The Ubiquitin Ligase Ubr2, a Recognition E3 Component of the N-End Rule Pathway, Stabilizes Tex19.1 during Spermatogenesis

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Abstract

Ubiquitin E3 ligases target their substrates for ubiquitination, leading to proteasome-mediated degradation or altered biochemical properties. The ubiquitin ligase Ubr2, a recognition E3 component of the N-end rule proteolytic pathway, recognizes proteins with N-terminal destabilizing residues and plays an important role in spermatogenesis. Tex19.1 (also known as Tex19) has been previously identified as a germ cell-specific protein in mouse testis. Here we report that Tex19.1 forms a stable protein complex with Ubr2 in mouse testes. The binding of Tex19.1 to Ubr2 is independent of the second position cysteine of Tex19.1, a putative target for arginylation by the N-end rule pathway R-transferase. The Tex19.1-null mouse mutant phenocopies the Ubr2-deficient mutant in three aspects: heterogeneity of spermatogenic defects, meiotic chromosomal asynapsis, and embryonic lethality preferentially affecting females. In Ubr2-deficient germ cells, Tex19.1 is transcribed, but Tex19.1 protein is absent. Our results suggest that the binding of Ubr2 to Tex19.1 metabolically stabilizes Tex19.1 during spermatogenesis, revealing a new function for Ubr2 outside the conventional N-end rule pathway.

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Introduction

The N-end rule pathway is a ubiquitin-dependent proteolytic system [1,2]. In this pathway, the stability of proteins is defined by their N-terminal amino acids that are distinguished into stabilizing and destabilizing residues. The latter constitute so-called N-degrons, which are signatures for degradation of short-lived proteins. Destabilizing residues include basic (Arg, Lys, and His) and bulky hydrophobic (Phe, Tyr, Trp, Leu, and Ile) residues. An N-degron can also be created by either endoproteolytic cleavage or modifications of a pre-N-degron (Cys, Asn, Asp, Gln, or Glu) through a series of N-terminal modifications [2]. Cysteine at position 2 (after methionine) is a unique type of destabilizing residue in mammalian cells. If N-terminally exposed, Cys can be oxidized to Cys-O2(H) or Cys-O3(H) before being arginylated by the arginine (R)-transferase ATE1 [3–5]. The N-degron is recognized by a family of UBR box-containing E3 ligases [6]. The mammalian genome encodes at least four UBR members (Ubr1, Ubr2, Ubr4 and Ubr5) characterized by the UBR box, a ~70-residue zinc finger-like domain [2,6].

N-end rule substrates known to contain N-degrons include a set of cardiovascular GPCR regulators (RGS4, RGS5, and RGS16) in mammals [3,5], D. melanogaster DIAP1 [7], and S. cerevisiae cohesin component Scc1 [8]. Substrates targeted through internal degradation signals include histone H2A in mouse spermatocytes [9], S. cerevisiae Cup9 [a transcriptional repressor of the peptide transporter Prt2] [10], and mammalian c-Fos [11].

Genetic studies have revealed that the N-end rule pathway plays an important role in many biological processes, including cardiac development, angiogenesis, and meiosis. Akt1-deficient mice die at fetal stages due to cardiovascular defects [4]. Mutations in the human UBR1 gene cause Johanson-Blizzard syndrome, which is characterized by exocrine pancreatic insufficiency, multiple malformations and mental retardation [12]. Disruption of Ubr2 in mice causes spermatogenic defects and female lethality [13]. Ubr2 localizes to meiotic chromatin regions and functions together with the ubiquitin conjugating (E2) enzyme HR6B in histone H2A ubiquitylation during male meiosis [9].

We previously identified Tex19.1 (also known as Tex19) as a gene with germ cell-specific expression in the testis [14]. Disruption of Tex19.1 causes defects in spermatogenesis [15]. Here we demonstrate that Tex19.1 forms a stable complex with Ubr2 during spermatogenesis. In Ubr2-deficient germ cells, Tex19.1 protein is absent despite abundant Tex19.1 mRNA, suggesting that Ubr2 is required for stabilization rather than degradation of Tex19.1 during spermatogenesis.
Results

Tex19.1 forms a stable complex with Ubr2

Mouse Tex19.1 is a small protein (351 aa) with a coiled-coil domain, which is known to mediate protein-protein interactions [14]. To identify potential binding partners of Tex19.1, we performed immunoprecipitation (IP) experiments with testicular protein extracts using a Tex19.1-specific antibody that we generated. One prominent protein band (~200 kD) was found in the co-immunoprecipitated proteins from wild type testes but not Tex19.1<sup>2/2</sup> testes (Fig. 1A). Mass spectrometry analysis identified this band as Ubr2, one of the recognition E3 components of the N-end rule pathway [13].

To verify the interaction between Tex19.1 and Ubr2, we performed co-immunoprecipitation experiments followed by Western blot analysis with specific antibodies. The abundance of Ubr2 in testes was too low to be detected in total testicular extract (Fig. 1B). However, Ubr2 was readily detectable in the protein fraction immunoprecipitated with the anti-Tex19.1 antibody from wild type but not from Tex19.1<sup>2/2</sup> testes (Fig. 1B). Co-immunoprecipitation and Western blot analysis further support the interaction between Tex19.1 and Ubr2 (Fig. 2A, Lane 1).

Interaction of Ubr2 with Tex19.1 is arginylation-independent

In the mammalian N-end rule pathway, Cys at position 2 (after Met) is a unique type of destabilizing residue. Cleavage of the first Met by Met aminopeptidase exposes Cys at position 2. Tex19.1 from all species examined bears an N-terminal Met<sup>1</sup>-Cys<sup>2</sup> sequence, a putative pre-degron that can destabilize a substrate through oxidation and arginylation [3–5]. To test whether the binding of Tex19.1 by Ubr2 requires the N-terminal arginylation of Tex19.1 Cys<sup>2</sup>, we generated two different mutant Tex19.1 proteins in which cysteine (C2) was replaced with either glycine (G2) or valine (V2). Co-transfection and co-IP experiments using NIH3T3 cells showed that Ubr2 binds to both Tex19.1 mutant proteins, demonstrating that the interaction between Ubr2 and Tex19.1 does not require Cys<sup>2</sup> (Fig. 2A, Lanes 2 and 3).

Ubr2 binds to the evolutionarily conserved N-terminal region of Tex19.1

The human genome contains only one TEX19 gene. Human TEX19 protein consists of only 164 aa and is homologous to the N-terminal part of mouse Tex19.1 protein (aa 1–162). This partial conservation suggests that the N-terminal half of Tex19 interacts with Ubr2. To test this hypothesis, we generated two partial murine Tex19.1 proteins consisting of either the N or the C-terminal half: Tex19.1N (aa 1–163) and Tex19.1C (aa 164–351). Co-IP analyses of these proteins with Ubr2 demonstrated that Ubr2 binds to the evolutionarily conserved N-terminal half but not the unconserved C-terminal half of Tex19 (Fig. 2B).

Ubr2 interacts with Tex19.2

Murine Tex19.1 forms a two-gene family with its sequence homologue Tex19.2, which is separated by only 27 kb from Tex19.1, but transcribed independently. While Tex19.1 is ex-
pressed specifically in germ cells, Tex19.2 expression is restricted to testicular somatic cells [16]. Using co-transfection and co-IP assays, we found that Ubr2 also binds to Tex19.2 (Fig. 3).

**Tex19.1-null mouse mutant phenocopies Ubr2-null mutant**

Ubr2<sup>−/−</sup> mice exhibit spermatogenic defects and embryonic lethality preferentially affecting females [13]. Because Tex19.1 and Ubr2 form a stable complex in the testis, we next investigated the consequence of loss of this interaction in the Tex19.1-null mice. We disrupted the Tex19.1 gene by homologous recombination in embryonic stem (ES) cells (Fig. 4A). Tex19.1 consists of three exons with the entire coding region (ORF) residing in the last exon. In the targeted allele, the ORF of Tex19.1 was replaced by the selection marker (Neo), rtTA and Lac<sub>Z</sub>. Western blot analysis confirmed the absence of the Tex19.1 protein in Tex19.1<sup>−/−</sup> testes (Fig. 4B). The testes of sterile XX<sup>++</sup> mice completely lack germ cells. The absence of Tex19.1 in XX<sup>++</sup> testes (Fig. 4B) demonstrates that Tex19.1 is germ cell-specific in the testis, consistent with previous studies [14–17]. In contrast with Tex19.1, Tex19.2 is expressed in somatic cells of the testis [16]. Therefore, our Western blot data (Fig. 4B) also shows that our antibody is specific to Tex19.1 and does not recognize Tex19.2, due to limited amino acid sequence homology (38% identity) between Tex19.1 and Tex19.2.

No gross somatic defects were observed in adult Tex19.1<sup>−/−</sup> mice. However, interbreeding of heterozygous mice yielded a sub-mendelian ratio of genotypes of offspring (+/+; +/−; −/−; males: 61:103:35; females: 43:84:8). Significantly fewer homozygous mice were produced than expected ($\chi^2 = 27.07$, two degrees of freedom, $p<0.0001$), suggesting embryonic lethality. In addition, the embryonic lethality preferentially affected females ($\chi^2 = 26.22$, two degrees of freedom, $p<0.0001$). As Tex19.1 protein is expressed in embryonic stem cells at substantial levels (detectable by Western blot analysis, data not shown), the observed embryonic lethality of Tex19.1-null mutant mice could be associated with consequences of lack of expression of Tex19.1 in pluripotent stem cells during embryogenesis [16].

Disruption of Tex19.1 resulted in sharply reduced testis size. The weight of Tex19.1<sup>−/−</sup> testes [104±48 mg/pair, $n = 6$ mice, $p<0.001$] from 2–3 month old mice was half (50%) that of Tex19.1<sup>+/−</sup> testes [210±34 mg/pair]. Notably, the testes from Tex19.1<sup>−/−</sup> males varied dramatically in size (Fig. 4C). Likewise, the sperm output in the cauda epididymus from Tex19.1<sup>−/−</sup> mice varied greatly, ranging from $3.0 \times 10^5$ to $1.2 \times 10^7$, but correlated well with the testis size. In most Tex19.1-deficient testes, spermatogenesis appeared to be blocked at the meiotic stage with abundant zygotene or early pachytene-like spermatocytes but few postmeiotic germ cells (Fig. 4E). However, the severity of meiotic defects varied greatly from mouse to mouse, and even from tubule to tubule in the same testis. The most severe meiotic defect was meiotic arrest at early pachytene stage. In testes with less severe defects, round and elongating spermatids were present but at a greatly reduced number (data not shown). It is intriguing but unclear why the Tex19.1-deficient mice exhibit such a large degree of phenotypic variation.

We next examined whether Tex19.1-deficient spermatocytes exhibit defects in chromosomal synopsis by immunostaining of SYCP1 (transverse filaments) and SYCP2 (lateral elements) [18–20]. The axial elements of the synaptonemal complex were assembled in Tex19.1-deficient spermatocytes. 48% of Tex19.1-deficient pachytene spermatocytes had normal synopsis (Fig. S1A). In the remaining pachytene spermatocytes, we observed variable synaptic failure; some chromosomes were completely asynapsed, whereas the majority of chromosomes were fully synapsed (Fig.
S1B-D). In contrast, only 3% of wild type pachytene nuclei (280 scored) had asynapsed chromosomes, which were all sex chromosomes. Specifically, of the Tex19.1-deficient pachytene spermatocytes, 16% contained only asynapsed sex chromosomes, 4% contained only one pair of asynapsed autosomes, in 27% 2–5 pairs of chromosomes were asynapsed and in 5% more than 5 pairs of chromosomes were affected (Fig. S1E). In conclusion, these data demonstrate that disruption of Tex19.1 causes defects in chromosomal synapsis.

In summary, Tex19.1−/− mouse mutant that we generated confirms the spermatogenic phenotypes of a previously reported Tex19.1 mutant [15]. In addition, our study has uncovered a novel
finding that the embryonic lethality in Tex19.1-null mice preferentially affects females. Collectively, the Tex19.1<sup>−/−</sup> mouse mutant exhibits abnormal spermatogenesis, defective chromosomal synapsis, and incomplete penetrance of embryonic lethality preferentially affecting females, all of which are present in the Ubr2<sup>−/−</sup> mouse mutant [13]. The heterogeneity of spermatogenic defects was also observed in Ubr2-deficient mice. The testis weight of Ubr2-deficient testis ranged from 30 to 70% of the wild type controls and some Ubr2-deficient males produced sperm [13]. Thus, consistent with the interaction between Tex19.1 and Ubr2, these two mouse null mutants phenocopy each other.

Depletion of the Tex19.1 protein in Ubr2-deficient testis

Ubr2 is an ubiquitin E3 ligase of the N-end rule pathway [13]. If Tex19.1 were an in vivo substrate of Ubr2 and thus targeted for degradation, one would expect increased abundance of Tex19.1 protein in Ubr2-deficient testes. To test this possibility, we first analyzed Tex19.1 transcript abundance in Ubr2<sup>−/−</sup> and wild type testes and found comparable levels (Fig. 5A). However, Tex19.1 protein was not detected in Ubr2<sup>−/−</sup> testes by Western blot analysis (Fig. 5B). The absence of Tex19.1 in Ubr2<sup>−/−</sup> testes was not due to loss of germ cells, since Sycp2, another meiosis-specific protein, was present in Ubr2<sup>−/−</sup> testes. Furthermore, we detected abundant Tex19.1 in Sycp2<sup>−/−</sup> testes exhibiting meiotic arrest (Fig. 5B) [20]. We then performed immunofluorescence analysis of testis sections to investigate the localization of Tex19.1 protein throughout meiosis in wild type compared to Ubr2-deficient germ cells. In early wild type germ cells, including meiotic spermatocytes, we observed that Tex19.1 localizes to the cytoplasm but not the nucleus. In contrast, Tex19.1 was not detected in post-meiotic spermatids, which express Acrv1, a component of the acrosomes (Fig. 5C) [21]. Consistent with our Western blot data, Tex19.1 protein was clearly absent from Ubr2-deficient germ cells at all stages (Fig. 5C). Therefore, the absence of Tex19.1 may be a major causative mechanism underlying spermatogenic defects in Ubr2-deficient mice. These results suggest that Ubr2 causes stabilization rather than degradation of the Tex19.1 protein in testes.

Discussion

Ubr2 is the recognition E3 component of the N-end rule pathway, in which an ubiquitin ligase recognizes a destabilizing N-terminal residue as an essential component of N-degron. It has been shown that Ubr2 plays a role in transcriptional silencing of meiotic chromosomes through ubiquitination of histone H2A [9,13]. Here, we provide several lines of evidence that Ubr2 plays a novel function outside the N-end rule pathway: protein stabilization. Firstly, we demonstrate that Ubr2 forms a stable complex with Tex19.1 in testis. If Tex19.1 were an enzymatic substrate of Ubr2 and thus destined for ubiquitin-dependent degradation, its association with Ubr2 might be too transient to be detected by co-IP. In fact, although Ubr2 protein was not detectable in the total testicular extracts by Western blot, it was readily co-immunoprecipitated with Tex19.1, suggesting the

![Figure 5. Depletion of the Tex19.1 protein in Ubr2-deficient testis.](http://example.com/figure5.png)

(A) Detection of Tex19.1 transcript in Ubr2<sup>−/−</sup> testis by RT-PCR. Sycp2 and β-actin served as germ cell-specific and ubiquitous expression controls [20]. (B) Western blot analysis shows the absence of Tex19.1 protein in Ubr2<sup>−/−</sup> testis. Sycp2<sup>−/−</sup> testis exhibits meiotic arrest and serves as a control [20]. (C) Loss of Tex19.1 protein in Ubr2<sup>−/−</sup> germ cells. Testis sections from adult wild type and Ubr2<sup>−/−</sup> mice were immunostained with anti-Tex19.1 and anti-Acrv1 antibodies. Acrv1 is a component of acrosomes and thus is only present in the haploid germ cells - spermatids. Note the abundance of round spermatids in the Ubr2-deficient testis. Scale bar, 25 μm.

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formation of a stable protein complex. Secondly, Tex19.1 bears an N-terminal cysteine. Therefore, Tex19.1 might be a substrate of ATE1-dependent arginylation and Ubr2-dependent ubiquitylation. However, our results show that the Ubr2-Tex19.1 interaction does not require the evolutionarily conserved Cys residues, a putative arginylation substrate. Thirdly, the Tex19.1 mouse mutant phenocopies the Ubr2 null mutant, underscoring the physiological relevance of the Tex19.1-Ubr2 interaction. Lastly, the Tex19.1 protein is absent rather than more abundant in Ubr2-deficient testes. As Ubr2 binds to Tex19.1, the most parsimonious explanation is that Ubr2 stabilizes Tex19.1 metabolically. However, we cannot exclude the possibility that Ubr2 might also play a role in the translation of the Tex19.1 mRNA.

The notion that Ubr2 plays a non-canonical function in protein turnover is further supported by the study of RECLQ4 [22]. RECLQ4, a putative DNA helicase, is mutated in the Rothmund-Thomson and RAPADILINO syndromes [23, 24]. Although RECLQ4 forms a stable complex with both Ubr1 and Ubr2, RECLQ4 is not ubiquitylated and is a long-lived protein in Hela cells, suggesting that it might also be stabilized by Ubr1/Ubr2 under certain conditions [22]. In our study, Ubr2 is required for the stability of Tex19.1. However, it is unclear how Ubr2 prevents Tex19.1 from degradation. One possibility is that the stable binding of Ubr2 to Tex19.1 blocks the accessibility of Tex19.1 by other E3 ligases.

Materials and Methods

Generation of anti-Tex19.1 and anti-Ubr2 polyclonal antibody

The entire mouse Tex19.1 coding region was cloned into the pQE30 vector. The 6xHis-Tex19.1 fusion protein was expressed in M15 bacteria, affinity-purified with Ni-NTA beads, and eluted in 8 M urea according to the manufacturer's standard purification protocol. The N-terminal 100 aa of mouse Ubr2 was expressed as a GST fusion protein in E. coli using the pGEX4T-1 vector and affinity purified with glutathione Sepharose. Each of the recombinant proteins was used to immunize two rabbits according to the company's standard protocol (Cocalico Biologicals, Inc). The anti-Tex19.1 antiserum (serum 2109) was used for western blot (1:500). The anti-Ubr2 antiserum (serum 2186) was used for western blot (1:500).

Co-immunoprecipitation (IP), mass spectrometry and western blot analyses

To identify proteins coimmunoprecipitated with Tex19.1, testicular protein extracts were prepared from postnatal day 20 testes by homogenization in lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM PMSF). After centrifugation, 100 extracts were precleared by incubating with protein A agarose beads (Invitrogen) and centrifuged prior to use for IP with affinity-purified anti-Tex19.1 antibody. Immunoprecipitated proteins were washed four times in washing buffer (50 mM Tris HCl, pH 7.5, 250 mM NaCl, 0.1% NP-40, 0.05% deoxycholate) and were subject to 4–15% gradient SDS-PAGE analysis. The gel was stained with SYPRO Ruby (Bio-rad). Gel bands of interest were cut and sent to the Proteomics Core Facility at the University of Pennsylvania for protein identification by mass spectrometry. The following primary antibodies were used for co-IP and western blot analyses: anti-Ubr2 (Cat# H00023304-A01, Novus Biologicals; 1:500), anti-Myh11 (Cat# ab6683, Abcam; 1:1000) and anti-β-actin (Cat# A5441, Sigma-Aldrich; 1:5000). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Sigma) were used.

DNA constructs and transfection of cultured cells

The ORFs of mouse Tex19.1, Tex19.2, and Ubr2 were cloned into pcDNA3.1/V5-His-TOPO mammalian expression vectors (Invitrogen) respectively. The V5 epitope tag was in frame only for Tex19.1, Tex19.2, Tex19.1N, and Tex19.1G (Fig. 2). Ubr2 was not tagged with the V5 epitope. Tex19.1 point mutations (C2G and C2V) were introduced through targeted mutation using PCR primers. All DNA constructs were verified by sequencing. DNA constructs were transfected into NIH3T3 cells (ATCC catalogue No. CRL-1658) using FuGENE6 Transfection Reagent (Invitrogen). IP and western blot analyses were performed with anti-V5 (Cat# 46-0705, Invitrogen) (1:5000), anti-Tex19.1, and anti-Ubr2 antibodies.

Targeted inactivation of the Tex19.1 gene

In the Tex19.1 targeting construct, two homologous arms (2.1 kb each) were amplified from a Tex19.1-positive BAC clone (RP23-400P17) by high-fidelity PCR and were subcloned to flank the rtTA2S-M2-ires-LacZ-PGK-Neo knockin/selection cassette (Fig. 4). The rtTA2S-M2 fragment was PCR amplified from the pUHDrtTA2S-M2 plasmid [25]. Hybrid V6.5 XY ES cells (C57BL/6×129/sv) were electroporated with the linearized Tex19.1 targeting construct (pUP77-29A/Neo) and were cultured in the presence of G418 (350 µg/ml). Electroporation was performed in a 0.4-cm Bio-Rad cuvette with the Bio-Rad Gene Pulser Xcell unit (Voltage, 400 v; Capacitance, 25 µF; Resistance, infinite; Expected time constant, 0.4 msec). Seven days after electroporation, 384 G418-resistant ES cell clones were picked. Screening of 96 clones by PCR identified twelve ES cell clones in which homologous recombination had occurred. PCR was performed with a forward primer upstream of the left arm and a reverse primer in the knockin/Neo cassette or a forward primer in the knockin/Neo cassette and a reverse primer downstream of the right arm. One targeted ES cell clone (3H6) was injected into B6C3F1 (Taconic) blastocysts that were subsequently transferred to the uteri of pseudopregnant ICR female mice. Male chimeras were bred with C57BL/6j females and germ-line transmission of the Tex19.1 knockout/knockin allele was obtained. Mice from mixed strain backgrounds (C57BL/6×129/sv) were used in this study. In the Tex19.1 mutant mice, the expression of rtTA and LacZ is expected to be under the control of the endogenous Tex19.1 promoter. Offspring were genotyped by PCR of tail genomic DNA with the following primers: wild type (311 bp), ATGGATCCTGTCGCCCAGTGAGGTT and GGTGTCGACTTTGAGCACATATAAGGGACCCCAAT; mutant (450 bp), AGAGTCGACATGTCTAGACTGGACAAAGAG and CCTTCGAAATCCGCGACCCAGGTCAA. ~3 mm-long tail biopsy was digested in 200 µl proteinase K buffer (10 mM Tris, 10 mM EDTA, 10 mM NaCl, 0.2% SDS, pH 8.0) at 55°C overnight. Genomic DNA was precipitated with 200 µl isopropanol, washed once with 1 ml of 70% ethanol, air dried and resuspended in 200 µl 1xTE buffer. 1 µl of genomic DNA was used in a 20 µl PCR genotyping reaction (94°C, 30 seconds; 55°C, 30 seconds; 72°C, 45 seconds; 35 cycles). Full details of the study were approved by the Institutional Animal Care and Use Committee (IACUC protocol # 802780) of the University of Pennsylvania.

Histology, Surface spread, and immunofluorescent analyses

For histology, testes were fixed in Bouin's solution, embedded in paraffin, sectioned using the Leica RM2035 microtome, and stained with hematoxylin and eosin. Surface spread analyses of spermatocytes were performed as previous described [26]. For
immunofluorescent analyses, adult wild type and Ubr2−/− tests were fixed with 4% paraformaldehyde overnight, dehydrated in 30% (w/v) sucrose solution, embedded with TBS tissue freezing medium (Fisher Scientific), and frozen at temperatures below −20°C. Sections (0.5 μm) were cut using a Reichert-Jung cryomicrotome. Sections were immunostained with anti-Tex19.1 and anti-Acrv1 antibodies [21]. For immunostaining of testis sections, specific anti-Tex19.1 antibodies were affinity-purified using the immunoblot method [27]. Texas Red and FITC-conjugated secondary antibodies were used.

Supporting Information

Figure S1 TEX19 promotes chromosome synopsis. Spread nuclei of spermatocytes from 20-day-old Tex19-deficient mice were immunostained with anti-SYCP1 (green) and anti-SYCP2 (red) antibodies [19,21]. (A) Tex19-deficient pachynema with apparently normal synopsis. Note the 19 pairs of fully synapsed autosomal homologues (yellow) and partially synapsed X-Y chromosomes (red). (B) Tex19-deficient pachynema with asynapsed XY only. Even though X and Y occupy the same nuclear domain, they remain separated. (C) Tex19-deficient pachynema with asynapsed XY and one pair of asynapsed autosomes (indicated by arrows). (D) Tex19-deficient pachynema with four pairs of asynapsed homologous chromosomes (red). (E) Analysis of asynapsed chromosomes in Tex19-deficient pachytene spermatocytes. ~100 pachytene spermatocytes from each Tex19−/− mouse were examined for synopsis and divided into the five categories shown. Four 20-day-old Tex19−/− mice were analyzed. Values shown represent the mean ± standard deviation. Found at: doi:10.1371/journal.pone.0014017.s001 (0.71 MB TIF)

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Author Contributions

Conceived and designed the experiments: FY PJW. Performed the experiments: FY SE NAL KJM PJW. Analyzed the data: FY. Contributed reagents/materials/analysis tools: JYA YTK. Wrote the paper: FY YTK SE PJW.

References

1. Bachmair A, Finley D, Varshavsky A (1986) In vivo half-life of a protein is a function of its amino-terminal residue. Science 234(4773): 179–186.
2. Tasaki T, Kwon Y (2007) The mammalian N-end rule pathway: New insights into its components and physiological roles. Trends Biochem Sci 32(11): 520–528.
3. Hu RG, Sheng J, Qiu X, Xu Z, Takahashi TT, et al. (2005) The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators. Nature 437(7061): 981–986.
4. Kwon YT, Kashina AS, Davydov IV, Hu RG, An JY, et al. (2002) An essential role of N-terminal arginylation in cardiovascular development. Science 297(5578): 96–99.
5. Lee MJ, Tasaki T, Moroi K, An JY, Kimura S, et al. (2005) RGS4 and RGS5 are in vivo substrates of the N-end rule pathway. Proc Natl Acad Sci U S A 102(42): 15030–15035.
6. Tasaki T, Zakrzewska A, Dudić DD, Jiang Y, Lazo JS, et al. (2009) The substrate recognition domains of the N-end rule pathway. J Biol Chem 284(5): 1884–1895.
7. Ditzel M, Wilson R, Tenen V, Zachariou A, Paul A, et al. (2003) Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. Nat Cell Biol 5(4): 467–473.
8. Rao H, Uhlmann F, Namkung K, Varshavsky A (2001) Degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability. Nature 410(6831): 955–959.
9. An JY, Kim EA, Jiang Y, Zakrzewska A, Kim DE, et al. (2010) UBR2 mediates transcriptional silencing during spermatogenesis via histone ubiquitination. Proc Natl Acad Sci U S A 107(5): 1912–1917.
10. Byrd C, Turner GC, Varshavsky A (1998) The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. EMBO J 17(1): 269–277.
11. Sasaki T, Kojima H, Kishimoto R, Ikeda A, Kunimoto H, et al. (2006) Molecular defect of RAPADILINO syndrome expands the phenotype spectrum of Rothmund-thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. Hum Mol Genet 15(19): 2911–2918.
12. Schmelkel K, Meuwissen RL, Dietrich AJ, Vink AG, van Marle J, et al. (1996) Organization of SCP1 protein molecules within synaptonemal complexes of the rat. Exp Cell Res 226(1): 29–30.
13. de Vries FA, de Boer E, van den Bosch M, Baarends WM, Ooms M, et al. (2005) Mouse Sycp1 functions in synaptonemal complex assembly, meiotic recombination, and XY body formation. Genesis 43(1): 1376–1389.
14. Yang F, De La Fuente R, Leu NA, Baumann C, McLaughlin KJ, et al. (2006) Mouse SYCP2 is required for synaptonemal complex assembly and chromosomal synopsis during male meiosis. J Cell Biol 174(4): 497–507.
15. Reddi PP, Nashby-Hansen S, Agoulnik I, Tsai JY, Silver LM, et al. (1995) Complementary deoxyribonucleic acid cloning and characterization of msp-10: The mouse homologue of human acrosomal protein SP-10. Biol Reprod 53(4): 873–881.
16. Yiu J, Kwon YW, Varshavsky A, Wang W (2004) RECLQ4, mutated in the rotund-thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. Hum Mol Genet 13(20): 2421–2430.
17. Kikyo S, Shimamoto A, Goto M, Miller RW, Smithson WA, et al. (1999) Mutations in RECLQ4 cause a subset of cases of rotund-thomson syndrome. Nat Genet 22(2): 82–84.
18. Sironen HA, Korpela O, Kaariainen H, Haravuori H, Winter RM, et al. (2003) Molecular defect of RAPADILINO syndrome expands the phenotype spectrum of RECLQ4 diseases. Hum Mol Genet 12(21): 2837–2844.
19. Ullinger S, Baron U, Thellmann M, Hasan MT, Bujard H, et al. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci U S A 97(14): 7963–7968.
20. Yang F, Geil K, van der Heijden GW, Eckhardt S, Leu NA, et al. (2008) Meiotic failure in male mice lacking an X-linked factor. Genes Dev 22(5): 682–691.
21. Harlow E, Lane D (1998) Using antibodies: A laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.