TEX15 is an essential executor of MIWI2-directed transposon DNA methylation and silencing

Theresa Schöpp1,2, Ansgar Zoch1,2, Rebecca V. Berrens3, Tania Auchynnikava2, Yuka Kabayama1,2, Lina Vasiliauskaitė1, Juri Rappsilber2,4, Robin C. Allshire2 & Dónal O’Carroll1,2✉

The PIWI protein MIWI2 and its associated PIWI-interacting RNAs (piRNAs) instruct DNA methylation of young active transposable elements (TEs) in the male germline. piRNAs are proposed to recruit MIWI2 to the transcriptionally active TE loci by base pairing to nascent transcripts, however the downstream mechanisms and effector proteins utilized by MIWI2 in directing de novo TE methylation remain incompletely understood. Here, we show that MIWI2 associates with TEX15 in foetal gonocytes. TEX15 is predominantly a nuclear protein that is not required for piRNA biogenesis but is essential for piRNA-directed TE de novo methylation and silencing. In summary, TEX15 is an essential executor of mammalian piRNA-directed DNA methylation.
he mammalian germline is derived from somatic cells during early development which necessitates the erasure and resetting of genomic DNA methylation patterns. In the mouse male germline, the process of de novo DNA methylation occurs in foetal gonocytes during late gestation. Many young active long interspersed nuclear element-1 (LINE1) and intracisternal A-particle (IAP) copies escape the first round of de novo genome methylation. These active TEs are silenced through post-transcriptional and transcriptional silencing mechanisms by PIWI proteins and their associated piRNAs. The PIWI protein MIWI1 (PIWIL2) initiates effector piRNA production through the piRNA-guided endonucleolytic cleavage and destruction of cytoplasmic TE transcripts. Effector piRNAs are loaded into the PIWI protein MIWI2 (PIWIL4) that licences its entry to the nucleus and the ribonucleoprotein particle (RNP) is proposed to guide de novo methylation by tethering to nascent TE transcripts and the recruitment of effector proteins. The P0 domain containing 1 (PODC1) was recently identified that links MIWI2 to the de novo methylation machinery but the full complement of MIWI2 effector proteins remains unknown. Here we show that TEX15 interacts with MIWI2 in foetal gonocytes and is required for piRNA-directed de novo DNA methylation of transposons.

**Results**

**TEX15 interacts with MIWI2 in foetal gonocytes.** We hypothesised that the tethering of the MIWI2 RNP to the nascent transcript could be used to devise a strategy to enrich for proteins that are required for the execution of nuclear MIWI2 function. We performed immunofluorescence (IF) on thinly cut unfixed foetal testis cryosections. The width of the section is less than the diameter of a gonocyte so the cells are effectively sliced open and material can diffuse into the surrounding solution unless it is anchored through an interaction. The treatment of cryosections with RNase A prior to fixation dramatically reduced MIWI2’s nuclear staining in gonocytes (Fig. 1a). In addition, the inclusion of RNase A during extraction increased MIWI2’s solubilisation in foetal testis lysates (Fig. 1b). We performed immunoprecipitation coupled with quantitative mass spectrometry (IP-MS) of MIWI2 from E16.5 testes extracts with or without RNase A treatment using the fully functional Miwi2HA allele that encodes an endogenously HA-epitope tagged MIWI2 protein. The addition of RNase A greatly increased the number of MIWI2 interacting proteins (Fig. 1c, d, Supplementary Tables 1 and 2). Among the RNase A-dependent interactions TEX15 immediately struck our attention as a putative executor of nuclear MIWI2 function because it contains a nuclear localisation sequence (Fig. 1a), and most importantly Tex15 deficiency in the mouse leads to the exact same phenotype observed in piRNA pathway or de novo methylation machinery mutants, namely sterility due to early meiotic arrest. Mutations in the human TEX15 are also associated with male infertility. Furthermore, the MIWI2-TEX15 interaction was confirmed from the analysis of an independent HA-MIWI2 IP-MS published dataset where the interaction is observed only in extracts prepared with Benzonase (Supplementary Fig. 1b), a nuclease that is commonly used to solubilise chromatin-bound proteins. Tex15 encodes a large protein encompassing 3059 amino acids of unknown molecular function that contains a DUF3715 domain and two TEX15 domains (Fig. 1e). We generated a fully functional C-terminal HA epitope tagged Tex15 (Tex15HA) allele (Supplementary Fig. 2a–c) and found that TEX15 expression in foetal testis is restricted to germ cells where it is predominantly localised to the nucleus (Fig. 1f).

**TEX15 is required for TE silencing in the male germline.** The association of MIWI2 with TEX15, its nuclear localisation in foetal gonocytes and telling phenotype prompted us to explore if Tex15 is required for TE silencing and de novo DNA methylation. We thus generated a Tex15 null (Tex15−/−) allele in the mouse by CRISPR/Cas9-mediated genome editing of exon 5 that encodes the conserved DUF3715 domain (Supplementary Fig. 3a–d). The modified allele contains a 70 bp insertion in exon 5 that introduces in frame stop codons and should result in nonsense-mediated decay, and indeed a dramatic reduction of the Tex15 transcript is observed in Tex15−/− E16.5 foetal gonocytes (Supplementary Fig. 3e). In addition, the residual mutant transcript would encode a highly truncated TEX15 polypeptide encompassing the first 136 amino acids lacking any of its conserved domains. Most importantly, homozygosity of our Tex15−/− allele fully recapitulated the published Tcx15-deficient phenotype of male sterility, meiotic arrest coupled with extensive DNA damage and apoptosis (Fig. 2a–c, Supplementary Fig. 3f–i). TEX15 is required for both LINE1 and IAP silencing in the adult testis.
TEX15 is required for TE silencing. a Representative images of testis sections from adult Tex15−/− and Tex15+/− mice stained with PAS and Haematoxylin (n = 3). Scale bar 50 μm. b, c Representative images of testis sections of (n = 3) adult Tex15+/− and Tex15−/− mice stained for (b) γH2AX showing DNA damage response or (c) TUNEL visualising apoptotic cells. DNA stained with DAPI. Scale bar 50 μm. d, e Adult Tex15+/− and Tex15−/− testis sections (n = 3) stained for (d) LINE1 ORF1p or (e) IAP-GAG protein. DNA stained with DAPI. Scale bar 50 μm. f Heat map from P20 testes and E16.5 gonocytes RNA-seq as indicated showing fold change of the ten most upregulated LINEs and ERVKs in Tex15−/− and Miwi2−/− P20 testis compared to control (n = 3). P < 0.01, Benjamini–Hochberg adjusted two-sided Wald test.

TEX15 is required for piRNA directed de novo DNA methylation. The demonstration that TEX15 interacts with Miwi2, localises to the nucleus and is not required for piRNA processing collectively indicates that TEX15 could be required for Miwi2-directed TE methylation. We therefore isolated genomic DNA from Tex15−/−/P14 spermatogonia and performed whole genome methylation sequencing (Methyl-seq) that we compared to Wildtype and Miwi2−/−/P14 spermatogonia methyomes8, generated using the same technique. As is the case for Miwi2-deficiency, no major changes in methylation of Tex15−/− spermatogonia were observed in genic, intergenic, CpG island, promoter regions or a conglomerate of all genomic transposons (Supplementary Fig. 5a). However, the young LINE1 families regulated by the piRNA pathway represented by L1Md_A, L1Md_T or L1Md_Gf as well as IAPEy and MMERVK10C failed to be fully methylated in Tex15−/− spermatogonia (Fig. 3d). Methylation specifically at TE promotor elements and in young TEs is a hallmark of piRNA-directed methylation2,8,19 and DNMT3C, which has a specialised function in germline de novo TE methylation20,21. Metaplot analysis revealed defective de novo methylation specifically at TE promotor elements in Tex15−/− spermatogonia of young LINE1 families exemplified by L1Md_T and L1Md_Gf compared to the older L1Md_F; as observed in Miwi2−/− spermatogonia (Fig. 3e, Supplementary Fig. 5b). Furthermore, the loss of methylation was especially evident in young elements within the respective families (Fig. 3f, Supplementary Fig. 5c). Miwi2 function is essential for the de novo methylation of a single imprinted locus, Rasgrf122. Consistently,

(FIG. 2d, e). To explore the full repertoire of TEs regulated by TEX15, we performed RNA-seq from post-natal day 20 (P20) mouse testes and found many TE families deregulated (Supplementary Fig. 4a, Supplementary Table 3). Importantly, we found that precisely the same families of TEs are deregulated in Tex15−/− and Miwi2−/− tests8 (Fig. 2f, Supplementary Fig. 4b). RNA-seq revealed that many of the TEs deregulated in P20 Tex15−/− and Miwi2−/− tests are also deregulated in Tex15−/− E16.5 foetal gonocytes (Fig. 2f) which demonstrates a function for TEX15 in the foetal piRNA-pathway.

TEX15 is not required for piRNA biogenesis. The dependency of young active TE silencing on TEX15 could indicate that TEX15 is required for execution of nuclear Miwi2 function or piRNA biogenesis amplification and loading; as the phenotypic outcome is identical in both scenarios. Sequencing small RNA from Tex15+/− and Tex15−/− E16.5 foetal testes did not reveal any major impact of Tex15-deficiency on small RNA length distribution (Fig. 3a), annotation of mapped piRNAs (Fig. 3b), piRNA amplification (Supplementary Fig. 4c, d) or piRNAs mapping to TEs (Supplementary Fig. 4e). The loss of piRNA biogenesis, amplification or loading results in the pronounced reduction of Miwi2’s nuclear localisation18. Thus, the normal localisation of Miwi2 in the absence of Tex15 (Fig. 3c) corroborates the fact that TEX15 is not required for piRNA processing. RNA-seq from E16.5 foetal gonocytes excludes the possibility that TEX15 may function as a transcription factor required for gene expression, as a total of only 13 genes exhibited altered expression levels in the absence of Tex15. With the exception of Tex15, none of the subtly deregulated genes are associated with the de novo methylation or piRNA pathways (Supplementary Fig. 4f, Supplementary Table 4). In summary, these data do not support a role for TEX15 being a piRNA biogenesis nor a transcription factor.
Rasgrf1 methylation alone requires TEX15 function among imprinted loci (Fig. 3g).

**Discussion**

A very recent study identified TEX15 as a regulator of TE silencing and connected TEX15 to MILI during spermatogenesis23. Here, we show that TEX15 interacts with MIWI2 in foetal gonocytes that are undergoing de novo DNA methylation thus directly link TEX15 to the process of piRNA-directed DNA methylation. Interestingly, the detection of the MIWI2–TEX15 interaction is dependent upon using either RNase A or Benzonase in the protein extraction procedure which likely indicates that the association is occurring in the context of chromatin. The localisation of MIWI2 to the nucleus coupled with normal piRNA biogenesis in the absence of TEX15 also clearly demonstrates that TEX15 is required for MIWI2’s...
nuclear function. Indeed, we unequivocally show that TEX15 is required for de novo DNA methylation of precisely the same TEs that are regulated by the Miwi2-piRNA pathway. Interestingly, TEX15 was not found to interact with SPOC1D in foetal gonocytes, which places a function for TEX15 upstream or in parallel to SPOC1D in the recruitment of the de novo methylation machinery. The DUF3715 domain of TEX15 is found in two other proteins, TASOR (FAM208A) and TASOR2 (FAM208B), of which TASOR is a critical component of the HUSH complex that mediates TE silencing in somatic cells.1,28, TASOR functions through the recruitment of MORC2 that stimulates the deposition of the repressive heterochromatin associated H3K9me3 mark.25,27 MORC1 is an orthologue of MORC2 expressed in the developing male germ line and essential for de novo methylation of active TEs26. It is tempting to speculate that TEX15 may contribute to transcriptional TE silencing, possibly through H3K9me3. Indeed, transcriptional TE silencing is a prerequisite for de novo methylation at DNM3TL1, a key component of the de novo methylation machinery, cannot interact with the transcriptionally active H3K4me3-marked chromatin.29,30,31,32 We have identified TEX15 as an essential executor of piRNA-directed DNA methylation whose future study holds great promise in unravelling the molecular mechanisms underpinning this epigenetic event that is essential for the immortality of the germ line.

Methods

Mouse strains and experimentation. The Miwi2Δ15 allele and Miwi2Tomato (Miwi2ΔTomato) allele have been previously generated in the O’Carroll laboratory and were kept on a C57BL/6N genetic background. Mouse alleles Tex15−/− and Tex15HA were generated using CRISPR/Cas9 gene editing technology.31,32 For Tex15−/− a single sgRNA (sgRNA-exb5 5′-ACATCTACCATCGCGCCGTC-3′) was injected together with Cas9 mRNA. For Tex15HA a single sgRNA (sgRNA-C2 5′-AAGACATCGCTTACGACA-3′) and a 194 bp single stranded DNA donor with 71 and 70 bp left and right homology arms respectively and a PAM site mutation (CTGGCTTATATTTCAATCTAAGTTGTGTTTTTTCCAGGGCTTGTCGACACAGGACATCGCTTACGACA-3′) were injected together with Cas9 mRNA. For Tex15HA and Miwi2HA/TA-RV1 5′-ACAAGCCTCTTTATAACTGCATGG-3′ the lysate was cleared for 10 min at 21,000 rcf, the supernatant utilised as the soluble fraction and the pellet resuspended for 5 min at 95 °C. Equal volumes of soluble and pellet fraction were then separated on 4%–12% Bis-Tris Polyacrylamide gels (Invitrogen) according to the manufacturer’s instructions. Proteins were transferred onto 0.45 µm nitrocellulose membrane (Amersham, Protran XL), blocked in 3% skimmed milk and stained with primary antibody (anti-HA (C29F4 rabbit polyclonal, 1:2000) diluted 1:10,000. Images were acquired and analysed using a Li-COR Odyssey Imager. Ratio between soluble and pellet fraction was calculated from signal intensity of each band as measured by Image Studio Lite (version 5.2.5).

Immunoprecipitation-coupled mass-spectrometry (IP-MS). E16.5 isolated testes were snap frozen in liquid nitrogen. A total of 30 testes per replicate were pooled, lysed and homogenised in 1 ml IP buffer (100 mM KCl, 5 mM MgCl2, 0.5% Triton X-100 untreated or treated with RNase A (10 µg ml−1; Sigma Aldrich). The lysate was cleared for 10 min at 21,000 rcf, the supernatant taken as the soluble fraction and the pellet resuspended for 5 min at 95 °C with an equal volume of 2% sodium dodecyl sulfate (SDS), 50 mM Tris pH 8. Equal volumes of soluble and pellet fraction were then separated on 4–12% Bis-Tris Polyacrylamide gels (Invitrogen) according to the manufacturer’s instructions. Proteins were transferred onto 0.45 µm nitrocellulose membrane (Amersham, Protran XL), blocked in 3% skimmed milk and stained with primary antibody (anti-HA (C29F4 Cell Signalling Technologies) diluted 1:5000 in blocking solution containing cold PBS) for 1 h, washed and incubated with Li-COR Fluoroacetic acid (v/v) and separated using ultra-high resolution nano-liquid chromatography nanoLC Ultimate3000 (Thermo Fisher Scientific). Raw data was processed with Max Quant (version 1.2.0.6.0) using Label-free quantitation (LFQ) IP-MS pipeline as described33 and peptides searched with standard settings against mouse Uniprot Database (July 2020).
Fluorescence activated cell sorting (FACS) were de-hydrated using a reverse series of graded alcohols and Xylene, then mounted with Pertex mounting media (Pioneer Research Chemicals).

Fluorescein activated cell sorting (FACS) Gonocytes were FACS purified from control Tex15−/−, Miw2tdTom/+ and experimental Tex15−/−; Miw2tdTom/+ E16.5 tests using tdTomato expression as marker of gonocytes. The isolated tests were dissected in a drop of Goni-MEM (DMEM (Life Technologies) supplemented with penicillin-streptavadin (Life Technologies), NEAA (Life Technologies), sodium pyruvate (Life Technologies) and sodium lactate (Sigma-Aldrich), transferred into 500 μl Tryptis-EDTA (0.25%, Gibco) and digested for 12 min at 37 °C, shaking. Digestion was stopped by the addition of 20% foetal calf serum (FCS) and 10 μl 5 mg ml−1 Dnase I (Sigma-Aldrich), incubated 5 min at 37 °C and pelleted for 3 min at 1000 g. The pellet was again treated with 10 μl Dnase I (as above) and the cells resuspended in PBS with 2% FCS by pipetting at least 50 times. A 1 μg ml−1 DAPI was added to the cell suspension and tdTomato+ cells were sorted on a BD Fusion into PBS at 4 °C (Supplementary Fig. 6a) and snap frozen in liquid nitrogen.

For sorting of P14 CD9+ spermatogonia, tests were isolated, deagglutinated and digested with collagenase (0.5 mg ml−1 Sigma) for 10 min at 32 °C, shaking. Cells were resuspended in PBS with 2% FCS by pipetting at least 50 times. A 1 μg ml−1 DAPI was added to the cell suspension and tdTomato+ cells were sorted on a BD Fusion into PBS at 4 °C (Supplementary Fig. 6a) and snap frozen in liquid nitrogen.

RNA sequencing and analysis. For RNA-seq of FACS-purified E16.5 gonocytes total RNA was extracted with QIAzol reagent (Qiagen) following the manufacturer’s recommendation. Libraries were prepared with Ribonome and the SMARTer Stranded RNA-seq kit for low input RNA-seq from Clontech and sequenced on a HiSeq 4000 (Illumina) in 75 bp paired-end mode. SMARTer Stranded RNA-seq kit for low input RNA-seq from Clontech and sequenced on a HiSeq 4000 (Illumina) in 75 bp paired-end mode. For RNA-seq of P20 testis total RNA was extracted from one testis with Qiagen RNeasy Mini spin columns (Corning) plugged with filter paper (Whatman) and were centrifuged at 10,000 rpm with a freeze/thaw step on dry ice in between. Samples were transferred onto spin columns (Corning) plugged with filter paper (Whatman) and were centrifuged 1 min at max speed. RNA precipitation of the flow through was done with 2.5 volumes ethanol 100%, 1/10 volume 3 M NaAc and 1 μl GlycoBlue (Life Technologies) overnight at −20 °C. RNA was washed in 80% ethanol and dissolved in 10 μl nuclease-free water. NEBnext Multiplex Small RNA Library Prep Set for Illumina (NEB) was used for library preparation following the manufacturer’s instructions with 4 μl size-selected RNA starting material, 1:2 diluted adapters and 16 cycles PCR amplification. Qubit high sensitivity dsDNA kit on a Qubit fluorometer (Life Technologies) was used to measure the DNA concentration. A pool of 100 μl or 500 μl DNA in 10 mM Tris-HCl pH 8.5, 5 mM EDTA, 1% SDS, 0.3 M Na-aceatate at 55°C overnight, followed by two successive rounds of phenol/chloroform/isooamylalcohol (25:24:1, Sigma-Aldrich) extraction and one round of chloroform extraction. DNA precipitation was done by addition of 1/10 volume 3 M NaAc-aceatate, 10 μg linear acrylamide (Invitrogen) and 1 volume of isopropanol and incubated at -20°C overnight, washed two times with 70% ethanol and solubilized in 5 mM Tris-HCl pH 8. Libraries were prepared using the NEBnext Enzymatic Methyl-seq kit (NEB) according to the manufacturer’s protocol and sequenced on a HiSeq 4000 (Illumina) in 150 bp paired-end read mode.

Analysis was performed as described previously36. Genic regions were defined as probes overlapping genes and promoter as probes overlapping 2000 bp upstream of mRNA transcripts, as annotated by Ensembl (GRCh38.p6). CpG islands (CGIs) were defined as probes overlapping the Ensembl (GRCh38.p6) CGI annotation. reads overlapping transposons were removed for genic, promoters and CGIs genome. Transposon analysis was performed by unique mapping in the genome excluding any repetitive sequence. Level of methylation was expressed as mean percentage of individual CG sites. Statistical information. Statistical testing was performed with R (3.3.1) using the R Studio software and with Perseus for the mass-spectrometry data. Unpaired, two-sided Student’s t-tests were used to compare differences between groups and adjusted for multiple testing using Bonferroni correction where indicated, except for RNA-seq data analysis where Wald tests and Benjamini–Hochberg correction were used. Averaged data are presented as mean ± s.e.m. (standard error of the mean), unless otherwise indicated. No statistical methods were used to pre-determine sample size. The experiments were not randomised and the investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The Methyl-seq data generated in this study have been deposited at ArrayExpress under the accession number E-MTAB-9090. The published Methyl-seq data of Miw2−/− and Wildtype spermatogonia was retrieved from E-MTAB-7598. The RNA-seq and RNA-seq data generated in this study have been deposited at Gene Expression Omnibus under the accession number GSE150350. Published RNA-seq data of P20 testis Miw2−/− tests was retrieved from GSE131377. Data from the IP-MS experiments re-analysed in this manuscript were retrieved from ProteomeXchange under the accession number PXD019087. The published IP-MS experiments re-analysed in this manuscript were retrieved from ProteomeXchange PXD016701. The Affymetrix microarray datasets for spermatogonia38 and spermatocytes67 and gonocytes8 were retrieved from ArrayExpress: E-MTAB-4828, E-MTAB-7067 and E-MTAB-7985, respectively. Full scans of the gels and blots are available in Supplementary Fig. 7. The source data underlying Figs. 1c, d, 2f, 3a, b, d-g and Supplementary Figs. 1a, 3e, f, 4a, c-f and 5a–c are provided in a Source Data file. All data are available from the corresponding author upon reasonable request. The optionData are provided with this paper.

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**Author contributions**

T.S. contributed to the design, execution and analysis of most experiments. A.Z. and T.S. designed and generated the Tex15−/− mice. D.O. supervised the project and generated the microarray data. D.O. and T.S. wrote the manuscript. D.O. and T.S. contributed to the design, execution and analysis of most experiments. A.Z. and T.S. designed and generated the published maps and institutional affiliations.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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