDDDDK 1:2000 (GenScript, A00170), mouse
229 anti-THOC1 1:1000 (Abcam, ab487), rabbit anti-SCML2 1:500 (Sigma-Aldrich, HPA064713), rabbit
230 anti-ACLY 1:1200 (Thermo Fisher Scientific, PA5-29495). Secondary antibodies used: goat anti-mouse
231 Alexa Fluor 594 1:500 (Thermo Fisher Scientific, A-11005), goat anti-rabbit Alexa Fluor 488 1:1000
232 (Abcam, ab150077), donkey anti-mouse Alexa Fluor 488 1:1000 (Thermo Fisher Scientific, A-21202),
233 goat anti-rabbit Alexa Fluor 594 1:500 (Thermo Fisher Scientific, A-11012).

234 2.9. Confocal microscopy and image analysis

235 All images were acquired using Leica DMI8 confocal microscope with Leica STELLARIS STED sys-
236 tem equipped with Leica HyD photodetectors (Leica Microsystems). Immunofluorescence images were
237 captured and processed using Leica Application Suite X (LAS X) software (v4.4.0.24861) and the HC
238 PL APO CS2 objective (100X magnification, 1.4 NA).

239 In order to investigate the localization of the DDX53 protein acquired images were analyzed for as-
240 sessing the percentage of red channel intensities in each nucleus to the whole cell by manually selecting
241 the nucleus area in each optical section of the height scan (z-stack). Each optical section was 0.2 um
242 thick and for every one the intensity sum in the nucleus and intensity sum in the whole cell was calcu-
243 lated. For each cell, at least 4 optical sections were selected for analysis. Subsequently, the ratio of

intensity sum in the nucleus to the whole cell was assessed by adding values obtained for each optical section. Finally, the average ratio for all cells selected for analysis was calculated.

2.10. Immunohistochemistry

Immunohistochemistry (IHC) procedure was performed as we described previously [40]. For the IHC staining, commercial human adult testis paraffin sections were used (Zyagen, HP-401). Slides were incubated in 60°C for one hour. Immunohistochemical stainings were performed using specific DDX53 antibody (Sigma-Aldrich SAB1402037). To determine the appropriate antibody dilution, eliminate false positive results, and reduce the background staining, a series of positive control reactions were performed using slides with normal testis cells present in section derived from patient with seminoma. The immunohistochemistry procedure was performed using EnVisionFlex+ visualization system kit (Dako, Agilent Technologies, K8002). Slides were then dewaxed and epitopes were unmasked in PT-Link device (Dako, Agilent Technologies) with the use of EnVisionFlex Target Retrieval Solution Low pH (Dako, Agilent Technologies, K8005). Tissue sections were then incubated for 20 mins at RT with primary antibody. The antigen-antibody complexes were visualized using EnVision FLEX+ Rabbit LINKER (Dako, Agilent Technologies, K8009) for 20 min at RT and localized using 3-3'diaminobenzidine (DAB) as chromogen. Slides were counterstained with hematoxylin, dehydrated in ethanol, incubated in a series of xylenes, and cover-slipped using mounting medium (Dako, Agilent Technologies). Protein expression was assessed under the Leica DMi8 light microscope.

2.11. Western blot

TCam-2 cells were lysed on a rotator for 30 min in RIPA buffer (Sigma-Aldrich, R0278) supplemented with protease inhibitors (Thermo Fisher Scientific, 87786). Samples were subjected to three rounds of sonication at 4°C [20 s on, 30 s off] using Bioruptor Pico (Diagenode, Belgium). Then, protein samples were centrifuged at 15000 x g for 10 min at 4°C and supernatants were mixed with 4X Laemmli Sample Buffer (Bio-Rad, 1610747) with 340 mM DTT (Thermo Fisher, R0861), denatured for 10 min at 95°C, separated on 8% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, 1620097). Membranes were blocked in 5% non-fat milk in TBS buffer with 0.1% Tween-20 for 1 hour, and incubated overnight at 4 °C with primary antibodies diluted in the blocking solution. Washing steps

(3x10min. with TBS buffer with 0.1% Tween 20) were followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1h at RT. Blots were washed twice with TBS buffer with 0.1% Tween-20 for 10 min and twice with TBS for 10 min. The chemiluminescent signal was detected using the Clarity Western ECL Substrate (Bio-Rad, 1705061) using ChemiDoc MP Imaging System (Bio-Rad). Antibodies used in this study were as follows: mouse anti-FLAG 1:1000 (Sigma Aldrich, F1804), mouse anti-GAPDH 1:10 000 (Santa Cruz, sc-365062), mouse anti-TUBB 1:5000 (Abcam, ab131205), mouse anti-SNRNP70 1:5000 (Santa Cruz, sc-390899), rabbit anti-H3C1 1:5000 (Abcam, ab18521), HRP-conjugated anti-rabbit 1:5000 (Abcam, ab6721), HRP-conjugated anti-mouse 1:10 000 (Santa Cruz Biotechnology, sc-516102).

2.12. Subcellular fractionation

Protein fractionation of TCam-2 cells (lentiviral-modified and WT) was performed according to the previously described method [51]. Briefly, cell pellets were lysed on ice using 1× lysis buffer (10 mM Tris-HCl pH 8, 300 mM sucrose, 10 mM NaCl, 2 mM MgAc₂, 3 mM CaCl₂, 0.1% Nonidet P-40, 0.5 mM DTT), followed by centrifugation at 1,000×g for 5 min at 4°C to obtain the cytoplasmic fraction. The nuclear pellet was subsequently washed once with glycerol buffer (50 mM Tris-HCl pH 8.0, 25% glycerol, 5 mM MgAc₂, 0.1 mM EDTA, 5 mM DTT). To further separate nucleoplasmic and chromatin fractions, nuclei were lysed in urea buffer (20 mM HEPES pH 7.5, 7.5 mM MgCl₂, 0.1 mM EGTA, 0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 1 mM DTT) and centrifuged at 14,000×g for 10 min at 4°C. All buffers were supplemented immediately before use with 20 U/mL RNaseOUT (Thermo Fisher Scientific, 10777019) and 1× Complete™ EDTA-free protease inhibitor cocktail (Thermo Fisher Scientific, 87786).

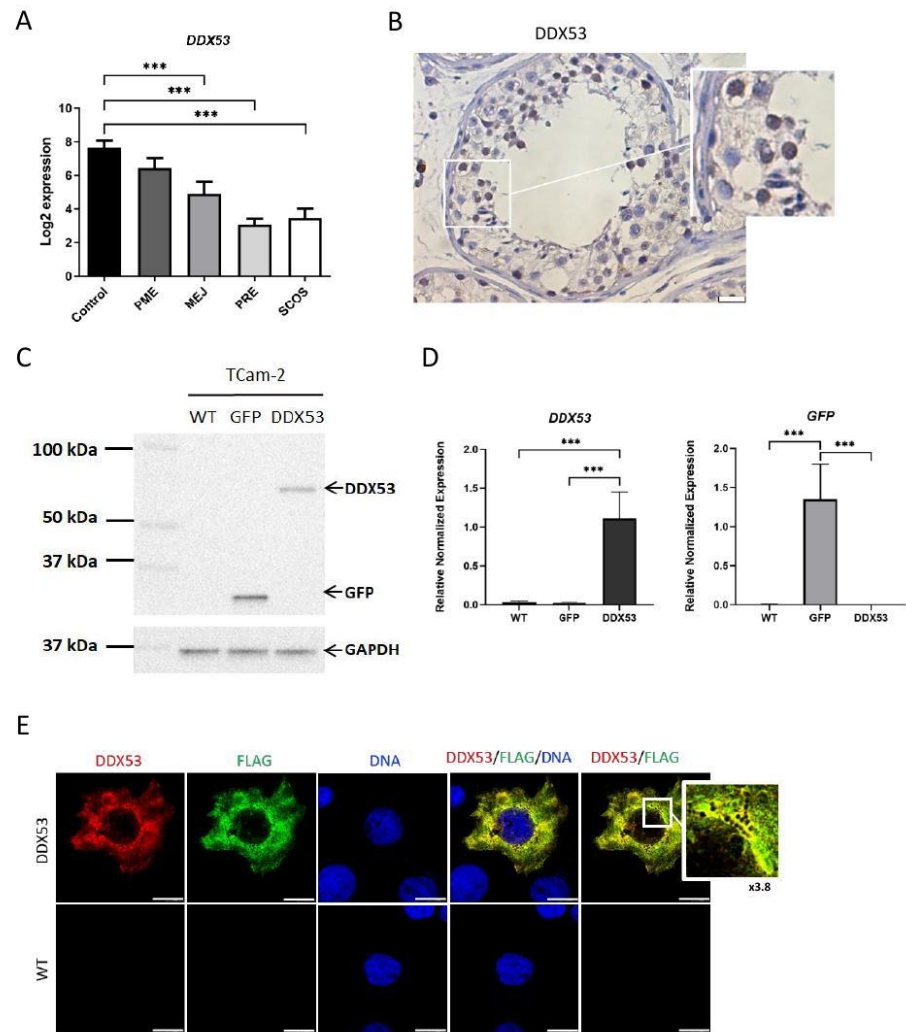
3. Results

3.1. Detection of DDX53 protein in the human testis and generation of in vitro germ cell model with DDX53 overexpression

The gene expression profile analysis, based on independent data set [52,53], showed differences in the expression of *DDX53* in infertile men with idiopathic non-obstructive azoospermia (NOA) compared to

men with normal spermatogenesis (Figure 1A). In order to confirm the presence of DDX53 in normal adult human testis we performed immunohistochemical staining for DDX53 using tissue section (Figure 1B). This experiment showed that DDX53 is present in spermatocytes but it is the most visible in spermatids. The detection of DDX53 in normal human testis, lower expression level in azoospermic patients, and possible association of *DDX53* with spermatogenesis failure based on the literature [1,2] encouraged us to investigate its function in the context of spermatogenesis. In our study, we used the human seminoma cell line (TCam-2), which in terms of gene expression resembles human primordial germ cells (hPGC), the embryonic precursors of gametes [37]. For the purpose of investigating the DDX53 protein function, we modified WT TCam-2 cells to obtain cells expressing the FLAG-tagged *DDX53* and FLAG-tagged *GFP* (as a control). Successful experimental model generation was confirmed by RT-qPCR and Western Blot (Figure 1C, D). Moreover, co-immunofluorescence staining using anti-FLAG and specific anti-DDX53 antibodies demonstrated complete signal co-localization coming from the *DDX53-FLAG* (Figure 1E).

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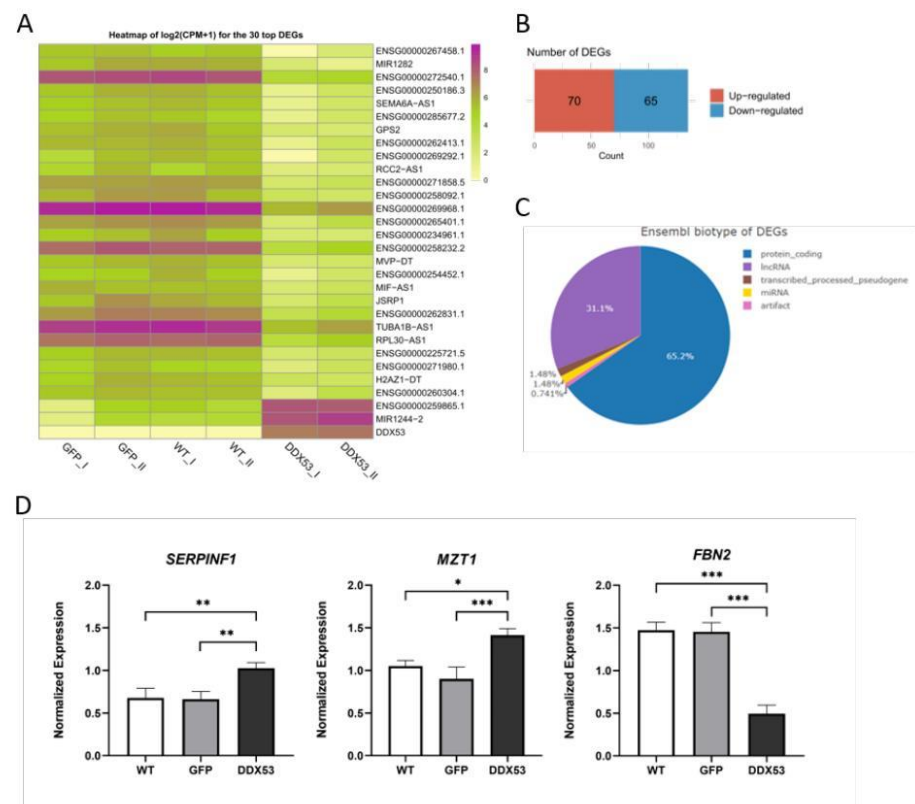
Figure 1. The *DDX53* expression in men with idiopathic non-obstructive azoospermia (NOA), *DDX53* in human testis with maintained spermatogenesis, and lentiviral transduction for stable expression of *DDX53* in TCam-2 cells. **(A)** Gene expression level of *DDX53* in men with normal spermatogenesis (control, n=5) compared to men with different types of azoospermia (PME - postmeiotic arrest, n = 6; MEJ - meiotic arrest, n = 5; PRE - premeiotic arrest, n = 3; SCOS - Sertoli cell-only syndrome, n = 5) analyzed by microarrays analysis using published data sets [52,53]. Error bars represent standard deviation (SD), ***p < 0.001. The p-values were calculated by ordinary one-way ANOVA non-parametric test with Tukey's multiple comparisons test. **(B)** Immunohistochemistry of

DDX53 using specific DDX53 antibody in the normal adult human testis. Scale bar 25 μ m. (C) Representative Western Blot analysis for proteins tagged with FLAG (DDX53 and GFP) using TCam-2 cells after transduction and wild type (WT, as control) using FLAG antibody. GAPDH served as a loading control. (D) The mRNA expression level of the *DDX53-FLAG* and *GFP-FLAG* in TCam-2 cells modified with lentiviral transduction, and with *GAPDH* and *ACTB* as reference genes. Error bars represent standard deviation (SD) with $n = 3$, *** $p < 0.001$. The p-values were calculated by ordinary one-way ANOVA non-parametric test with Tukey's multiple comparisons test. (E) Co-immunofluorescence staining using anti-FLAG and specific anti-DDX53 antibody in the TCam-2 cells expressing DDX53-FLAG and wild type (WT). The zoom window illustrates signal colocalization of DDX53 and FLAG, the magnification factor ($\times 3.8$) is indicated below. Scale bar 20 μ m.

3.2. DDX53 protein regulates the human transcriptome and influences alternative splicing of RNA transcripts

Subsequently, in order to investigate the impact of DDX53 protein overexpression on the TCam-2 transcriptome we performed RNA-sequencing (RNA-seq) using cells stably expressing either the *DDX53-FLAG* or *GFP-FLAG* as well as wild-type (WT) cells (Figure 2A, Supplementary Table S1; Supplementary Figure S1A). Since both the GFP and WT TCam-2 cells were used as controls we focused on genes that were the differentially expressed genes (DEGs) in both comparisons i.e. 'DDX53 vs. GFP' and 'DDX53 vs. WT' (see Materials and Methods section for more details). Employing a threshold of $|\log_2FC| \geq 1$ and $FDR < 0.05$, we identified 70 up-regulated genes and 65 down-regulated genes induced by *DDX53* overexpression (Figure 2B, Supplementary Table S2, Supplementary Figure S1B, C). The majority of up-regulated DEGs belonged to protein-coding transcripts, however, in the group of DEGs there were also long non-coding RNAs (lncRNAs) (Figure 2C). In order to validate our RNA-seq results, we selected three genes expressed in human testis (*SERPINF1*, *MZT1*, and *FBN2*) and confirmed their mRNA expression pattern in TCam-2 cells overexpressing *DDX53* via RT-qPCR with respect to both controls, *GFP* and WT cells (Figure 2D, Supplementary Figure S1D).

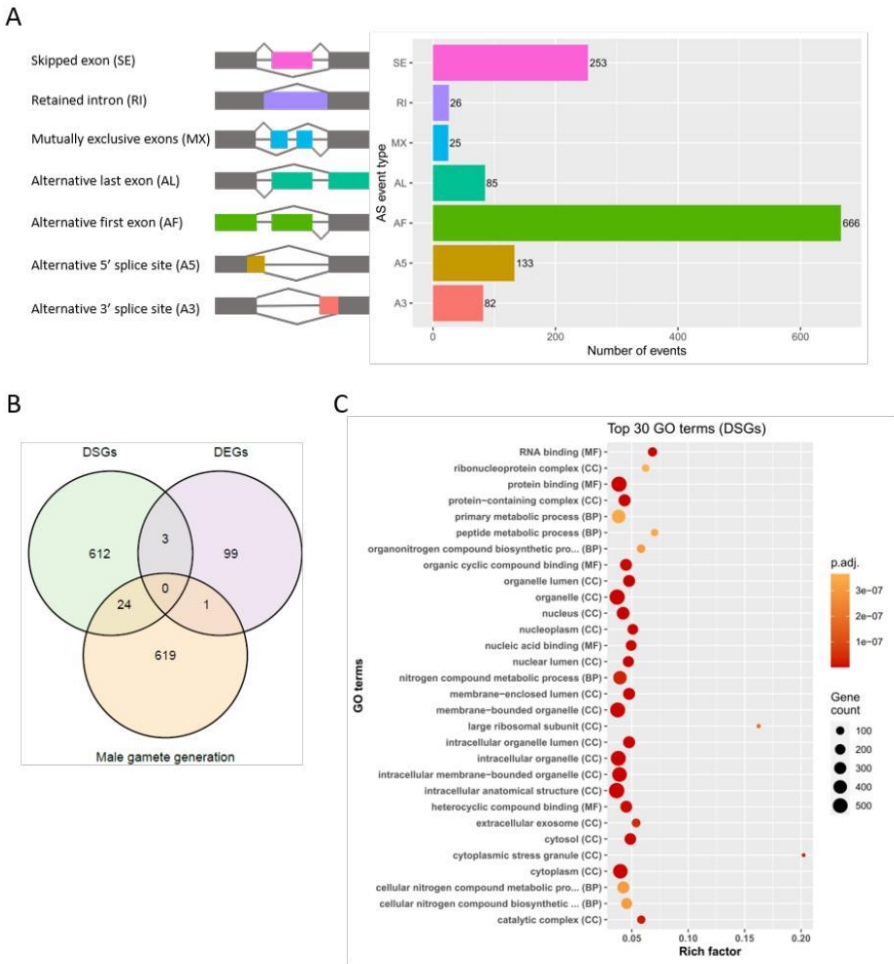
343



344

345 **Figure 2.** Identification of differentially expressed genes upon *DDX53* overexpression in TCam-2 cells. (A)
346 Heatmap of log2(CPM+1) for the top 30 differentially expressed genes (DEGs) based on RNA-seq analysis of
347 TCam-2 cells overexpressing *DDX53-FLAG*, *GFP-FLAG*, and WT ($|\log_2FC| \geq 1$, FDR < 0.05). The top 30 DEGs
348 were selected based on the highest $|\log_2FC|$ and the lowest FDR values derived from the ‘*DDX53* vs. *GFP*’ com-
349 parison (Supplementary Table S1, 2). (B) The number of down-regulated and up-regulated differentially expressed
350 genes (DEGs) based on RNA-seq analysis (Supplementary Table S2), $|\log_2FC| \geq 1$, FDR < 0.05. (C) Piechart
351 showing the Ensembl biotype of DEGs based on RNA-seq analysis of TCam-2 cells ($|\log_2FC| \geq 1$, FDR < 0.05).
352 (D) Validation results of selected (*SERPINF1*, *MZT1*, and *FBN2*) DEGs via RT-qPCR confirmed RNA-seq ex-
353 pression pattern from cells overexpressing *DDX53-FLAG*, *GFP-FLAG*, and WT. Error bars represent standard
354 deviation (SD) with n = 3, ***p < 0.001, **p < 0.01, *p < 0.05. The p-values were calculated by ordinary one-
355 way ANOVA non-parametric test with Tukey's multiple comparisons test.

Due to the fact that some of RNA helicases are thought to participate in RNA splicing, we analyzed the RNA-seq data using SUPPA2 software which allows for the identification of different alternative splicing (AS) events. To detect potentially more specific splicing events, we focused on significant splicing events present in both comparisons ('DDX53 vs. GFP' and 'DDX53 vs. WT', see Materials and Methods section for more details). According to SUPPA2 analysis, 1270 splicing events in 646 transcripts ($p \leq 0.05$ and $|\Delta PSI| \geq 0.15$) were predicted to occur in TCam-2 cells upon expression of DDX53 protein (Supplementary Table S3, 4). The majority of AS events were alternative first exon ($n=666$) and exon skipping ($n=253$) (Figure 3A). Then we extracted the transcripts associated with human male gamete generation by using the genes list annotated to the GO term (GO:0048232) and compared them with differentially spliced genes (DSGs) and DEGs. This integrative analysis identified 24 genes, with *PAFAH1B1*, *BAX*, *CDK16*, *SEPTIN7*, *RAN* and *YTHDF2*, among others, that potentially were differentially spliced due to DDX53 protein presence in TCam-2 cells (Figure 3B, Supplementary Table S5). Gene ontology (GO) enrichment analysis performed among the DSGs (Supplementary Table S6) showed that they are associated with terms such as spindle microtubule, mitotic cell cycle process, chromosome, DNA-templated transcription, sex chromosome but also regulation of RNA splicing, mRNA transport and mRNA processing. To further explore the potential involvement of DDX53 targets in spermatogenesis, the Reactome pathway analysis was carried out among the DSGs. This identified terms such as Nonsense-Mediated Decay (NMD), mRNA splicing, and translation, pathways crucial during the differentiation of germ cells (Supplementary Figure S2; Supplementary Table S6).



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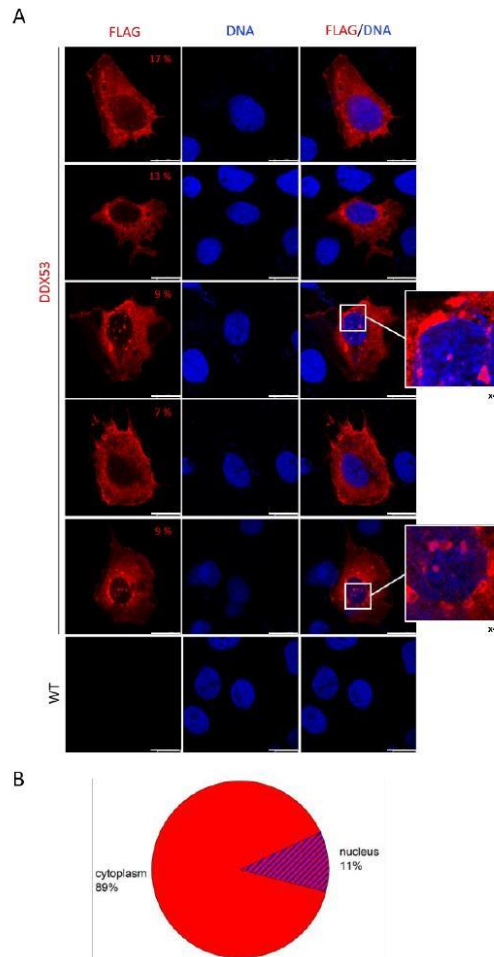
376 **Figure 3.** Global identification of alternative splicing (AS) events upon *DDX53* overexpression. (A) The total
377 number (1270) of differential alternative splicing (AS, $p \leq 0.05$ and $|dPSI| \geq 0.15$) events detected by SUPPA2 anal-
378 ysis of RNA-seq data from TCam-2 cells with *DDX53-FLAG* overexpression (Supplementary Table S4). (B) Venn
379 diagram illustrating the total number of DSGs and DEGs based on RNA-seq analysis compared to the list of genes
380 associated with human male gamete generation (Supplementary Table S5). The male gamete generation genes list
381 annotated to GO:0048232 was retrieved from AMIGO2 (accession date 2024-02-02). Intersection was prepared
382 using gene symbols. (C) GO terms significantly (adjusted p-value < 0.05) enriched for the differentially spliced
383 genes (DSGs) due to overexpression of the *DDX53* protein in TCam-2 cells. For the complete list of GO terms
384 see Supplementary Table S6.

385 3.3. DDX53 protein is predominantly cytoplasmic but also exhibits partial nuclear localization in 386 human TCam-2 cells

387 Previous studies have reported positive DDX53 chromatin immunoprecipitation (ChIP) assay signals,
388 suggesting its binding at promoter regions of protein-coding genes and microRNAs [12,15,23,27,54].
389 Surprisingly, in our hands, performed ChIP-seq based on FLAG-tagged protein did not show any DNA
390 sequences enrichment (unpublished data). This discrepancy may reflect technical differences between
391 studies, including potential limitations related to the specificity or performance of the antibodies used
392 to detect DDX53 in previous reports. However, an indirect or transient association of DDX53 with
393 chromatin, potentially mediated through protein-protein interactions, cannot be excluded. To examine
394 the localization of DDX53 in human TCam-2 cell line, we performed a series of immunofluorescence
395 stainings using FLAG antibody in cells with transient overexpression of the DDX53 protein. The de-
396 tailed analysis of DDX53 localization using confocal microscopy and confirmed its presence not only
397 in the cytoplasm but also in the nucleus of TCam-2 cells, albeit at a very limited level (Figure 4A). We
398 calculated the percentage of DDX53 protein presence in the nucleus for each cell separately and obtained
399 the average nuclear localization with respect to the whole cells, which remained low, reaching 11%
400 (Figure 4B). Our immunofluorescence staining images demonstrated similar DDX53 staining pattern
401 consistent with previous report [27]. Additionally, to test the conclusion regarding the partial nuclear
402 localization of DDX53, we performed subcellular fractionation using WT and DDX53-expressing
403 TCam-2 cells (Supplementary Figure S3A). This experiment showed that DDX53 is predominantly lo-
404 calized in the cytoplasm, with a small fraction present in the nucleus of TCam-2 cells. We acknowledge
405 that these findings are based on a single cell line and that it would be valuable to examine DDX53
406 cellular localization in additional cell lines.

407 Interestingly, using immunofluorescence staining and confocal imaging, we detected DDX53-positive
408 structures that may resemble “germ granules”, nonmembrane-bound organelles unique to the germline.
409 A visible foci of DDX53-positive signals were present only in a subset of TCam-2 cells (Figure 4A and
410 Figure 7 B,C), typically had perinuclear localization, but in certain instances appeared to localize also
411 within the nucleus (intranuclear granules). Germ granules are evolutionarily conserved structures com-
412 posed of RNAs and RNA-binding proteins that participate in posttranscriptional regulation in

413 developing male germ cells and have essential role in the control of spermatogenesis [55–57]. Although
414 this is an interesting observation, a more in-depth characterization would be essential to determine this
415 with certainty.



416
417 **Figure 4.** DDX53 protein is predominantly expressed in cytoplasm, but is also present in the nucleus of TCam-2
418 cells. **(A)** Confocal immunofluorescence staining images of FLAG used for the analysis of DDX53 localization in
419 TCam-2 cells after transfection with a plasmid coding for *DDX53-FLAG*. The numbers in the corner represent the
420 percentage of red channel intensity in the nucleus with respect to the whole cell (for each cell separately). Magni-
421 fied insets show DDX53-positive condensates; the magnification factor ($\times 4$) is indicated below. Immunostaining
422 for FLAG on TCam-2 WT cells was performed as a negative control. Scale bar 20 μm . **(B)** Piechart demonstrating

DDX53 localization in a human testicular cancer cell line (TCam-2). The percentage was calculated as the average (from 5 cells shown in panel A) of red channel intensities in each nucleus with respect to the whole cell.

3.4. Transcriptome-wide discovery of RNAs directly interacting with DDX53 by eCLIP

As DEAD-box domain, present in DDX53 protein, is known to contribute to the binding of RNA molecules, we performed the enhanced crosslinking and immunoprecipitation followed by sequencing (eCLIP-seq). For this purpose, TCam-2 WT cells were transfected with vector encoding *DDX53-FLAG* and expression of DDX53 was confirmed on both RNA and protein levels (Supplementary Figure S3B,C). The eCLIP experiment was performed using two biological replicates of TCam-2 cells transfected with *DDX53-FLAG* (Figure 5A) and eCLIP data were analyzed using very conservative cutoffs, $|\log_2\text{FC}| \geq 3$ and adjusted p-value ≤ 0.001 . This approach allowed for the identification of ~1,000 DDX53-binding peaks in over 500 human RNA transcripts, detected in each sample (Supplementary Table S7). Further analysis of DDX53-binding sites (Figure 5B-D) focused on 134 transcripts with reproducible peaks identified across DDX53 replicates. However, as protein-RNA interactions are dynamic, DDX53-binding sites on specific RNAs may differ between replicates (e.g. LIN28B, Supplementary Table S7). Therefore, we prepared the extended list of 181 DDX53-bound transcripts detected in both replicates, regardless of the exact binding site position (Supplementary Table S8). The majority of enriched peaks were found on CDS, 3'UTRs, and distal introns (Figure 5B-D). Additionally, HOMER results showed enrichment of CA motifs (Figure 6A). In order to identify transcripts of differentially spliced genes (DSGs) that were directly bound by DDX53 further analysis was conducted (Figure 6B, Supplementary Table S9). This led to identification of twelve transcripts that might be direct targets of DDX53 protein, *REST*, *TFAM*, *NCBP2*, *CAMSAP2*, *LRP1*, *LRRC8A*, *THAP12*, *LRIG3*, *ESCO1*, *CDH2*, *PEG3*, and *ZNF480*. Subsequently, performed gene ontology (GO) analysis of the detected RNAs revealed that the DDX53 targets are involved in processes such as regulation of RNA metabolic process, transcription by RNA polymerase II, DNA-templated transcription, DNA-binding transcription factor activity, but also cell-cell junction, cell differentiation, and cell population proliferation (Figure 6C, for the complete list of GO terms see Supplementary Table S10). Moreover, evidence for DDX53 binding to its own mRNA was observed, aligning with self-regulatory mechanisms reported for other RNA-

binding proteins [58–60]. In conclusion, eCLIP-seq experiment confirmed the functional identity of DDX53 as an RNA-binding protein and identified its RNA targets, highlighting its impact on human transcriptome regulation.

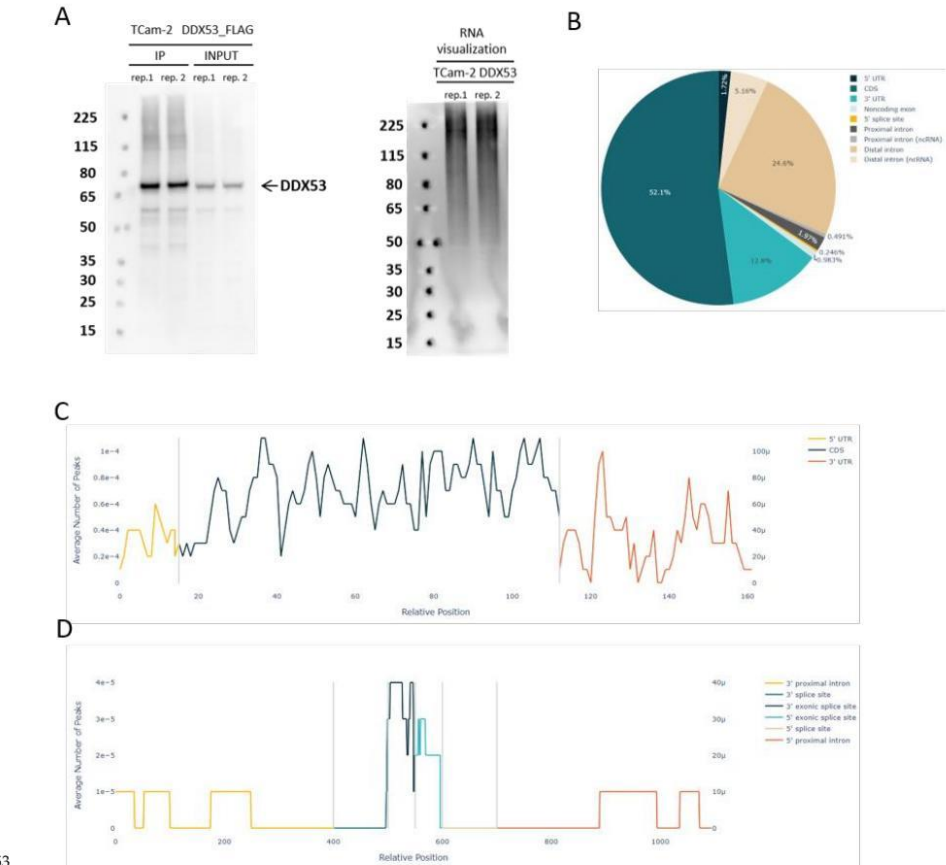


Figure 5. Identification of DDX53-bound RNA transcripts by eCLIP and their global profiling. **(A)** Western Blot analysis using anti-FLAG antibody performed on TCam-2 cells expressing *DDX53-FLAG* (left) and visualization of immunoprecipitated RNA (right) from TCam-2 cells. **(B)** Pie chart showing the relative frequency of peaks that map to each feature type, with a $|\log_2FC| \geq 3$ and adjusted p -value ≤ 0.001 . Based on reproducible peaks, identified across DDX53 replicates (Supplementary Table S7). **(C)** Peak metagene plot depicting the average number of peaks mapped to certain genomic regions. The number of peaks was calculated for each region of every gene, the lengths of the regions were normalized, and the average number of peaks for a set number of positions along the

regions was calculated. Prepared based on reproducible peaks ($|\log_2FC| \geq 3$ and adjusted p-value ≤ 0.001), identified across DDX53 replicates (Supplementary Table S7). (D) Peak metatrain plot depicting the average number of peaks mapped to certain intronic regions. The number of peaks was calculated for each region of every gene, the lengths of the regions were normalized, and the average number of peaks for a set number of positions along the regions was calculated. Prepared based on reproducible peaks ($|\log_2FC| \geq 3$ and adjusted p-value ≤ 0.001), identified across DDX53 replicates (Supplementary Table S7).

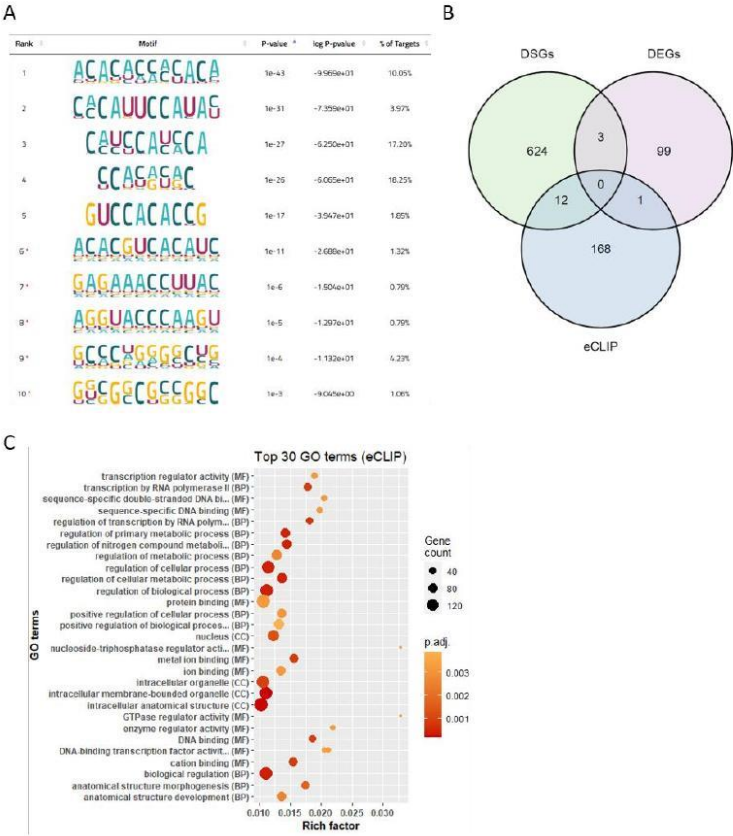


Figure 6. Identification and characterization of DDX53 RNA targets by eCLIP. (A) Significant binding motifs of DDX53 binding sites, sorted by p-value and identified by HOMER de novo motif analysis. (B) Venn diagram illustrating the total number of DSGs and DEGs based on RNA-seq data in comparison to the list of transcripts detected in DDX53 eCLIP, thus directly bound by DDX53 (Supplementary Table S9). Intersection was prepared using Ensembl IDs. (C) Gene ontology (GO) analysis for the transcripts bound by DDX53 based on eCLIP data.

473 The abbreviations in the brackets represent the three GO categories: biological process (BP), molecular function
474 (MF), and cellular component (CC). For the complete list of significant GO terms see Supplementary Table S10
475 (adjusted p-value < 0.05).

476 3.5. DDX53 potential protein interactors are important players during male gamete generation

477 To identify the protein interaction network of DDX53 we performed co-immunoprecipitation followed
478 by mass spectrometry analysis (Co-IP-MS) on TCam-2 cells with transient overexpression of FLAG-
479 tagged DDX53 and GFP (as control) using FLAG antibody (Figure 7A). This experiment enabled us to
480 identify 35 potential DDX53 interactors (Supplementary Table S11, Supplementary Figure S4A). These
481 proteins represent a diverse group, but members are involved in transcriptional and epigenetic regulation
482 (MTA3, SCML2, SUPT6H), RNA processing and nuclear transport (NUP54, PRCC), and cellular stress
483 response (HSPA9, DNAJB1), collectively contributing to the maintenance of cellular homeostasis. Ad-
484 ditional STRING analysis showed that some of the Co-IP-MS-identified proteins are functionally inter-
485 connected (Supplementary Table S12). Gene ontology enrichment analysis performed using the whole
486 list of potential DDX53 interactors showed that they are associated with pathways such as citrate meta-
487 bolic process (ACLY, ACO2) and chaperone cofactor-dependent protein refolding (DNAJB1, HSPA9),
488 processes important in phases of sperm development (Supplementary Figure S4B, Supplementary Table
489 S13) [61,62]. Then, we selected three proteins that are expressed in the human testis at a high level and
490 are present particularly in the germ cells where DDX53 is also expressed (spermatogonia, spermato-
491 cytes, and spermatids; according to HPA) and performed further interaction validation using confocal
492 microscopy (Figure 7B-D). Firstly, we took a closer look at the ACLY (also known as ACL/ATPCL),
493 enzyme responsible for the synthesis of acetyl-CoA, important in the context of fatty acids and the acet-
494 ylation of proteins and histones. Moreover, it has been shown that the ortholog of this gene in *Drosophila*
495 *melanogaster* is required for proper male spermatogenesis and that in germ cells of rats ACLY is a
496 potential heat sensitive target [63,64]. The immunofluorescence co-staining showed signal co-localiza-
497 tion (Figure 7B), especially at the structures near the nucleus which may indicate that ACLY and
498 DDX53 cooperate during human spermatogenesis. Next, we investigated the interaction of DDX53 with
499 THOC1 (known also as P84/HPR1) and record co-localization signal coming from the structures within

and outside the nucleus (Figure 7C). The THOC1 is a subunit of THO complex required for transcription elongation and mRNAs nuclear export [65,66] and has been shown to bind pachytene piRNAs precursors in mouse testis. This feature is especially important in the context of spermatogenesis as pachytene piRNAs emerge when spermatocytes enter the pachytene phase of meiosis [67]. The co-localization of THOC1 and DDX53 underlines the possible role of DDX53 in these processes. It is worth noting that we detected DDX53-positive condensates (Figure 7B,C) and observed colocalization with ACLY and THOC1 in some of these structures. This may suggest that DDX53 and these two interactors participate in germ granules, but this observation requires deeper characterization. Lastly, we investigated the possible interaction of DDX53 with germline-specific Polycomb protein, an epigenetic regulator SCML2 (Supplementary Figure S4C), which has established role in human spermatogenesis supported by other reports [68–70]. Based on co-immunofluorescence staining, we were not able to observe the co-localization of SCML2 and DDX53, thus the potential interaction between these two proteins, based on our Co-IP-MS, requires further validation.

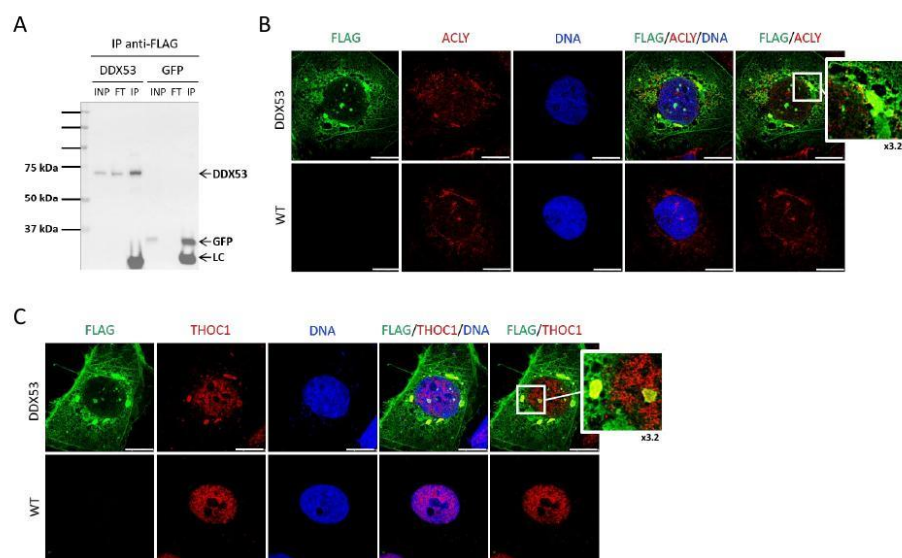


Figure 7. DDX53 potential protein interactors identified by Co-IP-MS. (A) Representative Western Blot after immunoprecipitation of FLAG-tagged DDX53 and GFP (as a control) using magnetic beads coated with anti-FLAG M2 antibody. Input (INP, 0.8%), flow-through (FT, 0.8%) and immunoprecipitated (IP, 0.8%) fractions

517 probed with FLAG antibody are shown. **(B, C)** Representative confocal images of TCam-2 cells after transient
518 transfection (DDX53) and WT, which were double immunostained for ACLY and FLAG **(B)** or THOC1 and
519 FLAG **(C)**, respectively. The FLAG antibody detects the DDX53 protein tagged with FLAG sequence. Signal
520 colocalization of DDX53 and its potential protein interactors is shown in the magnified inset ($\times 3.2$). Scale bar 15
521 μm .

522 **4. Discussion**

523 DEAD-box helicases are crucial enzymes involved in various aspects of RNA metabolism. RNA hel-
524 icases participate in nearly all major RNA-related processes, including RNA modification (such as m⁶A)
525 [71,72] pathways, splicing, RNA degradation, nuclear export, translation, RNP granule dynamics, chro-
526 matin-RNA coupling, and RNA quality control [34,73,74]. Their primary function is to remodel struc-
527 tured RNAs and dynamically reorganize RNP complexes, efficient recruitment and activity of RNA-
528 processing enzymes and cofactors. These activities are vital for the proper development of germ cells
529 and sperm maturation [30,73,74]. Although current studies have clarified specific functions of selected
530 DEAD-box helicases in the context of spermatogenesis [75–77], there is a continued need for evaluation
531 of other protein family members to uncover their unknown roles, protein interactors, and targets. Recent
532 reports mentioned the *DDX53* as a new candidate gene possibly associated with spermatogenesis failure
533 based on WGS/WES genetic screening of infertile patients with non-obstructive azoospermia (NOA)
534 [1,2]. The variant identified in infertile male patients results in a missense mutation that alters a residue
535 at the C-terminal end of the KH RNA-binding domain located in the N-terminal region of DDX53. This
536 potentially could modulate RNA substrate specificity as this domain favors single-stranded RNA recog-
537 nition and binding. Here, for the first time, we describe novel findings on DDX53 in the context of
538 human spermatogenesis, highlighting its role in RNA metabolism. In our research, we used the TCam-
539 2 cells overexpressing *DDX53-FLAG* as an *in vitro* male germline model. This approach enabled us to
540 uncover the coordinated network of RNA targets and protein interactors associated with DDX53 in a
541 cellular environment that mimics early germline status. While the TCam-2 cell line does not entirely
542 capture the full spectrum of germ cell differentiation occurring in the human testis, it remains a useful
543 system for investigating germline-associated mechanisms [37,78]. Therefore, our findings should be

544 regarded as indicative, with emphasis placed on those gene and protein targets that are likewise detected
545 in human male germ cells expressing *DDX53*, as reported by the Human Protein Atlas. Our results
546 demonstrate the importance of *DDX53* protein in human transcriptome regulation, which may be com-
547 promised in male patients with rare genetic variants of *DDX53* gene.

548 4.1. *DDX53* shapes the TCam-2 transcriptome

549 Firstly, we investigated the influence of *DDX53* on TCam-2 cells' transcriptome by performing RNA
550 sequencing. Although the TCam-2 cell line represents a model of germ cells, it should be noted that
551 these cells originate from a testicular germ cell tumor (seminoma) and harbor multiple cancer-related
552 features. In our study, among the differentially expressed genes (DEGs), we detected the *SERPINF1*,
553 upregulated in TCam-2 cells with *DDX53* protein overexpression. *SERPINF1* (also known as PEDF) is
554 a multifunctional protein that exerts anti-angiogenic properties, which are especially important given
555 the avascularity of the testicular seminiferous tubules, but also cancer metastasis. In humans, *SERPINF1*
556 is present in testicular tissue, particularly in Sertoli, Leydig, and peritubular cells, and extracellular ma-
557 trix, but not present in germ cells [28]. Previous studies have suggested its involvement in spermatogen-
558 esis [79,80] and underscored its relevance for diverse cancers [81]. Another example of a gene whose
559 expression is positively regulated by *DDX53* in TCam-2 cells is the *MZT1* (also known as *MOZART1*),
560 coding a protein that is a component of the gamma-tubulin large complex, primarily localized to the
561 centrosome and spindle. Although *MZT1* has not yet been studied in human spermatogenesis, it was
562 reported that *Drosophila* Mzt1 is required to maintain sperm motility and male fertility during aging
563 [82]. In theory, *DDX53* may underlie proper *MZT1* regulation, functionally significant also for human
564 male germ cells differentiation.

565 4.2. *DDX53* modulates alternative splicing of germline-relevant genes

566 In this work, we identified multiple differentially spliced genes (DSGs) upon *DDX53* overexpression.
567 Splicing alterations were more prominent than changes in overall gene expression, which aligns with
568 the anticipated role of *DDX53* in RNA metabolism, rather than transcriptional regulation. Among the
569 DSGs was the *LRRC8A*, coding for a component of the volume-regulated anion channel, which helps to
570 maintain cell volume homeostasis and has fundamental significance for the physiological function of

sperm. Knockout of *Lrrc8a* in mice led to defective development of spermatids and male infertility [83]. Our eCLIP results indicate that DDX53 binds *LRRC8A* mRNA within CDS and 3'UTR, raising the possibility that sequence variants in DDX53 may perturb *LRRC8A* mRNA processing, with potential consequences for protein function and male germ cell biology. Another interesting transcript, bound by DDX53 within 3'UTR, and classified as differentially spliced in TCam-2 cells upon *DDX53* overexpression, is *TFAM*. *TFAM* is a key mitochondrial transcription factor, essential not only for the transcription but also maintenance of mitochondrial DNA (mtDNA). This protein reaches the highest expression level in spermatids (similarly to DDX53), and dysregulation of *TFAM* expression has been correlated with qualitative impairment of spermatogenesis [84]. Studies have shown that downregulation of *TFAM* protein levels leads to a decrease in mtDNA copy number during spermatogenesis, which is believed to be a mechanism preventing the transmission of paternal mtDNA to the offspring, ensuring predominant maternal inheritance of mitochondria [85,86].

The overlap between the number of potential DDX53-induced DSGs and identified DDX53-bound transcripts was relatively low (according to RNA-seq and eCLIP data comparison), but it is important to remember that DDX53 may influence alternative splicing of many key genes, important for spermatogenesis, without direct binding to their mRNA. It is well-known that proteins may influence AS by interacting with other regulatory proteins, rather than by directly binding to RNA transcripts. Notably, multiple proteins involved in alternative splicing have been found among the potential DDX53 protein interactors as shown by Co-IP-MS, such as CELF2 [87], RBM26 [88], and THOC1 [89]. We performed immunofluorescence co-staining for DDX53 and THOC1 and observed signal overlap, which indicates a possible functional interplay. Another noteworthy example of a gene classified as differentially spliced upon *DDX53* overexpression, but not directly bound by DDX53, is the *SRSF1*. *SRSF1* protein is expressed in various stages of male germ cell development. A recent study showed that it is essential for the proper expression and alternative splicing of spermatogonia-related genes in mouse testis [90]. Moreover, conditional knockout (cKO) mice with *SRSF1* deficiency were infertile and exhibited a Sertoli cell-only syndrome (SCOS) phenotype. Another group showed that *SRSF1* loss led to meiotic arrest at the pachytene stage, emphasizing that germ cell-specific *Srsf1* knockout results in spermatogenesis failure and thus male infertility [91]. Interestingly, the SEPTIN7 also emerged among the DSGs. This

599 gene codes for a cytoskeletal GTPase that plays a crucial role in maintaining cell shape and structural
600 integrity during spermatogenesis. SEPTIN7 is robustly expressed throughout all stages of human sper-
601 matogenesis. This protein migrates from the midpiece to the annulus as spermatozoa mature, suggesting
602 that it may be involved in regulating sperm maturation [92]. The annulus is a septin-based ring structure
603 at the junction of the sperm midpiece and principal piece that functions as a diffusion barrier and is
604 required for proper flagellar organization and sperm motility. Proper regulation of *SEPTIN7* expression
605 may be critical for preventing sperm defects, highlighting a potentially important, although secondary,
606 role of DDX53.

607 Spermatogenesis relies on highly specialized RNA processing programs, reflected by the exceptionally
608 high prevalence of alternative splicing observed in the testis, a feature shared with the human brain
609 [93,94]. In this context, recent studies have demonstrated DDX53 expression in both tissues and its role
610 in neuronal function [3,4], suggesting that DDX53 may contribute also to complex RNA regulatory
611 mechanisms in male germ cells. Despite the widespread occurrence of alternative splicing in the testis,
612 most transcript isoforms remain poorly annotated and functionally uncharacterized [95], underscoring
613 the need to define RNA-binding regulators such as DDX53.

614 4.3. DDX53 binds numerous germline-associated transcripts

615 Our findings support DDX53 as an upstream regulator of RNA metabolism based on its ability to bind
616 a broad range of RNA molecules in human TCam-2 cells, as demonstrated by eCLIP-seq data including
617 transcripts already discussed in the context of differentially spliced genes (DSG). Among the RNA tar-
618 gets of the DDX53, numerous transcripts coding proteins and previously recognized as important in the
619 context of spermatogenesis were identified. Below, we discuss selected examples highlighting the po-
620 tential role of DDX53 in male germ cells. An important example of mRNA targeted by DDX53, relevant
621 in the context of spermatogenesis, is the *TAF7* (bound within its coding sequence). This gene codes for
622 spermatogenic transcription regulator whose mutations increase the risk of azoospermia [96]. The intra-
623 cellular localization of TAF7 is dynamically regulated, as it is present in the nucleus in developing germ
624 cells until the late pachytene stage, after which it becomes cytoplasmic [97]. Precise regulation of *TAF7*
625 mRNA may be a key to its proper testis-specific expression pattern, which could be disrupted in patients

with *DDX53* mutations, leading to infertility. Another noteworthy example is the *LIN28B*, an RNA-binding protein that acts as a post-transcriptional regulator of genes involved in developmental timing and self-renewal through its interaction with miRNAs [98]. In humans, *LIN28B* is mainly expressed in the testis and placenta. Moreover, within the adult human testis, *LIN28B* transcripts are found in the germ cells and not in the somatic cells. A recent study suggested that *LIN28B* may act as a proliferation driver in the most undifferentiated spermatogonia, and that a decrease in its expression could impair spermatogenic capacity, resulting in oligozoospermia in adult men [98]. *DDX53* binding (within 3'UTR) to *LIN28B* mRNA suggest that *DDX53* may act upstream of *LIN28B*-mediated pathways. Of particular interest, *NCBP2* was identified as a potential mRNA target of *DDX53*. The product of this gene plays a vital role in mRNA processing events through its binding to the 5' cap of nascent pre-mRNAs. Results from single-cell RNA sequencing of testicular cell samples from NOA patients indicated that *NCBP2* is positively associated with spermatogenesis [99]. Although its precise role during male germ cell development has not yet been investigated, the *NCBP2* has been determined as a significant predictor of non-obstructive azoospermia (NOA) [99]. An important example might be also the *BRD3* gene, whose mRNA is bound by *DDX53* within 3'UTR. In mice, *Brd3* is enriched in round spermatids, demonstrating a tightly regulated expression pattern in the male germ cell lineage and suggesting its specific role for spermatogenesis [100]. While the precise role of *BRD3* in spermatogenesis is yet unknown, its membership in the bromodomain and extraterminal (BET) family proteins implies involvement in transcriptional regulation during germ cell development. BET proteins, including *BRD3*, are known to interact with acetylated histones, influencing chromatin structure and gene expression. The specific enrichment of *BRD3* transcripts in round spermatids points to a potential role in the transcriptional programs necessary for the maturation of spermatids into spermatozoa. In humans, *BRD3* transcripts are expressed in the spermatogonia, spermatocytes, and spermatids, but the protein was specifically observed in spermatids [101]. Additionally, *DDX53* protein also binds the mRNAs of *LIN7C* and *DLC1*, both within coding sequence. These proteins exhibit stage-specific expression in germ cells during spermatogenesis, which implies their testis-specific functions. *Lin7c* has been proposed as a potential marker of early spermatogenesis [102], and a role for *DLC1* in chromatin condensation and spermatid shaping has been suggested [103]. Although more studies are needed to elucidate their precise

function in the testis, it has been suggested that they are important in germ cell development. Based on eCLIP data, it is possible that DDX53 governs the stage-specific expression of these genes in the human testis. Importantly, eCLIP analysis also revealed that DDX53 binds to its own mRNA. Although this finding does not directly support a self-regulatory effect, it is consistent with feature commonly observed among RNA-binding proteins [58–60] and may suggest a potential autoregulatory feedback mechanism, which remains to be functionally validated.

4.4. Protein interactome supports an RNA-centric regulatory role

Analysis of the STRING interaction network based on DDX53 potential protein interactors identified by Co-IP-MS suggests that DDX53 operates within an integrated transcriptional and post-transcriptional regulatory framework. The interactome comprising POLR2B, SUPT6H, RPRD1A, THOC1, NUP54 and NUP210 forms a coherent axis spanning transcription by RNA polymerase II, co-transcriptional RNA maturation, and nuclear export of messenger ribonucleoprotein complexes. Among the potential DDX53-protein interactors, there are proteins involved in pre-mRNA splicing and RNP dynamics. Collectively, our data suggest that DDX53 is involved in mediating mRNA metabolism, which is critical for precisely controlled germline-specific gene expression.

4.5. DDX53-positive condensates suggest potential involvement in germline-specific granules

We investigated DDX53 subcellular localization, demonstrating its presence mostly in the cytoplasm with partial nuclear localization in TCam-2 cells. Although several studies have detected positive DDX53 ChIP-based signals [15,16,23,27,54] and we confirmed the partial nuclear localization of DDX53 in TCam-2 cells, our ChIP-seq analysis did not support direct DNA binding by DDX53 (unpublished data). Based on this, previously reported positive ChIP assay results for DDX53 should be interpreted with caution. While nonspecific antibody interactions may partially account for these observations, our data do not preclude an indirect or transient association of DDX53 with chromatin, potentially mediated through protein–protein interactions.

To further clarify the nuclear context of DDX53, we examined its intracellular organization and observed distinct DDX53-positive condensates in TCam-2 cells that resemble germline-type RNP granules. These specialized ribonucleoprotein (RNP) granules are a hallmark of all germ cells and are

composed of germline-specific proteins and RNAs [104,105]. Although over recent decades various types of RNA granules have been identified in germ cells across species, still very little is known about these germline-specific condensates in humans. In the *Drosophila* embryo, nuclear germ granules were shown to promote germ cell divisions thereby increasing PGC number for the next generation [106]. Furthermore, one of the conserved components of germ granule proteins is Ddx4 (known also as Vasa) [56,106]. Although not expressed in TCam-2 cells [107], DDX4 is an evolutionarily conserved germline protein that belongs to the DEAD-box family of RNA helicases, as does DDX53. Importantly, the identification of DDX53-positive condensates remains preliminary and requires further validation using canonical germ granule markers and additional experimental approaches, particularly under physiological conditions. This may represent a valuable direction for future research and could provide additional insight into the role of DDX53 in the human germline.

5. Conclusions

Collectively, the presence of DDX53 in human testicular tissue, together with its influence on transcriptome in TCam-2 cells and its capacity to bind a broad repertoire of RNAs, implies its role in male germ cell biology. Moreover, our data suggest that DDX53 protein may affect various testis-specific post-transcriptional gene regulation mechanisms that influence male germ cell differentiation. Further research is warranted to elucidate the precise mechanisms by which DDX53 influences spermatogenesis in diverse stages of germ cell development, but this study highlights the relevance of DDX53 as an upstream regulator of RNA metabolism, vital for proper germline development.

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723 **Abbreviations**

724 RNA-seq RNA sequencing
725 eCLIP Enhanced crosslinking and immunoprecipitation
726 Co-IP-MS Co-immunoprecipitation coupled with Mass Spectrometry
727 WES Whole-exome sequencing
728 WGS Whole-Genome sequencing
729 CTAs Cancer-testis antigens
730 NOA Non-obstructive azoospermia
731 HPA Human Protein Atlas
732 hPGCs Human primordial germ cells
733 ChIP Chromatin Immunoprecipitation
734 IHC Immunohistochemistry

735 DEGs Differentially expressed genes

736 AS Alternative splicing

737 DSGs Differentially spliced genes

738 GO Gene ontology

739 SCOS Sertoli cell-only syndrome

740

741 **Supplemental Tables**

742 Supplementary Table S1 Normalized counts (CPM) based on RNA-seq of TCam-2 cells.

743 Supplementary Table S2 Differentially expressed genes (DEGs) based on RNA-seq of TCam-2 cells.

744 Supplementary Table S3 Normalized transcript-level values (TPM) for TCam-2 cells quantified with Salmon from
745 RNA-seq data.

746 Supplementary Table S4 Differentially spliced genes (DSGs) based on SUPPA2 analysis of RNA-seq data.

747 Supplementary Table S5 Genes overlap results for DSGs, DEGs, and genes involved in male gamete generation.

748 Supplementary Table S6 Gene Ontology and pathway enrichment analysis of DSGs detected upon DDX53 over-
749 expression.

750 Supplementary Table S7 Detailed information on detected DDX53 eCLIP peaks.

751 Supplementary Table S8 List of transcripts bound by DDX53 identified by eCLIP.

752 Supplementary Table S9 Genes overlap results for DSGs, DEGs, and DDX53 eCLIP targets.

753 Supplementary Table S10 Gene Ontology analysis of DDX53 eCLIP targets.

754 Supplementary Table S11 Results of DDX53 co-immunoprecipitation followed by mass spectrometry.

755 Supplementary Table S12 DDX53 protein-protein interaction network analysis performed with the STRING da-
756 tabase.

757 Supplementary Table S13 Gene Ontology enrichment analysis based on proteins identified in DDX53 co-immuno-
758 precipitation experiment.

759 Supplementary Table S14 List of primers.

760 References

- 761 1. Hardy, J.; Pollock, N.; Gingrich, T.; Sweet, P.; Ramesh, A.; Kuong, J.; Basar, A.; Jiang, H.; Hwang, K.;
762 Vukina, J.; et al. Genomic Testing for Copy Number and Single Nucleotide Variants in Spermatogenic
763 Failure. *J. Assist. Reprod. Genet.* **2022**, *39*, 2103–2114, doi:10.1007/s10815-022-02538-5.
- 764 2. Malcher, A.; Stokowy, T.; Berman, A.; Olszewska, M.; Jedrzejczak, P.; Sielski, D.; Nowakowski, A.;
765 Rozwadowska, N.; Yatsenko, A.N.; Kurpisz, M.K. Whole-Genome Sequencing Identifies New Candidate
766 Genes for Nonobstructive Azoospermia. *Andrology* **2022**, *10*, 1605–1624, doi:10.1111/ANDR.13269.
- 767 3. Scala, M.; Bradley, C.A.; Howe, J.L.; Trost, B.; Salazar, N.B.; Shum, C.; Mendes, M.; Reuter, M.S.;
768 Anagnostou, E.; MacDonald, J.R.; et al. Genetic Variants in DDX53 Contribute to Autism Spectrum
769 Disorder Associated with the Xp22.11 Locus. *Am. J. Hum. Genet.* **2025**, *112*, 154–167,
770 doi:10.1016/J.AJHG.2024.11.003.
- 771 4. Yuan, X.; Wang, Y.; Li, X.; Zhong, S.; Zhou, D.; Lin, X.; Fang, H.; Yang, Y.; Wang, M. Loss-of-Function
772 Mutation in DDX53 Associated with Hereditary Spastic Paraplegia-like Disorder. *J. Mol. Med.* **2024**, *102*,
773 913–926, doi:10.1007/S00109-024-02454-4.
- 774 5. Simpson, A.J.G.; Caballero, O.L.; Jungbluth, A.; Chen, Y.T.; Old, L.J. Cancer/Testis Antigens,
775 Gametogenesis and Cancer. *Nat. Rev. Cancer* **2005**, *5*, 615–625, doi:10.1038/nrc1669.
- 776 6. Almeida, L.G.; Sakabe, N.J.; de Oliveira, A.R.; Silva, M.C.C.; Mundstein, A.S.; Cohen, T.; Chen, Y.T.;
777 Chua, R.; Gurung, S.; Gnjjatic, S.; et al. CTdatabase: A Knowledge-Base of High-Throughput and Curated
778 Data on Cancer-Testis Antigens. *Nucleic Acids Res.* **2009**, *37*, D816–19, doi:10.1093/NAR/GKN673.
- 779 7. Liggins, A.P.; Lim, S.H.; Soilleux, E.J.; Pulford, K.; Banham, A.H. A Panel of Cancer-Testis Genes

- 780 Exhibiting Broad-Spectrum Expression in Haematological Malignancies. *Cancer Immun.* **2010**, *10*, 1–12.
- 781 8. Hofmann, O.; Caballero, O.L.; Stevenson, B.J.; Chen, Y.T.; Cohen, T.; Chua, R.; Maher, C.A.; Panji, S.;
782 Schaefer, U.; Kruger, A.; et al. Genome-Wide Analysis of Cancer/Testis Gene Expression. *Proc. Natl.*
783 *Acad. Sci. U. S. A.* **2008**, *105*, 20422–20427, doi:10.1073/pnas.0810777105.
- 784 9. Cho, B.; Lim, Y.; Lee, D.Y.; Park, S.Y.; Lee, H.; Jeoung, D. II; Kim, W.H.; Yang, H.; Bang, Y.J.
785 Identification and Characterization of a Novel Cancer/Testis Antigen Gene CAGE. *Biochem. Biophys. Res.*
786 *Commun.* **2002**, *292*, 715–726, doi:10.1006/BBRC.2002.6701.
- 787 10. Dakshinamurthy, A.G.; Ramesar, R.; Goldberg, P.; Blackburn, J.M. Infrequent and Low Expression of
788 Cancer-Testis Antigens Located on the X Chromosome in Colorectal Cancer: Implications for
789 Immunotherapy in South African Populations. *Biotechnol. J.* **2008**, *3*, 1417–1423,
790 doi:10.1002/BIOT.200800144.
- 791 11. Hanahan, D.; Weinberg, R.A. Hallmarks of Cancer: The next Generation. *Cell* **2011**, *144*, 646–674,
792 doi:10.1016/j.cell.2011.02.013.
- 793 12. Kim, Y.M.; Park, D.; Kim, H.; Choi, M.; Lee, H.; Lee, Y.S.; Choe, J.; Kim, Y.M.; Jeoung, D. MiR-200b
794 and Cancer/Testis Antigen CAGE Form a Feedback Loop to Regulate the Invasion and Tumorigenic and
795 Angiogenic Responses of a Cancer Cell Line to Microtubule-Targeting Drugs. *J. Biol. Chem.* **2013**, *288*,
796 36502–36518, doi:10.1074/JBC.M113.502047.
- 797 13. Van Tongelen, A.; Lorient, A.; De Smet, C. Oncogenic Roles of DNA Hypomethylation through the
798 Activation of Cancer-Germline Genes. *Cancer Lett.* **2017**, *396*, 130–137,
799 doi:10.1016/J.CANLET.2017.03.029.
- 800 14. Por, E.; Byun, H.J.; Lee, E.J.; Lim, J.H.; Jung, S.Y.; Park, I.; Kim, Y.M.; Jeoung, D. II; Lee, H. The

- 801 Cancer/Testis Antigen CAGE with Oncogenic Potential Stimulates Cell Proliferation by Up-Regulating
- 802 Cyclins D1 and E in an AP-1- and E2F-Dependent Manner. *J. Biol. Chem.* **2010**, 285, 14475–14485,
- 803 doi:10.1074/JBC.M109.084400.
- 804 15. Yeon, M.; Lee, S.; Lee, J.E.; Jung, H.S.; Kim, Y.; Jeoung, D. CAGE-MiR-140-5p-Wnt1 Axis Regulates
- 805 Autophagic Flux, Tumorigenic Potential of Mouse Colon Cancer Cells and Cellular Interactions Mediated
- 806 by Exosomes. *Front. Oncol.* **2019**, 9, 1240, doi:10.3389/FONC.2019.01240.
- 807 16. Kim, H.; Kim, Y.; Jeoung, D. DDX53 Promotes Cancer Stem Cell-Like Properties and Autophagy. *Mol.*
- 808 *Cells* **2017**, 40, 54–65, doi:10.14348/MOLCELLS.2017.2258.
- 809 17. Park, S.Y.; Kim, W. jin; Byun, J. hwan; Lee, J.J.; Jeoung, D.; Park, S.T.; Kim, Y. Role of DDX53 in
- 810 Taxol-Resistance of Cervix Cancer Cells in Vitro. *Biochem. Biophys. Res. Commun.* **2018**, 506, 641–647,
- 811 doi:10.1016/J.BBRC.2018.10.145.
- 812 18. Nin, D.S.; Deng, L.W. Biology of Cancer-Testis Antigens and Their Therapeutic Implications in Cancer.
- 813 *Cells* **2023**, 12, 926, doi:10.3390/CELLS12060926.
- 814 19. Kim, Y.; Park, H.; Jeoung, D. CAGE, a Cancer/Testis Antigen, Induces c-FLIPL and Snail to Enhance
- 815 Cell Motility and Increase Resistance to an Anti-Cancer Drug. *Biotechnol. Lett.* **2009**, 31, 945–952,
- 816 doi:10.1007/s10529-009-9981-9.
- 817 20. Kim, Y.; Park, H.; Park, D.; Lee, Y.S.; Choe, J.; Hahn, J.H.; Lee, H.; Kim, Y.M.; Jeoung, D. Cancer/Testis
- 818 Antigen CAGE Exerts Negative Regulation on P53 Expression through HDAC2 and Confers Resistance
- 819 to Anti-Cancer Drugs. *J. Biol. Chem.* **2010**, 285, 25957–25968, doi:10.1074/JBC.M109.095950.
- 820 21. Cho, B.; Lee, H.; Jeong, S.W.; Bang, Y.J.; Lee, H.J.; Hwang, K.S.; Kim, H.Y.; Lee, Y.S.; Kang, G.H.;
- 821 Jeoung, D. II Promoter Hypomethylation of a Novel Cancer/Testis Antigen Gene CAGE Is Correlated

- 822 with Its Aberrant Expression and Is Seen in Premalignant Stage of Gastric Carcinoma. *Biochem. Biophys.*
- 823 *Res. Commun.* **2003**, 307, 52–63, doi:10.1016/S0006-291X(03)01121-5.
- 824 22. Kwon, Y.; Kim, Y.; Jung, H.S.; Jeoung, D. Role of HDAC3-MiRNA-CAGE Network in Anti-Cancer
- 825 Drug-Resistance. *Int. J. Mol. Sci.* **2018**, 20, 51, doi:10.3390/IJMS20010051.
- 826 23. Park, D.; Kim, H.; Kim, Y.; Jeoung, D. MiR-30a Regulates the Expression of CAGE and P53 and
- 827 Regulates the Response to Anti-Cancer Drugs. *Mol. Cells* **2016**, 39, 299,
- 828 doi:10.14348/MOLCELLS.2016.2242.
- 829 24. Kim, Y.; Yeon, M.; Jeoung, D. DDX53 Regulates Cancer Stem Cell-Like Properties by Binding to SOX-
- 830 2. **2017**, 40, 322–330, doi:10.14348/MOLCELLS.2017.0001.
- 831 25. Yeon, M.; Byun, J.; Kim, H.; Kim, M.; Jung, H.S.; Jeon, D.; Kim, Y.; Jeoung, D. CAGE Binds to Beclin1,
- 832 Regulates Autophagic Flux and CAGE-Derived Peptide Confers Sensitivity to Anti-Cancer Drugs in Non-
- 833 Small Cell Lung Cancer Cells. *Front. Oncol.* **2018**, 8, 599, doi:10.3389/FONC.2018.00599.
- 834 26. Kim, H.; Kim, Y.; Goh, H.; Jeoung, D. Histone Deacetylase-3/CAGE Axis Targets EGFR Signaling and
- 835 Regulates the Response to Anti-Cancer Drugs. *Mol. Cells* **2016**, 39, 229–241,
- 836 doi:10.14348/MOLCELLS.2016.2244.
- 837 27. Kim, Y.; Kim, H.; Park, D.; Han, M.; Lee, H.; Lee, Y.S.; Choe, J.; Kim, Y.M.; Jeoung, D. MiR-217 and
- 838 CAGE Form Feedback Loop and Regulates the Response to Anti-Cancer Drugs through EGFR and HER2.
- 839 *Oncotarget* **2016**, 7, 10297–10321, doi:10.18632/ONCOTARGET.7185.
- 840 28. The Human Protein Atlas Available online: <https://www.proteinatlas.org/> (accessed on 7 February 2025).
- 841 29. Schütz, P.; Karlberg, T.; van den Berg, S.; Collins, R.; Lehtiö, L.; Högbom, M.; Holmberg-Schiavone, L.;
- 842 Tempel, W.; Park, H.W.; Hammarström, M.; et al. Comparative Structural Analysis of Human DEAD-

- 843 Box RNA Helicases. *PLoS One* **2010**, 5, e12791, doi:10.1371/JOURNAL.PONE.0012791.
- 844 30. Tutak, K.; Rozwadowska, N. Discrete Roles of RNA Helicases in Human Male Germline and
845 Spermatogenesis. *J. Appl. Genet.* **2020**, 61, 415–419, doi:10.1007/S13353-020-00558-4.
- 846 31. De Bortoli, F.; Espinosa, S.; Zhao, R. DEAH-Box RNA Helicases in Pre-mRNA Splicing. *Trends Biochem.*
847 *Sci.* **2021**, 46, 225–238, doi:10.1016/j.tibs.2020.10.006.
- 848 32. Li, D.; Kishita, M.S.; Wang, J. Regulation of Pluripotency and Reprogramming by RNA Binding Proteins.
849 *Curr. Top. Dev. Biol.* **2020**, 138, 113–138, doi:10.1016/BS.CTDB.2020.01.003.
- 850 33. Abdelhaleem, M. RNA Helicases: Regulators of Differentiation. *Clin. Biochem.* **2005**, 38, 499–503,
851 doi:10.1016/J.CLINBIOCHEM.2005.01.010.
- 852 34. Bourgeois, C.F.; Mortreux, F.; Auboeuf, D. The Multiple Functions of RNA Helicases as Drivers and
853 Regulators of Gene Expression. *Nat. Rev. Mol. Cell Biol.* **2016**, 17, 426–438, doi:10.1038/nrm.2016.50.
- 854 35. Suri, A.; Jagadish, N.; Saini, S.; Gupta, N. Targeting Cancer Testis Antigens for Biomarkers and
855 Immunotherapy in Colorectal Cancer: Current Status and Challenges. *World J. Gastrointest. Oncol.* **2015**,
856 7, 492–502, doi:10.4251/WJGO.V7.I12.492.
- 857 36. CTDatabase Available online: <http://www.cta.lncc.br/> (accessed on 6 August 2025).
- 858 37. Irie, N.; Weinberger, L.; Tang, W.W.C.; Kobayashi, T.; Viukov, S.; Manor, Y.S.; Dietmann, S.; Hanna,
859 J.H.; Surani, M.A. SOX17 Is a Critical Specifier of Human Primordial Germ Cell Fate. *Cell* **2015**, 160,
860 253–268, doi:10.1016/j.cell.2014.12.013.
- 861 38. Mizuno, Y.; Gotoh, A.; Kamidono, S.; Kitazawa, S. [Establishment and Characterization of a New Human
862 Testicular Germ Cell Tumor Cell Line (TCam-2)]. *Nihon Hinyokika Gakkai Zasshi Japanese J. Urol.* **1993**,
863 84, 1211–1218, doi:10.5980/JPNJUROL1989.84.1211.

- 864 39. De Jong, J.; Stoop, H.; Gillis, A.J.M.; Hersmus, R.; Van Gurp, R.J.H.L.M.; Van De Geijn, G.J.M.; Van
865 Drunen, E.; Beverloo, H.B.; Schneider, D.T.; Sherlock, J.K.; et al. Further Characterization of the First
866 Seminoma Cell Line TCam-2. *Genes. Chromosomes Cancer* **2008**, *47*, 185–196, doi:10.1002/GCC.20520.
- 867 40. Barszcz, A.; Tutak, K.; Zypych-Walczak, J.; Dassi, E.; Ilaslan, E.; Dąbrowska, M.; Malcher, A.;
868 Olszewska, M.; Cysewski, D.; Nadel, A.; et al. Multi-Omic Screening Identifies RBMXL3 as a Primate-
869 Specific RNA-Binding Protein and Candidate Regulator of RNA Metabolism in Human Spermatogenesis.
870 *bioRxiv* **2025**, doi:10.1101/2025.09.23.677621.
- 871 41. Dobin, A.; Davis, C.A.; Schlesinger, F.; Drenkow, J.; Zaleski, C.; Jha, S.; Batut, P.; Chaisson, M.; Gingeras,
872 T.R. STAR: Ultrafast Universal RNA-Seq Aligner. *Bioinformatics* **2013**, *29*, 15–21,
873 doi:10.1093/BIOINFORMATICS/BTS635.
- 874 42. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R.
875 The Sequence Alignment/Map Format and SAMtools. *Bioinformatics* **2009**, *25*, 2078–2079,
876 doi:10.1093/BIOINFORMATICS/BTP352.
- 877 43. Love, M.I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq
878 Data with DESeq2. *Genome Biol.* **2014**, *15*, 550, doi:10.1186/s13059-014-0550-8.
- 879 44. Wu, T.; Hu, E.; Xu, S.; Chen, M.; Guo, P.; Dai, Z.; Feng, T.; Zhou, L.; Tang, W.; Zhan, L.; et al.
880 ClusterProfiler 4.0: A Universal Enrichment Tool for Interpreting Omics Data. *Innov.* **2021**, *2*, 100141,
881 doi:10.1016/J.XINN.2021.100141.
- 882 45. Patro, R.; Duggal, G.; Love, M.I.; Irizarry, R.A.; Kingsford, C. Salmon: Fast and Bias-Aware
883 Quantification of Transcript Expression Using Dual-Phase Inference. *Nat. Methods* **2017**, *14*, 417–419,
884 doi:10.1038/NMETH.4197.

- 885 46. Alamancos, G.P.; Pagès, A.; Trincado, J.L.; Bellora, N.; Eyra, E. Leveraging Transcript Quantification
886 for Fast Computation of Alternative Splicing Profiles. *RNA* **2015**, *21*, 1521–1531,
887 doi:10.1261/RNA.051557.115.
- 888 47. Trincado, J.L.; Entizne, J.C.; Hysenaj, G.; Singh, B.; Skalic, M.; Elliott, D.J.; Eyra, E. SUPPA2: Fast,
889 Accurate, and Uncertainty-Aware Differential Splicing Analysis across Multiple Conditions. *Genome Biol.*
890 **2018**, *19*, 40, doi:10.1186/s13059-018-1417-1.
- 891 48. Van Nostrand, E.L.; Pratt, G.A.; Shishkin, A.A.; Gelboin-Burkhart, C.; Fang, M.Y.; Sundararaman, B.;
892 Blue, S.M.; Nguyen, T.B.; Surka, C.; Elkins, K.; et al. Robust Transcriptome-Wide Discovery of RNA-
893 Binding Protein Binding Sites with Enhanced CLIP (ECLIP). *Nat. Methods* **2016**, *13*, 508–514,
894 doi:10.1038/nmeth.3810.
- 895 49. Kloska, D.; Kopacz, A.; Cysewski, D.; Aepfelbacher, M.; Dulak, J.; Jozkowicz, A.; Grochot-Przeczek, A.
896 Nrf2 Sequesters Keap1 Preventing Podosome Disassembly: A Quintessential Duet Moonlights in
897 Endothelium. *Antioxid. Redox Signal.* **2019**, *30*, 1709–1730, doi:10.1089/ARS.2018.7505.
- 898 50. Cox, J.; Mann, M. MaxQuant Enables High Peptide Identification Rates, Individualized p.p.b.-Range Mass
899 Accuracies and Proteome-Wide Protein Quantification. *Nat. Biotechnol.* **2008**, *26*, 1367–1372,
900 doi:10.1038/nbt.1511.
- 901 51. Winkle, M.; Tayari, M.M.; Kok, K.; Duns, G.; Grot, N.; Kazimierska, M.; Seitz, A.; de Jong, D.; Koerts,
902 J.; Diepstra, A.; et al. The LncRNA KTN1-AS1 Co-Regulates a Variety of Myc-Target Genes and
903 Enhances Proliferation of Burkitt Lymphoma Cells. *Hum. Mol. Genet.* **2022**, *31*, 4193–4206,
904 doi:10.1093/HMG/DDAC159.
- 905 52. Malcher, A.; Rozwadowska, N.; Stokowy, T.; Kolanowski, T.; Jedrzejczak, P.; Zietkowiak, W.; Kurpisz,

- 906 M. Potential Biomarkers of Nonobstructive Azoospermia Identified in Microarray Gene Expression
907 Analysis. *Fertil. Steril.* **2013**, *100*, 1686–1694.e7, doi:10.1016/j.fertnstert.2013.07.1999.
- 908 53. Spiess, A.N.; Feig, C.; Schulze, W.; Chalmel, F.; Cappallo-Obermann, H.; Primig, M.; Kirchhoff, C.
909 Cross-Platform Gene Expression Signature of Human Spermatogenic Failure Reveals Inflammatory-like
910 Response. *Hum. Reprod.* **2007**, *22*, 2936–2946, doi:10.1093/HUMREP/DEM292.
- 911 54. Kim, Y.; Kim, H.; Park, D.; Lee, H.; Lee, Y.S.; Choe, J.; Kim, Y.M.; Jeon, D.; Jeoung, D. The Pentapeptide
912 Gly-Thr-Gly-Lys-Thr Confers Sensitivity to Anti-Cancer Drugs by Inhibition of CAGE Binding to GSK3 β
913 and Decreasing the Expression of CyclinD1. *Oncotarget* **2017**, *8*, 13632–13651,
914 doi:10.18632/ONCOTARGET.14621.
- 915 55. Lehtiniemi, T.; Kotaja, N. Germ Granule-Mediated RNA Regulation in Male Germ Cells. *Reproduction*
916 **2018**, *155*, R77–R91, doi:10.1530/REP-17-0356.
- 917 56. Voronina, E.; Seydoux, G.; Sassone-Corsi, P.; Nagamori, I. RNA Granules in Germ Cells. *Cold Spring*
918 *Harb. Perspect. Biol.* **2011**, *3*, a002774, doi:10.1101/CSHPERSPECT.A002774.
- 919 57. Chuma, S.; Hosokawa, M.; Tanaka, T.; Nakatsuji, N. Ultrastructural Characterization of Spermatogenesis
920 and Its Evolutionary Conservation in the Germline: Germinal Granules in Mammals. *Mol. Cell.*
921 *Endocrinol.* **2009**, *306*, 17–23, doi:10.1016/J.MCE.2008.11.009.
- 922 58. Dassi, E. Handshakes and Fights: The Regulatory Interplay of RNA-Binding Proteins. *Front. Mol. Biosci.*
923 **2017**, *4*, 67, doi:10.3389/FMOLB.2017.00067/BIBTEX.
- 924 59. Quattrone, A.; Dassi, E. The Architecture of the Human RNA-Binding Protein Regulatory Network.
925 *iScience* **2019**, *21*, 706–719, doi:10.1016/J.ISCI.2019.10.058.
- 926 60. Pullmann, R.J.; Kim, H.H.; Abdelmohsen, K.; Lal, A.; Martindale, J.L.; Yang, X.; Gorospe, M. Analysis

- 927 of Turnover and Translation Regulatory RNA-Binding Protein Expression through Binding to Cognate
- 928 MRNAs. *Mol. Cell. Biol.* **2007**, 27, 6265–6278, doi:10.1128/MCB.00500-07.
- 929 61. Loggia, D.; O’Flaherty, C. Citrate Promotes Nitric Oxide Production during Human Sperm Capacitation.
- 930 *Antioxidants* **2024**, 13, 885, doi:10.3390/ANTIOX13080885/S1.
- 931 62. Dun, M.D.; Aitken, R.J.; Nixon, B. The Role of Molecular Chaperones in Spermatogenesis and the Post-
- 932 Testicular Maturation of Mammalian Spermatozoa. *Hum. Reprod. Update* **2012**, 18, 420–435,
- 933 doi:10.1093/humupd/dms009.
- 934 63. Di Giorgio, M.L.; Morciano, P.; Bucciarelli, E.; Porrazzo, A.; Cipressa, F.; Saraniero, S.; Manzi, D.; Rong,
- 935 Y.S.; Cenci, G. The Drosophila Citrate Lyase Is Required for Cell Division during Spermatogenesis. *Cells*
- 936 **2020**, 9, 206, doi:10.3390/CELLS9010206.
- 937 64. Yadav, S.K.; Pandey, A.; Kumar, L.; Devi, A.; Kushwaha, B.; Vishvkarma, R.; Maikhuri, J.P.; Rajender,
- 938 S.; Gupta, G. The Thermo-Sensitive Gene Expression Signatures of Spermatogenesis. *Reprod. Biol.*
- 939 *Endocrinol.* **2018**, 16, 1–22, doi:10.1186/s12958-018-0372-8.
- 940 65. Luna, R.; Rondón, A.G.; Aguilera, A. New Clues to Understand the Role of THO and Other Functionally
- 941 Related Factors in MRNP Biogenesis. *Biochim. Biophys. Acta - Gene Regul. Mech.* **2012**, 1819, 514–520,
- 942 doi:10.1016/J.BBAGRM.2011.11.012.
- 943 66. Jimeno, S.; Aguilera, A. The THO Complex as a Key MRNP Biogenesis Factor in Development and Cell
- 944 Differentiation. *J. Biol.* **2010**, 9, 1–3, doi:10.1186/jbiol217.
- 945 67. Yu, T.; Fan, K.; Özata, D.M.; Zhang, G.; Fu, Y.; Theurkauf, W.E.; Zamore, P.D.; Weng, Z. Long First
- 946 Exons and Epigenetic Marks Distinguish Conserved Pachytene PiRNA Clusters from Other Mammalian
- 947 Genes. *Nat. Commun.* **2021**, 12, 73(1-17), doi:10.1038/s41467-020-20345-3.

- 948 68. Hasegawa, K.; Sin, H.S.; Maezawa, S.; Broering, T.J.; Kartashov, A. V.; Alavattam, K.G.; Ichijima, Y.;
949 Zhang, F.; Bacon, W.C.; Greis, K.D.; et al. SCML2 Establishes the Male Germline Epigenome through
950 Regulation of Histone H2A Ubiquitination. *Dev. Cell* **2015**, *32*, 574–588,
951 doi:10.1016/j.devcel.2015.01.014.
- 952 69. Menon, D.U.; Shibata, Y.; Mu, W.; Magnuson, T. Mammalian SWI/SNF Collaborates with a Polycomb-
953 Associated Protein to Regulate Male Germline Transcription in the Mouse. *Development* **2019**, *146*,
954 dev174094, doi:10.1242/DEV.174094.
- 955 70. Maezawa, S.; Hasegawa, K.; Alavattam, K.G.; Funakoshi, M.; Sato, T.; Barski, A.; Namekawa, S.H.
956 SCML2 Promotes Heterochromatin Organization in Late Spermatogenesis. *J. Cell Sci.* **2018**, *131*,
957 jcs217125, doi:10.1242/JCS.217125.
- 958 71. Shah, A.; Rashid, F.; Awan, H.M.; Hu, S.; Wang, X.; Chen, L.; Shan, G. The DEAD-Box RNA Helicase
959 DDX3 Interacts with M6A RNA Demethylase ALKBH5. *Stem Cells Int.* **2017**, *2017*,
960 doi:10.1155/2017/8596135.
- 961 72. Kapadia, B.; Roychowdhury, A.; Kayastha, F.; Lee, W.S.; Nanaji, N.; Windle, J.; Gartenhaus, R. M6A
962 Eraser ALKBH5/TreRNA1/DDX46 Axis Regulates BCR Expression. *Neoplasia* **2025**, *62*, 101144,
963 doi:10.1016/J.NEO.2025.101144.
- 964 73. Linder, P.; Jankowsky, E. From Unwinding to Clamping — the DEAD Box RNA Helicase Family. *Nat.*
965 *Rev. Mol. Cell Biol.* **2011**, *12*, 505–516, doi:10.1038/nrm3154.
- 966 74. Putnam, A.A.; Jankowsky, E. DEAD-Box Helicases as Integrators of RNA, Nucleotide and Protein
967 Binding. *Biochim. Biophys. Acta* **2013**, *1829*, 884–893, doi:10.1016/J.BBAGRM.2013.02.002.
- 968 75. Ramathal, C.; Angulo, B.; Sukhwani, M.; Cui, J.; Durruthy-Durruthy, J.; Fang, F.; Schanes, P.; Turek, P.J.;

Orwig, K.E.; Reijo Pera, R. DDX3Y Gene Rescue of a Y Chromosome AZFa Deletion Restores Germ
Cell Formation and Transcriptional Programs. *Sci. Rep.* **2015**, *5*, 1–13, doi:10.1038/srep15041.

76. Medrano, J. V.; Ramathal, C.; Nguyen, H.A.N.; Simon, C.; Reijo Pera, R.A. Divergent RNA-binding
Proteins, DAZL and VASA, Induce Meiotic Progression in Human Germ Cells Derived in Vitro. *Stem
Cells* **2012**, *30*, 441–451, doi:10.1002/STEM.1012.

77. Kavarthapu, R.; Anbazhagan, R.; Raju, M.; Morris, C.H.T.; Pickel, J.; Dufau, M.L. Targeted Knock-in
Mice with a Human Mutation in GRTH/DDX25 Reveals the Essential Role of Phosphorylated GRTH in
Spermatid Development during Spermatogenesis. *Hum. Mol. Genet.* **2019**, *28*, 2561–2572,
doi:10.1093/HMG/DDZ079.

78. Looijenga, L.H.J.; Stoop, H.; Biermann, K. Testicular Cancer: Biology and Biomarkers. *Virchows Arch.*
2014, *464*, 301–313, doi:10.1007/S00428-013-1522-1/FIGURES/7.

79. Windschüttl, S.; Kampfer, C.; Mayer, C.; Flenkenthaler, F.; Fröhlich, T.; Schwarzer, J.U.; Köhn, F.M.;
Urbanski, H.; Arnold, G.J.; Mayerhofer, A. Human Testicular Peritubular Cells Secrete Pigment
Epithelium-Derived Factor (PEDF), Which May Be Responsible for the Avascularity of the Seminiferous
Tubules. *Sci. Rep.* **2015**, *5*, doi:10.1038/SREP12820.

80. Bagdadi, N.; Sawaied, A.; Abumadighem, A.; Lunenfeld, E.; Huleihel, M. The Expression Levels and
Cellular Localization of Pigment Epithelium Derived Factor (PEDF) in Mouse Testis: Its Possible
Involvement in the Differentiation of Spermatogonial Cells. *Int. J. Mol. Sci.* **2021**, *22*, 1147,
doi:10.3390/IJMS22031147.

81. Abooshahab, R.; Al-Salami, H.; Dass, C.R. The Increasing Role of Pigment Epithelium-Derived Factor in
Metastasis: From Biological Importance to a Promising Target. *Biochem. Pharmacol.* **2021**, *193*, 114787,

- doi:10.1016/J.BCP.2021.114787.
82. Tovey, C.A.; Tubman, C.E.; Hamrud, E.; Zhu, Z.; Dyas, A.E.; Butterfield, A.N.; Fyfe, A.; Johnson, E.; Conduit, P.T. γ -TuRC Heterogeneity Revealed by Analysis of Mozart1. *Curr. Biol.* **2018**, *28*, 2314–2323.e6, doi:10.1016/j.cub.2018.05.044.
83. Lück, J.C.; Puchkov, D.; Ullrich, F.; Jentsch, T.J. LRRC8/VRAC Anion Channels Are Required for Late Stages of Spermatid Development in Mice. *J. Biol. Chem.* **2018**, *293*, 11796–11808, doi:10.1074/jbc.RA118.003853.
84. Faja, F.; Carlini, T.; Coltrinari, G.; Finocchi, F.; Nespoli, M.; Pallotti, F.; Lenzi, A.; Lombardo, F.; Paoli, D. Human Sperm Motility: A Molecular Study of Mitochondrial DNA, Mitochondrial Transcription Factor A Gene and DNA Fragmentation. *Mol. Biol. Rep.* **2019**, *46*, 4113–4121, doi:10.1007/s11033-019-04861-0.
85. Jiang, M.; Kauppila, T.E.S.; Motori, E.; Li, X.; Atanassov, I.; Folz-Donahue, K.; Bonekamp, N.A.; Albarran-Gutierrez, S.; Stewart, J.B.; Larsson, N.G. Increased Total MtDNA Copy Number Cures Male Infertility Despite Unaltered MtDNA Mutation Load. *Cell Metab.* **2017**, *26*, 429–436.e4, doi:10.1016/J.CMET.2017.07.003.
86. Rantanen, A.; Jansson, M.; Oldfors, A.; Larsson, N.G. Downregulation of Tfam and MtDNA Copy Number during Mammalian Spermatogenesis. *Mamm. Genome* **2001**, *12*, 787–792, doi:10.1007/s00335-001-2052-8.
87. Ladd, A.N.; Charlet-B., N.; Cooper, T.A. The CELF Family of RNA Binding Proteins Is Implicated in Cell-Specific and Developmentally Regulated Alternative Splicing. *Mol. Cell. Biol.* **2001**, *21*, 1285–1296, doi:10.1128/MCB.21.4.1285-1296.2001.

- 1011 88. Huang, L.; Li, G.; Du, C.; Jia, Y.; Yang, J.; Fan, W.; Xu, Y.; Cheng, H.; Zhou, Y. The PolyA Tail
1012 Facilitates Splicing of Last Introns with Weak 3' Splice Sites via PABPN1. *EMBO Rep.* **2023**, *24*, e57128,
1013 doi:10.15252/EMBR.202357128.
- 1014 89. Masuda, S.; Das, R.; Cheng, H.; Hurt, E.; Dorman, N.; Reed, R. Recruitment of the Human TREX
1015 Complex to mRNA during Splicing. *Genes Dev.* **2005**, *19*, 1512–1517, doi:10.1101/GAD.1302205.
- 1016 90. Sun, L.; Lv, Z.; Chen, X.; Ye, R.; Tian, S.; Wang, C.; Xie, X.; Yan, L.; Yao, X.; Shao, Y.; et al. Splicing
1017 Factor SRSF1 Is Essential for Homing of Precursor Spermatogonial Stem Cells in Mice. *Elife* **2024**, *12*,
1018 doi:10.7554/ELIFE.89316.
- 1019 91. Lei, W.L.; Li, Y.Y.; Du, Z.; Su, R.; Meng, T.G.; Ning, Y.; Hou, G.; Schatten, H.; Wang, Z.B.; Han, Z.; et
1020 al. SRSF1-Mediated Alternative Splicing Is Required for Spermatogenesis. *Int. J. Biol. Sci.* **2023**, *19*, 4883,
1021 doi:10.7150/IJBS.83474.
- 1022 92. Chao, H.C.A.; Lin, Y.H.; Kuo, Y.C.; Shen, C.J.; Pan, H.A.; Kuo, P.L. The Expression Pattern of SEPT7
1023 Correlates with Sperm Morphology. *J. Assist. Reprod. Genet.* **2010**, *27*, 299, doi:10.1007/S10815-010-
1024 9409-4.
- 1025 93. Naro, C.; Cesari, E.; Sette, C. Splicing Regulation in Brain and Testis: Common Themes for Highly
1026 Specialized Organs. *Cell cycle* **2021**, *20*, 480–489, doi:10.1080/15384101.2021.1889187.
- 1027 94. Mazin, P. V.; Khaitovich, P.; Cardoso-Moreira, M.; Kaessmann, H. Alternative Splicing during
1028 Mammalian Organ Development. *Nat. Genet.* **2021**, *53*, 925–934, doi:10.1038/S41588-021-00851-W.
- 1029 95. Liu, W.; Lu, X.; Zhao, Z.-H.; SU, R.; Li, Q.-N.L.; Xue, Y.; Gao, Z.; Sun, S.-M.S.; Lei, W.-L.; Li, L.; et al.
1030 SRSF10 Is Essential for Progenitor Spermatogonia Expansion by Regulating Alternative Splicing. *Elife*
1031 **2022**, *11*, doi:10.7554/ELIFE.78211.

- 1032 96. Pal, S.; Paladhi, P.; Dutta, S.; Bose, G.; Ghosh, P.; Chattopadhyay, R.; Chakravarty, B.; Saha, I.; Ghosh,
1033 S. Novel Variations in Spermatogenic Transcription Regulators RFX2 and TAF7 Increase Risk of
1034 Azoospermia. *J. Assist. Reprod. Genet.* **2021**, *38*, 3195–3212, doi:10.1007/S10815-021-02352-5.
- 1035 97. Kimmins, S.; Kotaja, N.; Davidson, I.; Sassone-Corsi, P. Testis-Specific Transcription Mechanisms
1036 Promoting Male Germ-Cell Differentiation. *Reproduction* **2004**, *128*, 5–12, doi:10.1530/REP.1.00170.
- 1037 98. Lardone, M.C.; Diaz-Fontdevila, M.; Ortiz, E.; Iñiguez, G.; Inostroza, P.; Espinoza, C.; Ebensperger, M.;
1038 Almstrup, K.; Castro, A. LIN 28B Expression Is Downregulated in Mature Spermatozoa of
1039 Oligozoospermic Men and Associates with Genetic Variants Previously Linked to Pubertal Onset. *Endocr.*
1040 *Connect.* **2025**, *14*, doi:10.1530/EC-25-0044.
- 1041 99. Peng, F.; Muhuitijiang, B.; Zhou, J.; Liang, H.; Zhang, Y.; Zhou, R. An Artificial Neural Network Model
1042 to Diagnose Non-Obstructive Azoospermia Based on RNA-Binding Protein-Related Genes. *Aging*
1043 *(Albany NY)* **2023**, *15*, 3120, doi:10.18632/AGING.204674.
- 1044 100. Shang, E.; Salazar, G.; Crowley, T.E.; Wang, X.; Lopez, R.A.; Wang, X.; Wolgemuth, D.J. Identification
1045 of Unique, Differentiation Stage-Specific Patterns of Expression of the Bromodomain-Containing Genes
1046 Brd2, Brd3, Brd4, and Brdt in the Mouse Testis. *Gene Expr. Patterns* **2004**, *4*, 513–519,
1047 doi:10.1016/J.MODGEP.2004.03.002.
- 1048 101. Klaus, E.S.; Gonzalez, N.H.; Bergmann, M.; Bartkuhn, M.; Weidner, W.; Kliesch, S.; Rathke, C. Murine
1049 and Human Spermatids Are Characterized by Numerous, Newly Synthesized and Differentially Expressed
1050 Transcription Factors and Bromodomain-Containing Proteins. *Biol. Reprod.* **2016**, *95*, 1–12,
1051 doi:10.1095/BIOLREPROD.115.137620.
- 1052 102. Kamijo, A.; Saitoh, Y.; Sakamoto, T.; Kubota, H.; Yamauchi, J.; Terada, N. Scaffold Protein Lin7 Family

- 1053 in Membrane Skeletal Protein Complex in Mouse Seminiferous Tubules. *Histochem. Cell Biol.* **2019**, *152*,
1054 333–343, doi:10.1007/s00418-019-01807-2.
- 1055 103. Wang, R.A.; Zhao, M.; Meistrich, M.L.; Kumar, R. Stage-Specific Expression of Dynein Light Chain-1
1056 and Its Interacting Kinase, P21-Activated Kinase-1, in Rodent Testes: Implications in Spermiogenesis. *J.*
1057 *Histochem. Cytochem.* **2005**, *53*, 1235–1243, doi:10.1369/jhc.5A6688.2005.
- 1058 104. Pham-Bui, H.A.; Lee, M. Germ Granule-Mediated mRNA Storage and Translational Control. *RNA Biol.*
1059 **2025**, *22*, 1–11, doi:10.1080/15476286.2025.2462276.
- 1060 105. Pamula, M.C.; Lehmann, R. How Germ Granules Promote Germ Cell Fate. *Nat. Rev. Genet.* **2024**, *25*,
1061 803–821, doi:10.1038/s41576-024-00744-8.
- 1062 106. Kistler, K.E.; Treck, T.; Hurd, T.R.; Chen, R.; Liang, F.X.; Sall, J.; Kato, M.; Lehmann, R. Phase
1063 Transitioned Nuclear Oskar Promotes Cell Division of Drosophila Primordial Germ Cells. *Elife* **2018**, *7*,
1064 e37949, doi:10.7554/ELIFE.37949.
- 1065 107. Malcher, A.; Smolibowski, M.; Stokowy, T.; Bauer, H.; Patyk, A.; Jedrzejczak, P.; Kostyk, J.; Graczyk,
1066 Z.; Ibrahim, R.; Bednarek-Rajewska, K.; et al. TKTL1: A New Candidate Gene in Non-Obstructive
1067 Azoospermia. *Reprod. Biomed. Online* **2025**, *51*, doi:10.1016/j.rbmo.2025.104895.
- 1068

Supplementary Material

The RNA helicase DDX53 (CAGE) contributes to RNA metabolism in a human germ cell model

Agata J.. Barszcz¹, Katarzyna Tutak^{1,2}, Agnieszka Malcher¹, Małgorzata Dąbrowska¹, Joanna
Zyprych-Walczak³, Erik Dassi⁴, Erkut Ilaslan^{1,5}, Marta Olszewska¹, Dominik Cysewski⁶,
Michał Hrab⁷, Tomasz Kolanowski¹, Alexander Yatsenko^{8,9,10}, Maciej Kurpisz¹ and Natalia
Rozwadowska^{1*}

¹ Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

² Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland

³ Department of Mathematical and Statistical Methods, Poznan University of Life Sciences, Poznan, Poland

⁴ Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

⁵ DNRF Center for Chromosome Stability, University of Copenhagen, DK-2200 Copenhagen, Denmark

⁶ Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

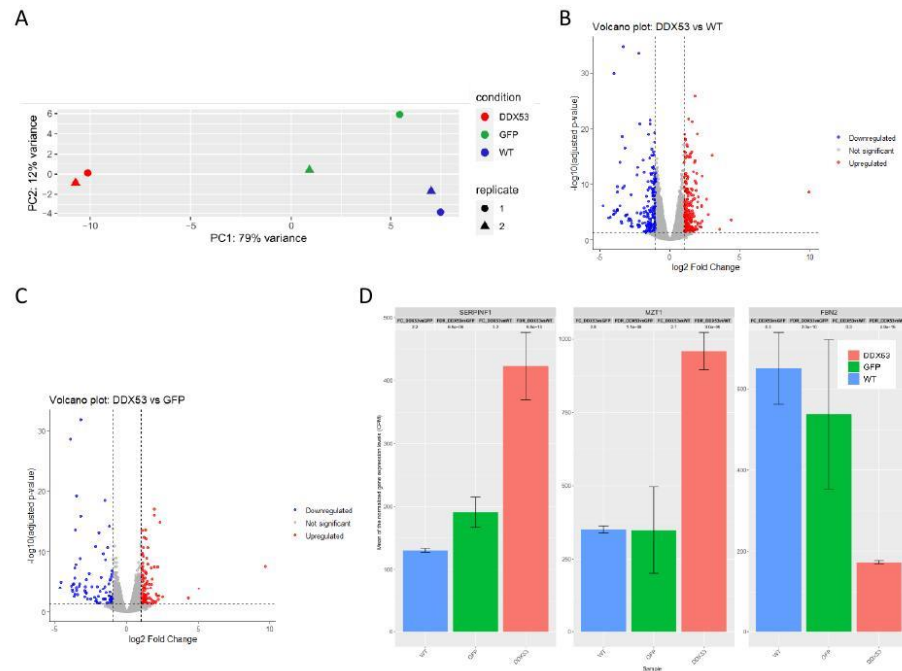
⁷ Department of Urology, Poznan University of Medical Sciences, Poznan, Poland

⁸ Department of Obstetrics, Gynecology, and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States

⁹ Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States

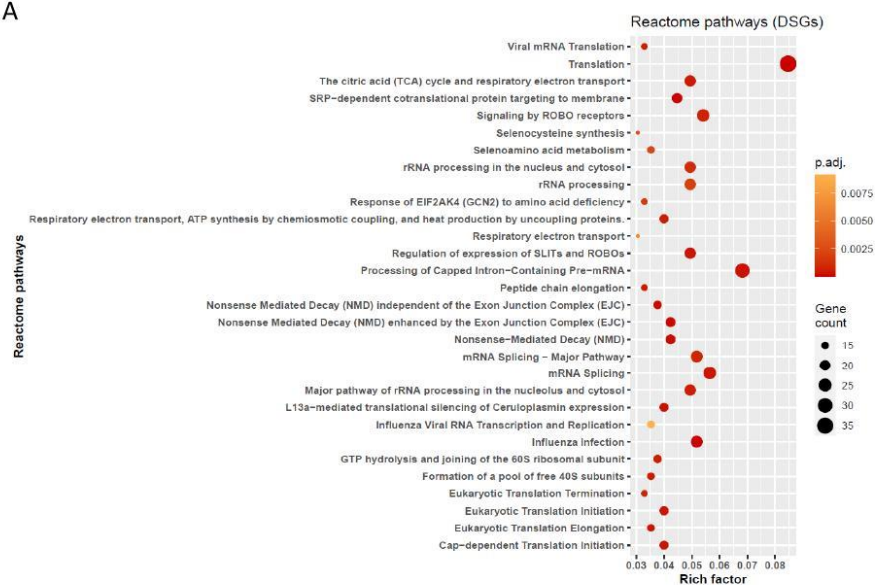
¹⁰ Department of Genetics, University of Pittsburgh School of Public Health, Pittsburgh, PA, United States

*Correspondence: natalia.rozwadowska@igcz.poznan.pl

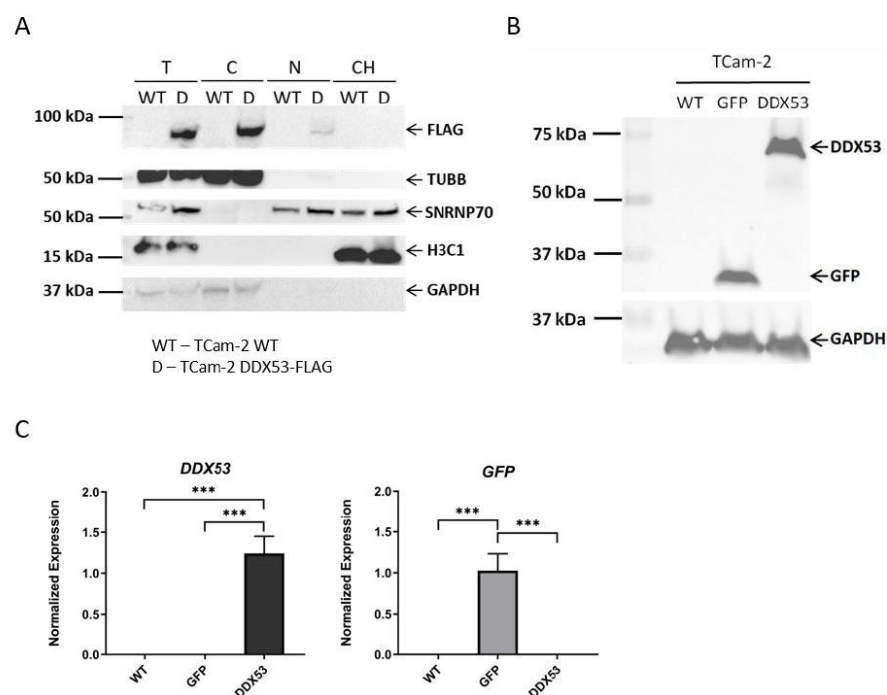


Supplementary Figure S1. Principal component analysis results of TCam-2 RNA-seq data, volcano plots, and expression results for three DEGs (SERPINF1, MZT1, and FBN2) selected for validation via qRT-PCR. (A) Principal component analysis (PCA) of TCam-2 RNA-seq samples (Supplementary Table S1). The TCam-2 with DDX53 overexpression samples are highlighted in red and the GFP and WT samples are highlighted in green and blue, respectively. Test samples (DDX53) separate from the control (GFP and WT) samples along principal component 1 (PC1). The two biological replicates are presented with different shapes. (B) Volcano plot of DEGs identified using DESeq2 from RNA-seq data of DDX53-overexpressing TCam-2 cells versus WT (Supplementary Table S2). Dashed lines represent twofold change in expression (vertical lines) and adjusted FDR < 0.05 cutoff (horizontal line). (C) Volcano plot of DEGs identified using DESeq2 from RNA-seq data of DDX53-overexpressing TCam-2 cells versus GFP (Supplementary Table S2). Dashed lines represent twofold change in expression (vertical lines) and adjusted FDR < 0.05 cutoff (horizontal line). (D) Barplots showing the mean expression level (CPM) of SERPINF1, MZT1, and FBN2 genes in TCam-2 cells with stable expression of DDX53 (red) and GFP (green) as well as WT (blue) based on RNA-seq analysis (Supplementary Table S1, 2). Additionally, fold change (FC) and adjusted p-value (FDR) for both comparisons (DDX53 vs. GFP and DDX53 vs. WT) are presented in the upper part of the plots. Error bars in black represent the standard deviation (SD) calculated for the CPM expression values from the two biological replicates.

A



Supplementary Figure S2. Potential pathways regulated by DSGs detected upon DDX53 overexpression and RNA-seq. (A) Reactome analysis for the DSGs based on the RNA-seq data. For the complete list of significant Reactome terms see Supplementary Table S6 (adjusted p-value < 0.05).



Supplementary Figure S3. Subcellular fractionation and validation of FLAG-tagged DDX53 and GFP expression in TCam-2 cells following transient transfection. (A) Western blot of TUBB (cytoplasmic marker), SNRNP70 (nuclear marker), and H3C1 (chromatin marker) as well as FLAG; following cellular fractionation of TCam-2 cells with DDX53-FLAG overexpression (denoted as D) and WT cells. The appropriate subcellular fractionation was confirmed by enrichment of the respective marker in each fraction. (B) Representative Western Blot analysis for proteins tagged with FLAG (DDX53 and GFP) on TCam-2 cells after transient transfection and wild type (WT, as control) using FLAG antibody. GAPDH was used as a loading control. (C) The mRNA expression level of the DDX53 and GFP with GAPDH and ACTB as reference genes. The TCam-2 cells were assayed 48h after transfection. Error bars represent standard deviation (SD) with $n = 3$, *** $p < 0.001$. The p values were calculated by ordinary one-way ANOVA non-parametric test with Tukey's multiple comparisons test.

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5. Wnioski

1. Systematyczne opracowanie aktualnego stanu wiedzy potwierdziło rosnące znaczenie modeli *in vitro* linii płciowej oraz spermatogenezy człowieka, w tym modeli bazujących na komórkach macierzystych, organoidów jąder oraz bioinżynieryjnych systemów mikro-przepływowych.

2. Na podstawie badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej sformułowano następujące wnioski dotyczące specyficznego dla naczelnych białka RBMXL3:

1. Wykazano jądrową lokalizację RBMXL3 w komórkach linii TCam-2 oraz ekspresję tego białka w męskiej gonadzie.
2. Wykazano wzbogacenie białka RBMXL3 we frakcji chromatynowej komórek TCam-2.
3. Stwierdzono wpływ RBMXL3 na poziom ekspresji oraz procesy alternatywnego splicingu genów takich jak *FOS*, *YY1*, *GJA1* oraz *HNRNPH1*, związanych z regulacją proliferacji komórkowej, utrzymaniem puli spermatogoniów, komunikacją pomiędzy komórkami Sertolego i komórkami germinalnymi.
4. Udowodniono, że RBMXL3 wiąże szeroki repertuar cząsteczek RNA, obejmujący między innymi transkrypty genów *BRCA1*, *BRCA2*, *MSH2*, *MSH6*, *EP300*, *PREP* oraz *FOXJ3*, związanych z utrzymaniem integralności genomu, regulacją chromatyny, przeżywalnością spermatogoniów oraz prawidłowym przebiegiem spermatogenezy.
5. Zidentyfikowano sieć interaktorów białkowych RBMXL3, obejmującą między innymi białka EIF4A3 oraz DDX39B, uczestniczące w procesach metabolizmu RNA, w tym alternatywnego splicingu.
6. Wykazano, że RBMXL3 hamuje proces retrotranspozycji ludzkich elementów LINE-1, co wskazuje na istotną rolę tego białka w utrzymaniu integralności genomu podczas rozwoju męskich komórek płciowych człowieka.

3. Na podstawie badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej sformułowano następujące wnioski dotyczące białka DDX53:

1. Wykazano głównie cytoplazmatyczną lokalizację DDX53 przy jednoczesnej częściowej obecności tego białka w jądrze komórkowym komórek linii TCam-2 oraz potwierdzono jego ekspresję na poziomie białka w męskiej gonadzie.
2. Wykazano wzbogacenie białka DDX53 we frakcji cytoplazmatycznej, a także potwierdzono jego częściową obecność we frakcji jądrowej linii komórek TCam-2.
3. Stwierdzono wpływ DDX53 na poziom ekspresji oraz procesy alternatywnego splicingu genów takich jak *MZT1*, *TFAM*, *SRSF1* oraz *SEPTIN7*, związanych między innymi z funkcją centrosomu, regulacją funkcji mitochondrialnych, procesami alternatywnego splicingu oraz dojrzewaniem i prawidłową morfologią plemników.
4. Udowodniono, że DDX53 wiąże szeroki repertuar cząsteczek RNA, obejmujący między innymi transkrypty genów *TAF7*, *LIN28B*, *NCBP2*, *BRD3*, *LIN7C* oraz *DLC1*, związanych z regulacją ekspresji genów, utrzymaniem funkcji spermatogoniów, metabolizmem RNA, organizacją chromatyny oraz rozwojem i dojrzewaniem męskich komórek płciowych.
5. Zidentyfikowano sieć interaktorów białkowych DDX53, obejmującą między innymi białka *ACLY* oraz *THOC1*, uczestniczące w procesach metabolizmu komórkowego oraz metabolizmu RNA, w tym eksporcie mRNA, istotnych dla prawidłowego przebiegu spermatogenezy.
6. Wykazano struktury wzbogacone o białko DDX53 przypominające granule germinalne, co wskazuje na potencjalny udział tego białka w funkcjonowaniu wyspecjalizowanych struktur związanych z metabolizmem RNA.

Podsumowanie:

W niniejszej rozprawie doktorskiej po raz pierwszy zidentyfikowano sieć powiązań oraz molekularne interaktory białek RBMXL3 oraz DDX53 w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka. W badaniach wykorzystano model *in vitro* ludzkich komórek germinalnych (linię komórkową TCam-2). W ramach realizacji pracy doktorskiej określono lokalizację RBMXL3 oraz DDX53

w komórkach TCam-2, oceniono ich wpływ na regulację transkryptomu i alternatywny splicing, a także zidentyfikowano pulę RNA wiązanych przez oba białka. Po raz pierwszy wytypowano również potencjalne interaktory białkowe obu badanych RBPs, a także potwierdzono obecność RBMXL3 oraz DDX53 w męskiej gonadzie dzięki wykorzystaniu specyficznych przeciwciał. Co istotne, wykazano także zdolność RBMXL3 do ograniczania retrotranspozycji elementów LINE-1, wskazując na jego potencjalną rolę w utrzymaniu integralności genomu komórek germinalnych. Dodatkowo zidentyfikowano struktury zawierające DDX53 przypominające granule germinalne, co może wskazywać na udział tego białka w tych wyspecjalizowanych strukturach, odgrywających istotną rolę w kontekście rozwoju męskich komórek płciowych.

Przeprowadzone badania dostarczają nowych danych dotyczących roli białek wiążących RNA - RBMXL3 oraz DDX53 - w regulacji rozwoju męskich komórek płciowych i spermatogenezy człowieka oraz poszerzają aktualny stan wiedzy na temat mechanizmów potranskrypcyjnych w komórkach ludzkich. Ponadto, uzyskane wyniki poszerzają wiedzę na temat złożonych i precyzyjnie regulowanych procesów warunkujących prawidłowy rozwój męskich gamet, których zaburzenia mogą prowadzić do niepłodności męskiej.

6. Streszczenie

Spermatogeneza człowieka jest wysoce zorganizowanym i dynamicznym procesem, którego odtworzenie w warunkach *in vitro* pozostaje dotychczas nieosiągnięte. Prawidłowy przebieg tego procesu wymaga precyzyjnej koordynacji czasowej i przestrzennej ekspresji genów, sygnalizacji molekularnej oraz rozległego przeprogramowania epigenetycznego. Niestety, wiele kluczowych regulatorów molekularnych mających znaczenie dla różnicowania męskiej linii płciowej wciąż pozostaje niepoznanych. Badania genetyczne wykorzystujące technologie sekwencjonowania całego genomu (WGS) oraz całego egzomu (WES) pozwoliły zidentyfikować geny *RBMXL3* oraz *DDX53* jako potencjalnie związane z procesem spermatogenezy człowieka.

Celem niniejszej rozprawy doktorskiej, która stanowi zbiór trzech artykułów naukowych, była identyfikacja sieci powiązań oraz molekularnych interaktorów białek *RBMXL3* oraz *DDX53* w kontekście rozwoju męskich komórek płciowych i spermatogenezy człowieka. We wstępie rozprawy doktorskiej opisano procesy związane z metabolizmem RNA oraz rolę przykładowych białek wiążących RNA w procesie tworzenia męskich gamet, które zostały już częściowo poznane. W pierwszej publikacji przedstawiono przegląd strategii badawczych przydatnych w kontekście badań nad rozwojem ludzkiej linii płciowej i spermatogenezy człowieka. Kolejne dwie publikacje wchodzące w skład rozprawy doktorskiej to prace oryginalne, których celem było zbadanie białek *RBMXL3* oraz *DDX53*. W celu potwierdzenia znaczenia tych białek dla rozwoju męskich komórek płciowych i spermatogenezy człowieka wykorzystano linię komórkową nowotworu jądra TCam-2. W zaprezentowanych badaniach przygotowano odpowiednie modele komórkowe z wykorzystaniem komórek TCam-2 w celu uzyskania ekspresji białek *RBMXL3*, *DDX53* oraz GFP (oznakowane epitopem FLAG). Ekspresję badanych białek potwierdzono na poziomie RNA i białka z wykorzystaniem technik molekularnych, takich jak RT-qPCR, Western blot oraz immunofluorescencji. W celu identyfikacji sieci powiązań oraz molekularnych interaktorów białek *RBMXL3* i *DDX53* przeprowadzono analizy RNA-seq, eCLIP oraz Co-IP-MS. Uzyskane wyniki umożliwiły identyfikację genów o zróżnicowanej ekspresji i alternatywnym splicingu, a także cząsteczek RNA i białek bezpośrednio oddziałujących z *RBMXL3* oraz *DDX53*. Interpretacja wyników koncentrowała się na molekularnych celach opisanych w literaturze jako związanych z różnicowaniem męskich komórek linii płciowej. Istotnym

elementem badań była również analiza lokalizacji subkomórkowej badanych białek w komórkach TCam-2, wykonana za pomocą frakcjonowania komórek połączonego z Western Blot, a także podwójnych barwień immunofluorecencyjnych. Dodatkowo obecność obu białek potwierdzono w skrawkach męskiej gonady metodą immunohistochemii z użyciem specyficznych przeciwciał.

Uzyskane wyniki pozwoliły na potwierdzenie roli białek RBMXL3 oraz DDX53 w regulacji procesów zachodzących w męskich komórkach płciowych człowieka.

7. Abstract

Human spermatogenesis is a highly organized and dynamic biological process, the full recapitulation of which under *in vitro* conditions has not yet been achieved. The proper progression of this process requires precise temporal and spatial coordination of gene expression, molecular signalling, and extensive epigenetic reprogramming. Despite significant advances, many key molecular regulators involved in male germ cell differentiation remain unidentified. Genetic studies using whole-genome sequencing (WGS) and whole-exome sequencing (WES) have identified the genes *RBMXL3* and *DDX53* as potentially associated with human spermatogenesis.

The aim of this doctoral thesis, comprising three scientific publications, was to investigate the network and molecular targets of the RBMXL3 and DDX53 proteins in the context of male germ cells development and human spermatogenesis. The introductory chapter describes processes related to RNA metabolism and the roles of selected proteins in male gamete formation that have been partially characterised to date. The first publication provides an overview of research strategies relevant to studies of human germ cells and spermatogenesis. The remaining two publications are original research articles focused on the investigation of RBMXL3 and DDX53 proteins. To assess the relevance of these proteins in the context of male germ cells development and human spermatogenesis, the TCam-2 human testicular germ cell tumour cell line was used. Appropriate cellular models were established using TCam-2 cells to achieve the expression of RBMXL3, DDX53, and GFP proteins (FLAG-tagged). The expression of the studied proteins was confirmed at both the RNA and protein levels using molecular techniques, including RT-qPCR, Western blotting, and immunofluorescence. Investigation of the interaction networks and molecular targets of RBMXL3 and DDX53 was performed using RNA-seq, eCLIP, and Co-IP-MS. The obtained results enabled the identification of differentially expressed genes, alternative splicing events, as well as RNA molecules and proteins directly interacting with RBMXL3 and DDX53. The interpretation of the results focused on molecular targets described in the literature as being involved in male germ cell differentiation. Subcellular localisation of the investigated proteins was also analysed using cell fractionation combined with Western blotting and double immunofluorescence staining. Additionally, the presence of both proteins was confirmed in human testicular sections by immunohistochemistry using specific antibodies.

Collectively, the findings of this doctoral thesis support the role of RBMXL3 and DDX53 proteins in the regulation of processes underlying human male germ cell development.

8. Bibliografia

- Amann, Rupert P. 2008. "The Cycle of the Seminiferous Epithelium in Humans: A Need to Revisit?" *Journal of Andrology* 29(5):469–87. doi: <https://doi.org/10.2164/jandrol.107.004655>.
- Anbazzhagan, Rajakumar, Raghuveer Kavarthapu, Maria L. Dufau, Rajakumar Anbazzhagan, Raghuveer Kavarthapu, and Maria L. Dufau. 2022. "Chromatoid Bodies in the Regulation of Spermatogenesis: Novel Role of GRTH." *Cells* 2022, Vol. 11, 11(4):613. doi: 10.3390/CELLS11040613.
- Chen, Qing, Safia Malki, Xiaojiang Xu, Jiajia Wang, Brian Bennett, Xiaofeng Zheng, Brad L. Lackford, Oleksandr Kirsanov, Christopher B. Geyer, and Guang Hu. 2025. "Cnot3 Is Required for Male Germ Cell Development and Spermatogonial Stem Cell Maintenance." *Development (Cambridge, England)* 152(15):dev204557. doi: 10.1242/DEV.204557.
- Choi, Jin Ouk, Jeong Hyeon Ham, and Soo Seok Hwang. 2022. "RNA Metabolism in T Lymphocytes." *Immune Network* 22(5). doi: 10.4110/IN.2022.22.E39.
- Chong, P. Andrew, Robert M. Vernon, and Julie D. Forman-Kay. 2018. "RGG/RG Motif Regions in RNA Binding and Phase Separation." *Journal of Molecular Biology* 430(23):4650–65. doi: 10.1016/j.jmb.2018.06.014.
- Corley, Meredith, Margaret C. Burns, and Gene W. Yeo. 2020. "How RNA-Binding Proteins Interact with RNA: Molecules and Mechanisms." *Molecular Cell* 78(1):9–29. doi: 10.1016/J.MOLCEL.2020.03.011.
- Dassi, Erik. 2017. "Handshakes and Fights: The Regulatory Interplay of RNA-Binding Proteins." *Frontiers in Molecular Biosciences* 4:67. doi: 10.3389/FMOLB.2017.00067.
- Ehrmann, Ingrid, James H. Crichton, Matthew R. Gazzara, Katherine James, Yilei Liu, Sushma Nagaraja Grellscheid, Tomaž Curk, Dirk de Rooij, Jannetta S. Steyn, Simon Cockell, Ian R. Adams, Yoseph Barash, and David J. Elliott. 2019. "An Ancient Germ Cell-Specific RNA-Binding Protein Protects the Germline from Cryptic Splice Site Poisoning." *ELife* 8:e39304. doi: 10.7554/eLife.39304.
- Eisenberg, Michael L., Sandro C. Esteves, Dolores J. Lamb, James M. Hotaling, Aleksander Giwercman, Kathleen Hwang, and Yu Sheng Cheng. 2023. "Male Infertility." *Nature Reviews Disease Primers* 9(1):49. doi: 10.1038/s41572-023-00459-w.

- Elliott, David J. 2000. “RBMV Genes and AZFb Deletions.” *Journal of Endocrinological Investigation* 23(10):652–58. doi: 10.1007/BF03343789.
- Gao, Ying, Yikun Zhou, Zhidan Hong, Binyu Ma, Xiaojie Wang, Linhang Nie, Ling Ma, Yuanzhen Zhang, Ming Zhang, and Mei Wang. 2025. “The Intricate Dance of RNA-Binding Proteins: Unveiling the Mechanisms behind Male Infertility.” *Human Reproduction Update* 32(1):58. doi: 10.1093/HUMUPD/DMAF023.
- Gehring, Niels H., and Jean Yves Roignant. 2021. “Anything but Ordinary – Emerging Splicing Mechanisms in Eukaryotic Gene Regulation.” *Trends in Genetics* 37(4):355–72. doi: 10.1016/J.TIG.2020.10.008.
- Gerstberger, Stefanie, Markus Hafner, and Thomas Tuschl. 2014. “A Census of Human RNA-Binding Proteins.” *Nature Reviews Genetics* 15(12):829. doi: 10.1038/NRG3813.
- Griswold, Michael D. 2016. “Spermatogenesis: The Commitment to Meiosis.” *Physiological Reviews* 96(1):1–17. doi: 10.1152/physrev.00013.2015.
- Guo, Jingtao, Xichen Nie, Maria Giebler, Hana Mlcochova, Yueqi Wang, Edward J. Grow, Robin Kim, Melissa Tharmalingam, Gabriele Matilonyte, Cecilia Lindskog, Douglas T. Carrell, Rod T. Mitchell, Anne Goriely, James M. Hotaling, and Bradley R. Cairns. 2020. “The Dynamic Transcriptional Cell Atlas of Testis Development during Human Puberty.” *Cell Stem Cell* 26(2):262–276.e4. doi: 10.1016/J.STEM.2019.12.005.
- Hardy, J., N. Pollock, T. Gingrich, P. Sweet, A. Ramesh, J. Kuong, A. Basar, H. Jiang, K. Hwang, J. Vukina, T. Jaffe, M. Olszewska, M. Kurpysz, and A. N. Yatsenko. 2022. “Genomic Testing for Copy Number and Single Nucleotide Variants in Spermatogenic Failure.” *Journal of Assisted Reproduction and Genetics* 39(9):2103–14. doi: 10.1007/s10815-022-02538-5.
- Hentze, Matthias W., Alfredo Castello, Thomas Schwarzl, and Thomas Preiss. 2018. “A Brave New World of RNA-Binding Proteins.” *Nature Reviews Molecular Cell Biology* 19(5):327–41. doi: 10.1038/nrm.2017.130.
- Hermann, Brian P., Keren Cheng, Anukriti Singh, Lorena Roa-De La Cruz, Kazadi N. Mutoji, I. Chung Chen, Heidi Gildersleeve, Jake D. Lehle, Max Mayo, Birgit Westernströer, Nathan C. Law, Melissa J. Oatley, Ellen K. Velte, Bryan A. Niedenberger, Danielle Fritze, Sherman Silber, Christopher B. Geyer, Jon M. Oatley, and John R. McCarrey. 2018. “The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids.” *Cell Reports*

- 25(6):1650-1667.e8. doi: 10.1016/J.CELREP.2018.10.026.
- Hermo, Louis, R. Marc Pelletier, Daniel G. Cyr, and Charles E. Smith. 2010. "Surfing the Wave, Cycle, Life History, and Genes/Proteins Expressed by Testicular Germ Cells. Part 2: Changes in Spermatid Organelles Associated with Development of Spermatozoa." *Microscopy Research and Technique* 73(4):279–319. doi: 10.1002/JEMT.20787.
- Irie, Naoko, Leehee Weinberger, Walfred W. C. Tang, Toshihiro Kobayashi, Sergey Viukov, Yair S. Manor, Sabine Dietmann, Jacob H. Hanna, and M. Azim Surani. 2015. "SOX17 Is a Critical Specifier of Human Primordial Germ Cell Fate." *Cell* 160(1–2):253–68. doi: 10.1016/j.cell.2014.12.013.
- De Jong, Jeroen, Hans Stoop, Ad J. M. Gillis, Remko Hersmus, Ruud J. H. L. M. Van Gorp, Gert Jan M. Van De Geijn, Ellen Van Drunen, H. Berna Beverloo, Dominik T. Schneider, Jon K. Sherlock, John Baeten, Sohei Kitazawa, E. Joop Van Zoelen, Kees Van Roozendaal, J. Wolter Oosterhuis, and Leendert H. J. Looijenga. 2008. "Further Characterization of the First Seminoma Cell Line TCam-2." *Genes, Chromosomes & Cancer* 47(3):185–96. doi: 10.1002/GCC.20520.
- Kaltsas, Aris, Maria-Anna Kyrgiagini, Eleftheria Markou, Andreas Koumenis, Zissis Mamuris, Fotios Dimitriadis, Athanasios Zachariou, Michael Chrisofos, Nikolaos Sofikitis, Aris Kaltsas, Maria-Anna Kyrgiagini, Eleftheria Markou, Andreas Koumenis, Zissis Mamuris, Fotios Dimitriadis, Athanasios Zachariou, Michael Chrisofos, and Nikolaos Sofikitis. 2025. "Artificial Gametogenesis and In Vitro Spermatogenesis: Emerging Strategies for the Treatment of Male Infertility." *International Journal of Molecular Sciences* 26(15). doi: 10.3390/IJMS26157383.
- Komeya, Mitsuru, Takuya Sato, and Takehiko Ogawa. 2018. "In Vitro Spermatogenesis: A Century-Long Research Journey, Still Half Way Around." *Reproductive Medicine and Biology* 17(4). doi: 10.1002/rmb2.12225.
- Kubota, Hiroshi, and Ralph L. Brinster. 2018. "Spermatogonial Stem Cells." *Biology of Reproduction* 99(1):52. doi: 10.1093/BIOLRE/IOY077.
- Lei, Wen Long, Yuan Yuan Li, Zongchang Du, Ruibao Su, Tie Gang Meng, Yan Ning, Guanmei Hou, Heide Schatten, Zhen Bo Wang, Zhiming Han, Fei Sun, Wei Ping Qian, Chenli Liu, and Qing Yuan Sun. 2023. "SRSF1-Mediated Alternative Splicing Is Required for Spermatogenesis." *International Journal of Biological Sciences* 19(15):4883. doi: 10.7150/IJBS.83474.
- Li, Haixin, Zhuqing Liang, Jian Yang, Dan Wang, Hanben Wang, Mengyi Zhu, Baobao

- Geng, and Eugene Yujun Xu. 2018. “DAZL Is a Master Translational Regulator of Murine Spermatogenesis.” *National Science Review* 6(3):455. doi: 10.1093/NSR/NWY163.
- Li, Xin, Dandan Cao, Yue Lu, Zexin Bian, Tingting Zhao, Wenbo Liu, Yan Zhao, Jun Tan, Lei Li, and Eugene Yujun Xu. 2025. “Loss of Full-Length DAZL Isoform Disrupts PABPC1-Dependent Translational Regulation and Meiosis.” *Cell Death & Disease* 16(1). doi: 10.1038/S41419-025-08179-7.
- Li, Yang, Yuanyuan Wang, Yue Qiu Tan, Qiuling Yue, Yueshuai Guo, Ruoyu Yan, Lanlan Meng, Huicong Zhai, Lingxiu Tong, Zihan Yuan, Wu Li, Cuicui Wang, Shenglin Han, Sen Ren, Yitong Yan, Weixu Wang, Lei Gao, Chen Tan, Tongyao Hu, Hao Zhang, Liya Liu, Pinglan Yang, Wanyin Jiang, Yiting Ye, Huanhuan Tan, Yanfeng Wang, Chenyu Lu, Xin Li, Jie Xie, Gege Yuan, Yiqiang Cui, Bin Shen, Cheng Wang, Yichun Guan, Wei Li, Qinghua Shi, Ge Lin, Ting Ni, Zheng Sun, Lan Ye, Anastasios Vourekas, Xuejiang Guo, Mingyan Lin, and Ke Zheng. 2024. “The Landscape of RNA Binding Proteins in Mammalian Spermatogenesis.” *Science* 386(6720). doi: 10.1126/science.adj8172.
- Looijenga, Leendert H. J., Hans Stoop, and Katharina Biermann. 2014. “Testicular Cancer: Biology and Biomarkers.” *Virchows Archiv* 464(3):301–13. doi: 10.1007/S00428-013-1522-1/FIGURES/7.
- Malcher, Agnieszka, Tomasz Stokowy, Andrea Berman, Marta Olszewska, Piotr Jedrzejczak, Dawid Sielski, Adam Nowakowski, Natalia Rozwadowska, Alexander N. Yatsenko, and Maciej K. Kurpisz. 2022. “Whole-Genome Sequencing Identifies New Candidate Genes for Nonobstructive Azoospermia.” *Andrology* 10(8):1605–24. doi: 10.1111/ANDR.13269.
- Mazin, Pavel V., Philipp Khaitovich, Margarida Cardoso-Moreira, and Henrik Kaessmann. 2021. “Alternative Splicing during Mammalian Organ Development.” *Nature Genetics* 53(6):925–34. doi: 10.1038/S41588-021-00851-W.
- McSwiggin, H. M., and A. M. O’Doherty. 2018. “Epigenetic Reprogramming during Spermatogenesis and Male Factor Infertility.” *Reproduction* 156(2):R9–21. doi: 10.1530/REP-18-0009.
- Meikar, Oliver, Vasily V. Vagin, Frédéric Chalmel, Karin Söstar, Aurélie Lardenois, Molly Hammell, Ying Jin, Matteo Da Ros, Kaja A. Wasik, Jorma Toppari, Gregory J. Hannon, and Noora Kotaja. 2014. “An Atlas of Chromatoid Body Components.” *RNA* 20(4):483. doi: 10.1261/RNA.043729.113.

- Miao, Xiaosu, and Guang Hu. 2025. "Poly(A) Tail Regulation in Stem Cells and Early Development." *Cell Regeneration* 14(1):48. doi: 10.1186/S13619-025-00250-0.
- Mitchell, Sarah F., and Roy Parker. 2014. "Principles and Properties of Eukaryotic MRNPs." *Molecular Cell* 54(4):547–58. doi: 10.1016/j.molcel.2014.04.033.
- Morgan, Marcos, Lokesh Kumar, Yin Li, and Marine Baptissart. 2021a. "Post-Transcriptional Regulation in Spermatogenesis: All RNA Pathways Lead to Healthy Sperm." *Cellular and Molecular Life Sciences* 78(24):8049. doi: 10.1007/S00018-021-04012-4.
- Morgan, Marcos, Lokesh Kumar, Yin Li, and Marine Baptissart. 2021b. "Post-Transcriptional Regulation in Spermatogenesis: All RNA Pathways Lead to Healthy Sperm." *Cellular and Molecular Life Sciences* 78(24):8049. doi: 10.1007/S00018-021-04012-4.
- Mruk, Dolores D., and C. Yan Cheng. 2015. "The Mammalian Blood-Testis Barrier: Its Biology and Regulation." *Endocrine Reviews* 36(5):564–91. doi: 10.1210/ER.2014-1101.
- Nettersheim, Daniel, Sina Jostes, Simon Schneider, and Hubert Schorle. 2016. "Elucidating Human Male Germ Cell Development by Studying Germ Cell Cancer." *Reproduction* 152(4):R101–13. doi: 10.1530/REP-16-0114.
- O'Bryan, Moira K., Brett J. Clark, Eileen A. McLaughlin, Rebecca J. D'Sylva, Liza O'Donnell, Jacqueline A. Wilce, Jessie Sutherland, Anne E. O'Connor, Belinda Whittle, Christopher C. Goodnow, Christopher J. Ormandy, and Duangporn Jamsai. 2013. "RBM5 Is a Male Germ Cell Splicing Factor and Is Required for Spermatid Differentiation and Male Fertility." *PLOS Genetics* 9(7):e1003628. doi: 10.1371/JOURNAL.PGEN.1003628.
- Ozturk, Saffet, and Fatma Uysal. 2018. "Potential Roles of the Poly(A)-Binding Proteins in Translational Regulation during Spermatogenesis." *The Journal of Reproduction and Development* 64(4):289. doi: 10.1262/JRD.2018-026.
- Pamula, Melissa C., and Ruth Lehmann. 2024. "How Germ Granules Promote Germ Cell Fate." *Nature Reviews Genetics* 25(11):803–21. doi: 10.1038/s41576-024-00744-8.
- Passmore, Lori A., and Jeff Collier. 2021. "Roles of mRNA Poly(A) Tails in Regulation of Eukaryotic Gene Expression." *Nature Reviews. Molecular Cell Biology* 23(2):93. doi: 10.1038/S41580-021-00417-Y.
- Pham-Bui, Hoang Anh, and Mihye Lee. 2025. "Germ Granule-Mediated mRNA Storage and Translational Control." *RNA Biology* 22(1):1–11. doi:

10.1080/15476286.2025.2462276.

- Putnam, Andrea A., and Eckhard Jankowsky. 2013. "DEAD-Box Helicases as Integrators of RNA, Nucleotide and Protein Binding." *Biochimica et Biophysica Acta* 1829(8):884–893. doi: 10.1016/J.BBAGRM.2013.02.002.
- Saitou, Mitinori, Masahiro Nagano, and Ken Mizuta. 2025. "Mechanisms of Human Germ Cell Development." *Nature Reviews Molecular Cell Biology* 27(2):153–71. doi: 10.1038/s41580-025-00893-6.
- Schmeing, Stefan, and Peter t. Hart. 2024. "Challenges in Therapeutically Targeting the RNA-Recognition Motif." *Wiley Interdisciplinary Reviews. RNA* 15(6). doi: 10.1002/WRNA.1877.
- Sendler, Edward, Graham D. Johnson, Shihong Mao, Robert J. Goodrich, Michael P. Diamond, Russ Hauser, and Stephen A. Krawetz. 2013. "Stability, Delivery and Functions of Human Sperm RNAs at Fertilization." *Nucleic Acids Research* 41(7):4104–17. doi: 10.1093/NAR/GKT132.
- Siebert-Kuss, Lara M., Henrike Krenz, Tobias Tekath, Marius Wöste, Sara Di Persio, Nicole Terwort, Margot J. Wyrwoll, Jann Frederik Cremers, Joachim Wistuba, Martin Dugas, Sabine Kliesch, Stefan Schlatt, Frank Tüttelmann, Jörg Gromoll, Nina Neuhaus, and Sandra Laurentino. 2022. "Transcriptome Analyses in Infertile Men Reveal Germ Cell-Specific Expression and Splicing Patterns." *Life Science Alliance* 6(2):e202201633. doi: 10.26508/LSA.202201633.
- Skinner, Michael K., ed. 2018. *Encyclopedia of Reproduction - 2nd Edition*. Elsevier.
- Smialek, Maciej J., Erkut Ilaslan, Marcin P. Sajek, Aleksandra Swiercz, Damian M. Janecki, Kamila Kusz-Zamelczyk, Tomasz Wozniak, Maciej Kotecki, Luiza Handschuh, Marek Figlerowicz, and Jadwiga Jaruzelska. 2020. "Characterization of RNP Networks of PUM1 and PUM2 Post-Transcriptional Regulators in TCam-2 Cells, a Human Male Germ Cell Model." *Cells* 9(4):984. doi: 10.3390/CELLS9040984.
- Song, Huibin, Ling Wang, Dake Chen, and Fenge Li. 2020. "The Function of Pre-mRNA Alternative Splicing in Mammal Spermatogenesis." *International Journal of Biological Sciences* 16(1):38–48. doi: 10.7150/IJBS.34422.
- Soumillon, Magali, Anamaria Necsulea, Manuela Weier, David Brawand, Xiaolan Zhang, Hongcang Gu, Pauline Barthès, Maria Kokkinaki, Serge Nef, Andreas Gnirke, Martin Dym, Bernard deMassy, Tarjei S. Mikkelsen, and Henrik Kaessmann. 2013. "Cellular Source and Mechanisms of High Transcriptome

- Complexity in the Mammalian Testis.” *Cell Reports* 3(6):2179–90. doi: 10.1016/J.CELREP.2013.05.031.
- Tanaka, Satomi S., Yayoi Toyooka, Ryuko Akasu, Yuko Katoh-Fukui, Yoko Nakahara, Rika Suzuki, Minesuke Yokoyama, and Toshiaki Noce. 2000. “The Mouse Homolog of Drosophila Vasa Is Required for the Development of Male Germ Cells.” *Genes & Development* 14(7):841–53. doi: 10.1101/GAD.14.7.841.
- Tang, Walfred W. C., Toshihiro Kobayashi, Naoko Irie, Sabine Dietmann, and M. Azim Surani. 2016. “Specification and Epigenetic Programming of the Human Germ Line.” *Nature Reviews Genetics* 17(10):585–600.
- Tao, Yining, Qi Zhang, Haoyu Wang, Xiyu Yang, and Haoran Mu. 2024. “Alternative Splicing and Related RNA Binding Proteins in Human Health and Disease.” *Signal Transduction and Targeted Therapy* 9(1):1–33. doi: 10.1038/s41392-024-01734-2.
- Teves, Maria Eugenia, and Eduardo R. S. Roldan. 2021. “Sperm Bauplan and Function and Underlying Processes of Sperm Formation and Selection.” *Physiological Reviews* 102(1):7. doi: 10.1152/PHYSREV.00009.2020.
- Tharmalingam, Melissa D., Anne Jorgensen, and Rod T. Mitchell. 2018. “Experimental Models of Testicular Development and Function Using Human Tissue and Cells.” *Molecular and Cellular Endocrinology* 468:95–110. doi: 10.1016/j.mce.2017.12.011.
- Valverde, Roberto, Laura Edwards, and Lynne Regan. 2008. “Structure and Function of KH Domains.” *The FEBS Journal* 275(11):2712–26. doi: 10.1111/J.1742-4658.2008.06411.X.
- Vasileva, Ana, Daniela Tiedau, Adriana Firooznia, Thomas Müller-Reichert, and Rolf Jessberger. 2009. “Tdrd6 Is Required for Spermiogenesis, Chromatoid Body Architecture, and Regulation of MiRNA Expression.” *Current Biology* 19(8):630–39. doi: 10.1016/j.cub.2009.02.047.
- Voronina, Ekaterina, Geraldine Seydoux, Paolo Sassone-Corsi, and Ippei Nagamori. 2011. “RNA Granules in Germ Cells.” *Cold Spring Harbor Perspectives in Biology* 3(12):a002774. doi: 10.1101/CSHPERSPECT.A002774.
- Wang, Eric T., Rickard Sandberg, Shujun Luo, Irina Khrebtkova, Lu Zhang, Christine Mayr, Stephen F. Kingsmore, Gary P. Schroth, and Christopher B. Burge. 2008. “Alternative Isoform Regulation in Human Tissue Transcriptomes.” *Nature* 456:470–76. doi: 10.1038/nature07509.
- Wanjari, Uddesh Ramesh, and Abilash Valsala Gopalakrishnan. 2024. “Blood-Testis

- Barrier: A Review on Regulators in Maintaining Cell Junction Integrity between Sertoli Cells.” *Cell and Tissue Research* 396(2):157–75. doi: 10.1007/S00441-024-03894-7.
- Wilkinson, Max E., Clément Charenton, and Kiyoshi Nagai. 2020. “RNA Splicing by the Spliceosome.” *Annual Review of Biochemistry* 89:359–88. doi: 10.1146/ANNUREV-BIOCHEM-091719-064225.
- Xu, Caoling, Yuzhu Cao, and Jianqiang Bao. 2021. “Building RNA-Protein Germ Granules: Insights from the Multifaceted Functions of DEAD-Box Helicase Vasa/Ddx4 in Germline Development.” *Cellular and Molecular Life Sciences* 79(1). doi: 10.1007/S00018-021-04069-1.
- Xu, Ning, Yixian Qin, Yu Liu, Yudong Guan, Hang Xin, Junwen Ou, and Yiqiao Wang. 2025. “An Integrated Transcriptomic Analysis Unveils the Regulatory Roles of RNA Binding Proteins during Human Spermatogenesis.” *Frontiers in Endocrinology* 16:1522394. doi: 10.3389/fendo.2025.1522394.
- Yang, Fang, and P. Jeremy Wang. 2016. “Multiple LINEs of Retrotransposon Silencing Mechanisms in the Mammalian Germline.” *Seminars in Cell & Developmental Biology* 59:118–25. doi: 10.1016/J.SEMCDB.2016.03.001.
- Yang, Juxiang, Sergey Medvedev, Junying Yu, Linda C. Tang, Julio E. Agno, Martin M. Matzuk, Richard M. Schultz, and Norman B. Hecht. 2005. “Absence of the DNA-/RNA-Binding Protein MSY2 Results in Male and Female Infertility.” *Proceedings of the National Academy of Sciences of the United States of America* 102(16):5755–60. doi: 10.1073/pnas.0408718102.
- Zagore, Leah L., Sarah E. Grabinski, Thomas J. Sweet, Molly M. Hannigan, R. Michael Sramkoski, Qin Li, and Donny D. Licatalosi. 2015. “RNA Binding Protein Ptbp2 Is Essential for Male Germ Cell Development.” *Molecular and Cellular Biology* 35(23):4030. doi: 10.1128/MCB.00676-15.
- Zamudio, N., and D. Bourc’his. 2010. “Transposable Elements in the Mammalian Germline: A Comfortable Niche or a Deadly Trap?” *Heredity* 105(1):92–104. doi: 10.1038/hdy.2010.53.
- Zhou, Shumin, Akihiko Sakashita, Shuiqiao Yuan, and Satoshi H. Namekawa. 2022. “Retrotransposons in the Mammalian Male Germline.” *Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation* 16(5–6):404. doi: 10.1159/000520683.

Zhou, Zhi, Takayuki Shirakawa, Kazuyuki Ohbo, Aiko Sada, Quan Wu, Kazuteru Hasegawa, Rie Saba, and Yumiko Saga. 2015. "RNA Binding Protein Nanos2 Organizes Post-Transcriptional Buffering System to Retain Primitive State of Mouse Spermatogonial Stem Cells." *Developmental Cell* 34(1):96–107. doi: 10.1016/j.devcel.2015.05.014.

9. Oświadczenia współautorów Artykułu 1



ul. Staszczńska 32
60-179 Poznań

tel. +48 (0) 61 657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

Poznań, 04.05.2026

mgr Agata Barszcz

OŚWIADCZENIE

Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „*In vitro* models for human spermatogenesis research: concepts and perspectives”

Agata J. Barszcz; Natalia Rozwadowska

był następujący

- współudział w opracowaniu koncepcji i stworzenie planu pracy
- przegląd i wybór prac omówionych w publikacji
- napisanie oryginalnej wersji publikacji oraz przygotowanie abstraktu w formie graficznej
- przygotowanie ostatecznej wersji publikacji

Z wyrazami szacunku,

Agata Barszcz

Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr hab. n. med. Natalia Rozwadowska, prof. IGC

OŚWIADCZENIE

Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „*In vitro* models for human spermatogenesis research: concepts and perspectives”

Agata J. Barszcz; Natalia Rozwadowska

był następujący

- opracowanie koncepcji i planu przeglądu literatury
- krytyczna rewizja oryginalnej wersji manuskryptu

Z wyrazami szacunku,



10. Oświadczenia współautorów Artykułu 2



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48 61 657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

mgr Agata Barszcz

OŚWIADCZENIE

Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis” (bioRxiv doi:10.1101/2025.09.23.677621)

Agata J. Barszcz; Katarzyna Tutak; Joanna Zypřych-Walczak; Erik Dassi; Erkut Ilaslan; Małgorzata Dąbrowska; Agnieszka Malcher; Marta Olszewska; Dominik Cysewski; Agnieszka Nadel; Tomasz Kolanowski; Zofia E. Madeja; Michał Hrab; Alexander Yatsenko; Maciej Kurpisz; Zbigniew Warkocki; Natalia Rozwadowska

był następujący

- udział w opracowaniu koncepcji i przygotowaniu planu badań
- prowadzenie hodowli komórkowych, wykonywanie transfekcji i transdukcji
- optymalizacja metodologii oraz wykonanie i interpretacja eksperymentów z wykorzystaniem linii komórkowej TCam-2
- wykonanie większości izolacji RNA i białek oraz przeprowadzenie analiz RT-qPCR, Western Blot
- optymalizacja i wykonanie barwień immunofluorescencyjnych oraz Co-IP
- analiza bioinformatyczna danych NGS oraz opracowanie graficznej prezentacji wyników
- napisanie oryginalnej wersji publikacji, przygotowanie większości załączników oraz rycin
- przygotowywanie odpowiedzi dla recenzentów w procesie publikacyjnym
- przygotowanie ostatecznej wersji publikacji

Z wyrazami szacunku,

Agata Barszcz

Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr hab. n. med. Natalia Rozwadowska, prof. IGC

OŚWIADCZENIE

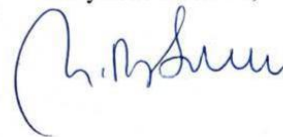
Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis” (bioRxiv doi:10.1101/2025.09.23.677621)

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był następujący

- opracowanie koncepcji i planu badań
- pozyskanie finansowania i opieka nad właściwą realizacją projektów NCN
- wsparcie merytoryczne podczas projektowania eksperymentów
- udział w interpretacji wyników badań
- krytyczna rewizja oryginalnej wersji manuskryptu
- zatwierdzenie ostatecznej wersji manuskryptu
- pełnienie nadzoru nad przygotowaniem odpowiedzi dla recenzentów w procesie publikacyjnym

Z wyrazami szacunku,



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr Agnieszka Malcher

OŚWIADCZENIE

Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis” (bioRxiv doi:10.1101/2025.09.23.677621)

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był następujący

- udział w opracowaniu koncepcji badań
- udział w projektowaniu eksperymentów oraz wsparcie merytoryczne

Z wyrazami szacunku

Agnieszka Malcher

Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr hab. n. med. i n. o zdr. Tomasz Kolanowski, prof. IGC

OŚWIADCZENIE

Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis” (bioRxiv doi:10.1101/2025.09.23.677621)

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był następujący

- edycja i korekty oryginalnej wersji manuskryptu

Z wyrazami szacunku,

Tomasz Kolanowski

Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr Dominik Cysewski

OŚWIADCZENIE

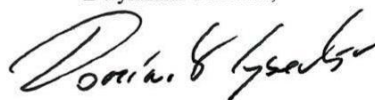
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był następujący

- przeprowadzenie analiz z wykorzystaniem spektrometrii mas (MS)

Z wyrazami szacunku,



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr Erkut Ilaslan

STATEMENT

I hereby declare that my contribution to the manuscript „Multi-omic screening identifies RBMXL3 as a primate-specific RNA-binding protein and candidate regulator of RNA metabolism in human spermatogenesis” (bioRxiv doi:10.1101/2025.09.23.677621)

Agata J. Barszcz; Katarzyna Tutak; Joanna Zypych-Walczak; Erik Dassi; Erkut Ilaslan; Małgorzata Dąbrowska; Agnieszka Malcher; Marta Olszewska; Dominik Cysewski; Agnieszka Nadel; Tomasz Kolanowski; Zofia E. Madeja; Michał Hrab; Alexander Yatsenko; Maciej Kurpisz; Zbigniew Warkocki; Natalia Rozwadowska

was as follows

- analysis of Co-IP-MS experimental data
- editing and revising the original manuscript

Regards,
Erkut Ilaslan



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań

tel. +48/61/657 91 00

e-mail: sekretariat@igcz.poznan.pl

www.igcz.poznan.pl

mgr Małgorzata Dąbrowska

OŚWIADCZENIE

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był następujący

- wsparcie podczas optymalizacji procedur barwień immunofluorescencyjnych
- wsparcie w prowadzeniu hodowli komórek TCam-2

Z wyrazami szacunku,

Małgorzata Dąbrowska

11. Oświadczenia współautorów Artykułu 3



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48 61 657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

mgr Agata Barszcz

OŚWIADCZENIE

Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „The RNA helicase DDX53 (CAGE) contributes to RNA metabolism in a human germ cell model” (bioRxiv doi: 10.64898/2026.02.03.703580)

Agata J. Barszcz, Katarzyna Tutak, Agnieszka Malcher, Małgorzata Dąbrowska, Joanna Zypych-Walczak, Erik Dassi, Erkut Ilaslan, Marta Olszewska, Dominik Cysewski, Michał Hrab, Tomasz Kolanowski, Alexander Yatsenko, Maciej Kurpisz, Natalia Rozwadowska

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- udział w opracowaniu koncepcji i przygotowaniu planu badań
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- wykonanie większości izolacji RNA i białek oraz przeprowadzenie analiz RT-qPCR, Western Blot
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- analiza bioinformatyczna danych NGS oraz opracowanie graficznej prezentacji wyników
- napisanie oryginalnej wersji publikacji, przygotowanie większości załączników oraz rycin
- przygotowywanie odpowiedzi dla recenzentów w procesie publikacyjnym
- przygotowanie ostatecznej wersji publikacji

Z wyrazami szacunku,

Agata Barszcz

Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań

tel. +48/61/657 91 00

e-mail: sekretariat@igcz.poznan.pl

www.igcz.poznan.pl

dr hab. n. med. Natalia Rozwadowska, prof. IGC

OŚWIADCZENIE

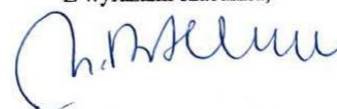
Niniejszym oświadczam, że mój wkład w powstanie artykułu pt. „The RNA helicase DDX53 (CAGE) contributes to RNA metabolism in a human germ cell model” (bioRxiv doi: 10.64898/2026.02.03.703580)

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był następujący

- opracowanie koncepcji i planu badań
- pozyskanie finansowania i opieka nad właściwą realizacją projektów NCN
- wsparcie merytoryczne podczas projektowania eksperymentów
- udział w interpretacji wyników badań
- krytyczna rewizja oryginalnej wersji manuskryptu
- zatwierdzenie ostatecznej wersji manuskryptu
- pełnienie nadzoru nad przygotowaniem odpowiedzi dla recenzentów w procesie publikacyjnym

Z wyrazami szacunku,



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr Agnieszka Malcher

OŚWIADCZENIE

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był następujący

- udział w opracowaniu koncepcji badań
- udział w projektowaniu eksperymentów oraz wsparcie merytoryczne
- wykonanie eksperymentu frakcjonowania
- edycja i korekty oryginalnej wersji manuskryptu

Z wyrazami szacunku,

Agnieszka Malcher

Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań

tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr hab. n. med. i n. o zdr. Tomasz Kolanowski, prof. IGC

OŚWIADCZENIE

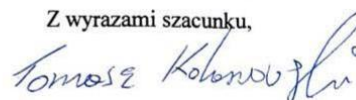
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był następujący

- wsparcie podczas analizy danych
- edycja i korekty oryginalnej wersji manuskryptu

Z wyrazami szacunku,



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
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dr Dominik Cysewski

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był następujący

- przeprowadzenie analiz z wykorzystaniem spektrometrii mas (MS)
- edycja i korekty oryginalnej wersji manuskryptu

Z wyrazami szacunku,



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
www.igcz.poznan.pl

dr Erkut Ilaslan

STATEMENT

I hereby declare that my contribution to the manuscript „The RNA helicase DDX53 (CAGE) contributes to RNA metabolism in a human germ cell model” (bioRxiv doi: 10.64898/2026.02.03.703580)

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was as follows

- analysis of Co-IP-MS experimental data
- editing and revising the original manuscript

Regards,
Erkut Ilaslan



Poznań, 04.05.2026

ul. Strzeszyńska 32
60-479 Poznań
tel. +48/61/657 91 00
e-mail: sekretariat@igcz.poznan.pl
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mgr Małgorzata Dąbrowska

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był następujący

- wykonanie barwień immunofluorescencyjnych wybranych interaktorów (THOC1 oraz SCML2)
- optymalizacja primerów oraz walidacja poziomu ekspresji genów zidentyfikowanych na podstawie wyników z RNA-seq
- edycja i korekty oryginalnej wersji manuskryptu

Z wyrazami szacunku,

Małgorzata Dąbrowska