Degradation of the Separase-cleaved Rec8, a Meiotic Cohesin Subunit, by the N-end Rule Pathway

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ABSTRACT

The Ate1 arginyltransferase (R-transferase) is a component of the N-end rule pathway, which recognizes proteins containing N-terminal degradation signals called N-degrons, polyubiquitylates these proteins and thereby causes their degradation by the proteasome. Ate1 arginylates N-terminal Asp, Glu or (oxidized) Cys. The resulting N-terminal Arg is recognized by ubiquitin ligases of the N-end rule pathway. In the yeast Saccharomyces cerevisiae, the separase-mediated cleavage of the Scc1/Rad21/Mcd1 cohesin subunit generates a C-terminal fragment that bears N-terminal Arg and is destroyed by the N-end rule pathway without requirement for arginylation. In contrast,
the separase-mediated cleavage of Rec8, the mammalian meiotic cohesin subunit, yields a fragment bearing N-terminal Glu, a substrate of the Ate1 R-transferase. Here we constructed and used a germ cells-confined Ate1−/− mouse strain to analyze the separase-generated C-terminal fragment of Rec8. We show that this fragment is a short-lived N-end rule substrate, that its degradation requires N-terminal arginylation, and that male Ate1−/− mice are nearly infertile, owing to massive apoptotic death of Ate1−/− spermatocytes during the metaphase of meiosis I. These effects of Ate1 ablation are inferred to be caused, at least in part, by the failure to destroy the C-terminal fragment of Rec8 in the absence of N-terminal arginylation.

The N-end rule pathway is a set of proteolytic systems whose unifying feature is the ability to recognize and polyubiquitylate proteins containing degradation signals called N-degrons, thereby causing degradation of these proteins by the proteasome (Fig. 1A, B) (1-8). The main determinant of an N-degron is either an unmodified or chemically modified destabilizing N-terminal residue of a protein. The identity of the next residue, at position 2, is often important as well. A second determinant of an N-degron is a protein’s internal Lys residue. It functions as the site of a protein’s polyubiquitylation and tends to be located in a conformationally disordered region (4,9,10). Recognition components of the N-end rule pathway are called N-recognins. In eukaryotes, N-recognins are E3 ubiquitin (Ub) ligases that can target N-degrons (Fig. 1A, B). Bacteria also contain the N-end rule pathway, but Ub-independent versions of it (11-16).

Regulated degradation of proteins and their natural fragments by the N-end rule pathway has been shown to mediate a strikingly broad range of biological functions, including the sensing of heme, nitric oxide (NO), oxygen, and short peptides; the control, through subunit-selective degradation, of the input stoichiometries of subunits in oligomeric protein complexes; the elimination of misfolded or otherwise abnormal proteins; the degradation of specific proteins after their retrotranslocation to the cytosol from mitochondria or other membrane-enclosed compartments; the regulation of apoptosis and repression of neurodegeneration; the regulation of DNA repair, transcription, replication, and chromosome cohesion/segregation; the regulation of G proteins, autophagy, peptide import, meiosis, immunity, fat metabolism, cell migration, actin filaments, cardiovascular development, spermatogenesis, and neurogenesis; the functioning of adult organs, including the brain, muscle, testis, and pancreas; and the regulation of leaf and shoot development, leaf senescence, and many other processes in plants (Figs. 1A, B) ((4-7) and references therein).

In eukaryotes, the N-end rule pathway consists of two branches. One branch, called the Ac/N-end rule pathway, targets proteins for degradation through their Nα-terminally acetylated (Nt-acetylated) residues (Fig. 1B) (2,3,17-19). Degradation signals and E3 Ub ligases of the Ac/N-end rule pathway are called Ac/N-degrons and Ac/N-recognins, respectively. Nt-acetylation of cellular proteins is apparently irreversible, in contrast to acetylation-deacetylation of proteins’ internal Lys residues. About 90% of human proteins are cotranslationally Nt-acetylated by ribosome-associated Ac/N-acetylases (20). Posttranslational Nt-acetylation is known to occur as well. Many, possibly most, Nt-acetylated proteins contain Ac/N-degrons (Fig. 1B).

The pathway’s other branch, called the Arg/N-end rule pathway, targets specific
unacetylated N-terminal residues (Fig. 1A) (3,21-25). The “primary” destabilizing N-terminal residues Arg, Lys, His, Leu, Phe, Tyr, Trp, and Ile are directly recognized by N-recognins. The unacetylated N-terminal Met, if it is followed by a bulky hydrophobic (Φ) residue, also acts as a primary destabilizing residue (Fig. 1A) (3). In contrast, the unacetylated N-terminal Asn, Gln, Asp, and Glu (as well as Cys, under some metabolic conditions) are destabilizing owing to their preliminary enzymatic modifications, which include N-terminal deamidation (Nt-deamidation) of Asn and Gln and Nt-arginylation of Asp, Glu and oxidized Cys (Fig. 1A) (4-6,26). In the yeast S. cerevisiae, the Arg/N-end rule pathway is mediated by the Ubr1 N-recognin, a 225 kDa RING-type E3 Ub ligase and a part of the targeting complex comprising the Ubr1-Rad6 and Ufd4-Ubc4/5 E2-E3 holoenzymes (4,27). In multicellular eukaryotes, several E3 Ub ligases, including Ubr1, function as N-recognins of the Arg/N-end rule pathway (Fig. 1A).

Nt-arginylation is mediated by the Ate1-encoded arginyltransferase (Arg-tRNA-protein transferase; R-transferase), a component of the Arg/N-end rule pathway and one subject of the present study (Fig. 1A) (28-36). Alternative splicing of mouse Ate1 pre-mRNAs yields at least six R-transferase isoforms, which differ in their Nt-arginylation activity (28,31). R-transferases are sequelogous (similar in sequence (37)) throughout most of their ~60 kDa spans from fungi to mammals (4). R-transferase can arginylate not only N-terminal Asp and Glu but also N-terminal Cys, if it has been oxidized to Cys-sulfinate or Cys-sulfonate, through reactions mediated by NO, oxygen, and N-terminal Cys-oxidases (5,6,30). Consequently, the Arg/N-end rule pathway functions as a sensor of NO and oxygen, through the conditional oxidation of N-terminal Cys in proteins such as the Rgs4, Rgs5 and Rgs16 regulators of G proteins in mammals (30,38) and specific transcriptional regulators in plants (reviewed in (5,6)). By now, more than 200 distinct proteins (including natural protein fragments) have been either shown or predicted to be Nt-arginylated (21,22,39-41). Many, possibly most, Nt-arginylated proteins are conditionally or constitutively short-lived substrates of the Arg/N-end rule pathway (Fig. 1A).

Identified and predicted substrates of the Arg/N-end rule pathway include cohesin subunits of the kleisin family. Rings of oligomeric cohesin keep sister chromatids together through a topological confinement (42-53). Physiological roles of cohesins include DNA replication, cohesion/segregation, repair, transcription; and control of apoptosis (54-61). In S. cerevisiae, the Scc1/Rad21/Mcd1 cohesin subunit is cleaved at a specific site, late in mitosis, by the nonprocessive Esp1 protease called separase, allowing the release of sister chromatids and the transition from metaphase to anaphase (48,62-64).

In mammals and other multicellular eukaryotes, the bulk of cohesin-mediated confinement of sister chromatids is removed during prophase through openings of cohesin rings that do not involve proteolytic cuts. Sister chromatids continue to be held together until the end of metaphase, to a large extent through still intact cohesin rings at the centromeres. Once the bipolar spindle attachment of chromosomes is achieved at the end of metaphase, the activated separase cleaves the mammalian Rad21 subunit (a sequelog of yeast Scc1) in the closed cohesin complexes, resulting in their opening and allowing the separation of sister chromatids (45,48,58,65). At least in mammals, the Rad21 cohesin subunit can be cleaved in vivo not only by separase but also by calpain-1 (a Ca$^{2+}$-activated protease) (58), and by caspases as well (56,57).

During meiosis, in which cohesins also play essential roles, the mitosis-specific
cohesin subunit Rad21 is replaced by the sequelogous (similar in sequence (37)), meiosis-specific Rec8 subunit (43,59,66-70). (A second meiosis-specific kleisin-type subunit, called Rad21L, is expressed during early meiosis and disappears afterward (71.) Meiotic DNA replication is followed by two rounds of cell division to produce four haploid daughter cells. During the first meiotic cell division cycle (meiosis I), replicated homologous chromosomes pair and recombine with each other. The pairs of modified (recombined) homologous chromosomes are separated at the end of meiosis I, yielding two diploid daughter cells. During meiosis II, the replicated sister chromatids of each chromosome are pulled apart to produce haploid daughter cells. In the testis of male mice, meiosis I and II take place in meiotic spermatocytes, leading to the formation of haploid spermatids and later mature sperm cells (43,59,66,74).

In *S. cerevisiae*, the separase-generated C-terminal fragment of the Scc1 cohesin subunit bears N-terminal Arg. This fragment of Scc1 forms late in mitosis upon the activation of separase, and is rapidly destroyed by the Arg/N-end end rule pathway (62). A failure to eliminate this (normally short-lived) Scc1 fragment, for example, in a *ubr1A* mutant that lacks the Arg/N-end rule pathway, results in chromosome instability (62). The C-terminal fragment of Scc1 retains a physical affinity for the rest of cohesin complex (62). Therefore the plausible (but not proven) cause of chromosome instability in *ubr1A* cells is an interference with cohesin mechanics by the metabolically stabilized, cohesin-bound C-terminal fragment of Scc1. Since the yeast Scc1 fragment bears N-terminal Arg, the fragment’s degradation by the Arg/N-end rule pathway does not require Nt-arginylation (62). In mammals, however, the separase-generated C-terminal fragments of the Rad21 subunit of mitotic cohesin and the Rec8 subunit of meiotic cohesin bear N-terminal Glu, a substrate of the Ate1 R-transferase (Fig. 1A, C) (47,75,76).

In the present work, we constructed an *Ate1* Δ mouse strain in which the ablation of *Ate1* was confined to germ cells. We show that the separase-generated C-terminal fragment of Rec8, a subunit of meiotic cohesin, is a short-lived physiological substrate of the Arg/N-end rule pathway, and that the degradation of this Rec8 fragment requires its Nt-arginylation. These and related results suggest that a failure to destroy this fragment in arginylation-lacking spermatocytes of *Ate1* Δ mice contributes to a greatly reduced male fertility of these mice, owing to the observed arrest and apoptotic death of *Ate1* Δ spermatocytes at the end of meiosis I. These results expand the already large set of functions of the Arg/N-end rule pathway. Together with earlier data about *Ate1* Δ and *Ubr2* Δ mice (33,77), the present findings also indicate that perturbations of the Arg/N-end rule pathway may be among the causes of infertility in humans.

**EXPERIMENTAL PROCEDURES**

**Mutant Mouse Strains** – Conditional (cre-lox-based) *Ate1* *^Boxflox^* mice were described (33). *Tnap-Cre* mice, in which the Cre recombinase is selectively expressed in primordial germ cells (78), were purchased from Jackson Laboratory (Bar Harbor, ME). Germ-cell specific *Ate1* Δ mice were constructed in the present study through matings of *Ate1* *^Boxflox^* mouse strains were constructed in the present study through matings of *Ate1* *^Boxflox^* mice (33) and *Tnap-Cre* mice (78). All animal experiments were approved by the Animal Research Panel of the Committee on Research Practice of the University of the Chinese Academy of Science.

**Antibodies** – Mouse anti-Sycp3 monoclonal antibody (SC-74569) and mouse anti-PLZF monoclonal antibody (SC-28319) were from Santa Cruz Biotechnology (Dallas, TX). Rabbit anti-Sycp3 polyclonal
antibody (ab150292) and Rabbit anti-Rec8 polyclonal antibody were from Abcam (Cambridge, MA). Mouse anti-MLH1 monoclonal antibody (51-1327GR) was from BD Pharmingen (San Jose, CA). Rabbit anti-Sycp1 polyclonal antibody-Cy5 (NB300-228c) was from Novus Biologicals (Littleton, CO). Mouse anti-Cyclin B1 monoclonal antibody (MA5-14319) was from Thermo Scientific (Waltham, MA). FITC-conjugated mouse monoclonal anti-α-tubulin antibody (F2168) was from Sigma-Aldrich (St. Louis, MO). FITC-conjugated goat anti-rabbit antibody, and TRITC-conjugated goat anti-mouse antibody, as well as horseradish peroxidase (HRP)-conjugated secondary antibody were from Zhong Shan Jin Qiao (Beijing, China). Alexa Fluor 680-conjugated goat anti-mouse antibody and Alexa Fluor 680-conjugated goat anti-rabbit antibody were from Invitrogen (Carlsbad, CA).

**Male Fertility Assays** – Breeding assays with wild-type and Ate1-/- male mice were carried out previously described (79). Briefly, each examined male mouse (8-9 weeks old) was caged with two wild-type (CDI strain) female mice (6-8 weeks old), and their vaginal plugs were checked every morning. The number of pups produced by each pregnant female was counted within a week after birth. Each male was tested through six cycles of this breeding assay.

**Epididymal Sperm Count** – The cauda epididymis was dissected from adult mice. Sperm was released by cutting the cauda epididymis into pieces, placing them in 1 ml of phosphate-buffered saline (PBS), and incubating for 10 min at 37°C. Thereafter, 10-μl samples were transferred to a hemocytometer for counting sperm cells.

**Immunoblotting and Related Procedures** – Extracts from testes were prepared using a Dounce homogenizer in a cold high-salt RIPA-like buffer (0.5 M NaCl, 1% Nonidet-P40, 1% Na-deoxycholate, 0.1% SDS, 2 mM Na-EDTA, 25 mM Tris-HCl, pH 7.6) supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF) and a protein inhibitor cocktail (Roche, Basel, Switzerland, 04693132001). Extracts were centrifuged at 13,000g for 15 min. Samples of supernatants were fractionated by SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel; Invitrogen) and electro-transferred to a nitrocellulose membrane, followed by incubation for 2-4 hrs at room temperature (RT) with a primary antibody, a further incubation with the (HRP)-conjugated secondary antibody, washes, and the detection of immunoblotting patterns using chemiluminescence, largely as described (18,80).

The Ubiquitin Reference Technique (URT) and Analyses of Rec8 in Reticulocyte Extract – The mouse Rec8 open reading frame (ORF) was amplified by PCR, using cDNA from GE Healthcare Dharmacon (Clone ID: 6335959) (Pittsburgh PA). The resulting DNA fragments were cut with SacII and Clai and cloned into SacII/Clai-cut pKP496, yielding the plasmids pBW442, pBW443, and pBW444, respectively. pKP496 was an AmpR, NeoR, pcDNA3-based plasmid encoding flag-DHFR-ha-UbK48R-MCS (multiple cloning site)-flag under the control of the P<sub>CMV</sub> promoter (21). pBW442, pBW443, and pBW444 expressed, respectively, fDHFR-ha-UbK48R-Glu<sup>455</sup>-Rec8<sup>f</sup>, fDHFR-ha-UbK48R-Val<sup>455</sup>-Rec8<sup>f</sup>, and fDHFR-ha-UbK48R-Arg-Glu<sup>455</sup>-Rec8<sup>f</sup> from the P<sub>CMV</sub> promoter. The plasmid pcDNA3MR8, expressing fDHFR-ha-UbK48R-Met<sup>206</sup>-Rec8<sup>f</sup>, was constructed similarly.

Rabbit reticulocyte-based degradation assays were carried out using the TNT T7 Coupled Transcription/Translation System (Promega, Madison, WI), largely as previously described (21-23). Nascent proteins translated in the extract were pulse-labeled with <sup>35</sup>S-L-methionine (0.55 mCi/mL, 1,000 Ci/mmol, MP Biomedicals, Santa Ana, CA) for 10 min at 30°C in the
total volume of 30 μl. Labeling was quenched by the addition of 0.1 mg/ml cycloheximide, and 5 mM unlabeled methionine, bringing the final reaction volume to 40 μl. Samples of 10 μl were removed at the indicated time points and the reaction was terminated by the addition of 80 μl of TSD buffer (1% SDS, 5 mM dithiothreitol (DTT), 50 mM Tris-HCl, pH 7.4), snap-frozen in liquid nitrogen, and stored at -80°C until use. Following the collection of all time points, samples were heated at 95°C for 10 min, then diluted with 1 ml of TNN buffer (0.5% NP40, 0.25 M NaCl, 5 mM Na-EDTA, 50 mM Tris-HCl, pH 7.4) containing 1X complete Protease Inhibitor Cocktail (Roche), and immunoprecipitated using 5 μl of anti-flag M2 magnetic beads (Sigma-Aldrich). Samples were incubated with rotation at 4°C for 4 hrs, followed by three washes in TNN buffer, a wash in 10 mM Tris-HCl, pH 8.5, and resuspension in 20 μl of SDS-sample buffer. Samples were heated at 95°C for 5 min, and fractionated SDS-10% PAGE, followed by autoradiography and quantification, using Typhoon-9500 Imager and ImageQuant (GE Healthcare).

Reticulocyte extract-based assays in which Rec8f, its derivatives, and the DHFR-ha-UbK48R reference protein were detected by immunoblotting were carried similarly, except that no 35S-Met/Cys labeling was involved. The synthesis-deubiquitylation-degradation of URT-based test fusions was allowed to proceed for 1 hr at 30°C either in the absence of added dipeptides, or in the presence of either Arg-Ala (1 mM) or Ala-Arg (1 mM), followed by SDS-PAGE and immunoblotting with anti-flag antibody (Fig. 5E).

Histology and Immunohistochemistry – For histological analyses, testes and epididymis were fixed, after dissection, in 4% paraformaldehyde (formaldehyde, HCHO) (Solarbio, Beijing, China, P1110) overnight at 4°C, followed by standard procedures (79). Briefly, tissues were dehydrated through a series of ethanol washes and thereafter embedded in paraffin wax. Sections (5 μm thick) were produced using a microtome. Deparaffinized and rehydrated sections were stained with hematoxylin and eosin for histological observations. For immunohistochemical assays, deparaffinized and rehydrated sections were rinsed 3 times at room temperature (RT) in PBS (pH 7.4), followed by antigen retrieval by boiling for 15 min in 10 mM Na-citrate, pH 6.0. Sections were then incubated for 10 min at RT with 3% H2O2, followed by blocking of each section by incubating them for 30 min at RT in 5% bovine serum albumin (BSA; Sigma). Sections were incubated with a primary antibody at 4°C overnight, followed by incubation with an HRP-conjugated secondary antibody at 37°C for 1 hr. Sections were then stained with 3, 3'-diaminobenzidine (DAB) according to manufacturer's instructions (Zhong Shan Jin Qiao, Beijing, China, ZL1-9018), and nuclei were stained with hematoxylin. Negative controls were processed identically but without the primary antibody. Sections were examined using a Nikon 80i inverted microscope with a charge-coupled camera.

Spreads of Spermatocyte Nuclei – They were prepared as previously described (81), with modifications. Briefly, testes were washed in PBS after dissection from mice. The tunicae were removed and adherent extratubular tissues were removed by rinsing the seminiferous tubules with PBS at room temperature. The tubules were placed in a hypotonic extraction buffer (50 mM sucrose, 17 mM Na-citrate, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 3 mM Tris-HCl, pH 8.2) for 30-60 min. Thereafter approximately 1 inch of tubule was shredded to pieces by fine-tipped forceps in 20 μl of 0.1 M sucrose (pH adjusted to pH 8.2 by NaOH) on a clean glass slide. Another 20 μl of sucrose solution
was then added, and a slightly cloudy suspension was prepared, using a 20-μl pipettor. Residual tubular tissues were removed. The suspension was transferred onto a new precoated (3-aminopropyltriethoxysilane (Zhong Shan Jin Qiao, Beijing, China, ZL1-9002)) glass slides, each of them containing on the surface 0.1 ml of freshly made (and filtered through a 0.22 μm Rephile filter (Rephile, Shanghai, China, RJP3222SH)) solution of 1% paraformaldehyde (HCHO), 0.15% Triton-X100 (with pH adjusted to 9.2 using 10 mM Na-borate, pH 9.2). Each slide was gently rocked to mix the initial suspension with HCHO solution, followed by drying for at least 2 hr in a closed box with high humidity. To stain the resulting nuclei spreads, slides were washed with 0.4% Photoflo (Kodak, Rochester, NY) 3 times and with PBS 3 times, then blocked in 5% BSA for 1 hr, incubated with a primary antibody in 1.5% BSA and 0.3% Triton-X100 overnight at 4°C, then incubated with secondary antibody in PBS for 1 hr at 37°C. The resulting slides were washed in PBS, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were examined using the LSM 780/710 microscope (Zeiss).

**RESULTS**

*Germ Cell-specific Ablation of the Ate1 R-transferase Strongly Decreases Fertility of Male Mice* – Probing sections of mouse testis with affinity-purified antibody to mouse Ate1 indicated the presence of the Ate1 R-transferase in the testis, particularly in spermatocytes and spermatogonia (precursors of spermatocytes) (Fig. 2A). These immunohistochemical results were in agreement with *in situ* hybridization data about Ate1 expression in spermatocytes (77). To produce mouse strains in which Ate1 was selectively ablated in primordial germ cells (PGCs), we mated the previously constructed Ate1<sup>flox/flox</sup> mice (33) with Tnap-Cre mice expressing Cre recombinase from the PGC-specific Tnap promoter (78) (see Experimental Procedures). Immunoblotting analyses of testis extracts from the resulting Tnap-Ate1<sup>-/-</sup> mice vs. Ate1<sup>flox/flox</sup> (wild-type) mice with anti-Ate1 antibody indicated a dramatic decrease of Ate1 in Tnap-Ate1<sup>-/-</sup> testes (Fig. 2B). Inasmuch as spermatogonia and spermatocytes (in which Tnap-Cre was selectively expressed) comprise a large fraction but not the entirety of testicular cells, these results (Fig. 2B) indicated that the Ate1 R-transferase was either completely or nearly completely absent from spermatogonia and spermatocytes of Tnap-Ate1<sup>-/-</sup> mice.

Fertility of Tnap-Ate1<sup>-/-</sup> and “wild-type” Ate1<sup>flox/flox</sup> males was assessed by mating three males of each strain with Ate1<sup>flox/flox</sup> females. For each male mouse, at least six plugged females were collected and the pregnancy rates were recorded. Only ~9% of plugged Ate1<sup>flox/flox</sup> females became pregnant after mating with Tnap-Ate1<sup>-/-</sup> male mice, in comparison to the pregnancy rate of ~78% after mating with Ate1<sup>flox/flox</sup> males (Fig. 2D). In addition, the average number of pups born to Ate1<sup>flox/flox</sup> females that were mated with Tnap-Ate1<sup>-/-</sup> males was only ~1.3, in contrast to ~7.3 pups that were born, on
average, to Ate1\(^{flox/flox}\) females mated to Ate1\(^{flox/flox}\) males (Fig. 2E).

In agreement with low fertility of Tnap-Ate1\(^{-/-}\) males (Fig. 2D, E), hematoxylin/eosin (HE)-stained sections of their epididymides contained few apparently mature spermatozoa (~1×10\(^6\) per epididymis), in contrast to a much larger number (~16×10\(^6\) per epididymis) of mature spermatozoa in Ate1\(^{flox/flox}\) males (Fig. 2G, I). In contrast to Ate1\(^{flox/flox}\) males, the epididymides of Tnap-Ate1\(^{-/-}\) males contained a number of round-shaped cells with large nuclei, possibly immature spermatids (Fig. 2F). In agreement with these results, Tnap-Ate1\(^{-/-}\) testes were considerably smaller and lighter than their Ate1\(^{flox/flox}\) counterparts (Fig. 2C, H). Histological assays also showed that the average diameter of seminiferous tubules in Tnap-Ate1\(^{-/-}\)testes was ~139 μm, in contrast to ~204 μm for Ate1\(^{flox/flox}\) tubules, and that Tnap-Ate1\(^{-/-}\) tubules contained a smaller average number of cells per section (~93 cells vs. ~177 cells, respectively) (Fig. 2K, L).

These results (Fig. 2) indicated that the Ate1 R-transferase was required for normal fertility levels in male mice. Given very low but still non-zero fertility of Ate1\(^{-/-}\) males (Fig. 2D, E) as well as complete or nearly complete absence of the Ate1 R-transferase from germ cells in Tnap-Ate1\(^{-/-}\) testes (Fig. 2B), it is formally possible that the total absence of the Ate1-mediated arginylation in spermatogonia and spermatocytes is still compatible with a low but non-zero probability of sperm maturation. The alternative and a priori more likely interpretation is that the low but detectable sperm maturation that underlies the residual fertility of Tnap-Ate1\(^{-/-}\) mice (Fig. 2D, E) is made possible by rare spermatocytes of Tnap-Ate1\(^{-/-}\) testes that retained at least one copy of the intact Ate1\(^{flox}\) gene.

**The Absence of Arginylation Is Compatible with Early Stages of Germ Cell Development** – Using an antibody to the promyelocytic leukemia zinc finger (Plzf), a spermatogonia-specific marker, we observed similar levels of Plzf in presumptive spermatogonia in either the absence or presence of Ate1 (~56 Plzf-positive cells, on average, per a section of seminiferous tubule in both Tnap-Ate1\(^{-/-}\) and Ate1\(^{flox/flox}\) mice) (Fig. 3A, C). Formation of the synaptonemal complex and synapsis of chromosomes during the prophase of meiosis I in spermatocytes is accompanied by expression of the synaptonemal complex proteins 1 and 3 (Sycp1 and Sycp3) (83-85). Antibodies to Sycp1 and Sycp3 stained meiotic chromosomes indistinguishably in chromosome spreads of either Tnap-Ate1\(^{-/-}\) or Ate1\(^{flox/flox}\) spermatocytes (Fig. 3B). Similar immunofluorescence assays with Mlh1, a marker for chromosome crossovers (86-89), also showed no significant differences between Tnap-Ate1\(^{-/-}\) and Ate1\(^{flox/flox}\) spermatocytes (Fig. 3B, D, E).

In addition, we could readily identify, cytologically, every major stage of the prophase of meiosis I in both Tnap-Ate1\(^{-/-}\) and Ate1\(^{flox/flox}\) spermatocytes, including leptotene, zygotene, pachytene and diplotene, and there were no statistical significant differences between the two genotypes vis-à-vis the percentages of each stage of prophase I (Figs. 3E, F and Fig. 4A, B). The average numbers of spermatocytes per a seminiferous tubule section in the prophase of meiosis I were also similar between Tnap-Ate1\(^{-/-}\) and Ate1\(^{flox/flox}\) mice (~61 and ~60 spermatocytes, respectively) (Fig. 3E). These results indicated that (at resolution levels of our assays) pre-metaphase stages of germ-cell development did not require the Ate1 R-transferase.

**Lack of Arginylation Causes Metaphase Arrest in Meiosis I** – In contrast to the absence of detectable defects in the progression of Ate1-lacking Tnap-Ate1\(^{-/-}\) spermatocytes through the prophase of meiosis I, we found these cells to be arrested...
at the metaphase of meiosis I, followed by their death through apoptosis (Figs. 3G, H and 4C, D, F). In stage-XII seminiferous tubules, metaphase-I spermatocytes could be identified by their highly condensed (hematoxylin-stained) chromatin (74) (Fig. 3G, H). Both metaphase and later-stage (anaphase) spermatocytes (indicated by arrowheads and arrows, respectively) could be observed in Ate1-containing tubules of Ate1^{flox/flox} testes (Fig. 3G). However, no anaphase spermatocytes could be detected in tubules of Ate1-lacking Tnap-Ate1^{-/-} testes, whose relative content of metaphase spermatocytes was ~9.9%, in contrast to ~2.6% of such cells in Ate1^{flox/flox} testes (Fig. 3G, H). In agreement with these results, it was easy to detect spindle bodies marked by α-tubulin (consistent with the arrest of metaphase I spermatocytes) in Tnap-Ate1^{-/-} testes (Fig. 4E). We also examined, by immunoblotting, the expression of cyclin B1, a marker of metaphase (90). The level of cyclin B1 was significantly increased in Tnap-Ate1^{-/-} testes (Fig. 2J), yet another indication of metaphase arrest of Ate1-lacking spermatocytes in meiosis I.

Apoptotic Death of Arginylation-lacking Spermatocytes in Metaphase of Meiosis I – The terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) was used to measure the extent of apoptosis of Tnap-Ate1^{-/-} vs. Ate1^{flox/flox} spermatocytes (Fig. 4C, D, F). In Tnap-Ate1^{-/-} testes, on average ~33% of spermatocytes were overtly apoptotic (TUNEL-positive), in comparison to ~9% of such cells in Ate1-containing Ate1^{flox/flox} testes (Fig. 4C). In addition, only ~1.8 apoptotic cells per a seminiferous tubule section were found, on average, in Ate1^{flox/flox} testes, vs. ~8.3 apoptotic cells in Tnap-Ate1^{-/-} testes (Fig. 4F). Together, these results are likely to account for the observed massive decrease in the content of mature sperm cells in the testes of Ate1-lacking Tnap-Ate1^{-/-} males and the resulting very low fertility of these mice (Fig. 2C-E). As described below, the metabolic stabilization of a natural fragment of the meiosis-specific Rec8 cohesin subunit is likely to be at least a significant, and possibly the major, reason for this functional consequence of Ate1 ablation in germ cells.

Arginylation-mediated Degradation of the Separate-produced Rec8 Fragment – Rec8 is the main meiosis-specific cohesin subunit of the kleisin family. Mouse Rec8 is sequelogous to both yeast and mammalian kleisin-type cohesin subunits (Fig. 1C and Introduction). The cleavage of mouse Rec8 by the separase would be expected to generate a 15-kDa C-terminal fragment bearing N-terminal Glu, a secondary destabilizing residue and a substrate of the Ate1 R-transferase (Fig. 1A, C). Although separase can cleave mouse Rec8 in vitro at more than one location, the cleavage between Arg-454 and Glu-455 is by far the predominant one (Fig. 1C) (76).

To determine whether the separase-generated Rec8 fragment was a substrate of the Arg/N-end rule pathway, we used both steady-state and pulse-chase assays. Immunoblotting of extracts from wild-type and Tnap-Ate1^{-/-} mouse testes with antibody to a C-terminal region of Rec8 showed the presence of both the full-length endogenous Rec8 protein and its fragment. The latter species migrated, upon SDS-PAGE, at a position close to the one expected for the 15 kDa Glu^{455}-Rec8 fragment (Figs. 1C and 5A, lane 2). Strikingly, however, while this endogenous Rec8 fragment was abundant in extracts from Tnap-Ate1^{-/-} testes, it was virtually absent in wild-type extracts (Fig. 5A, lane 1 vs. lane 2).

A parsimonious interpretation of these results is that the separase-generated 15 kDa Glu^{455}-Rec8 fragment (Fig. 1C) (76) was arginylated by the Ate1 R-transferase in wild-type cells and thereafter rapidly destroyed by the “downstream” part of the Arg/N-end rule pathway, whereas in
*Tnap-Ate1*−/− cells the Glu455-Rec8 fragment was long-lived, as it could not be arginylated (Figs. 1A and 5A). Hence the virtual absence of the endogenous Glu455-Rec8 fragment in wild-type testes at steady state (Fig. 5A, lane 1) and its accumulation in *Tnap-Ate1*−/− testes (Fig. 5A, lane 2). Interestingly, the level of the full-length Rec8 protein was also considerably higher in *Tnap-Ate1*−/− testes than in wild-type ones, at equal total protein loads (Fig. 5A and Discussion).

Degradation of the Glu455-Rec8 fragment was also assayed directly, using 35S-pulse-chases and the Ub reference technique (URT), derived from the Ub fusion technique (Fig. 5C) (4,21,22,91,92).

Cotranslational cleavage of a URT-based Ub fusion by deubiquitylases that are present in all eukaryotic cells produces, at the initially equimolar ratio, both a test protein with a desired N-terminal residue and the reference protein DHFR-UbR48, a flag-tagged derivative of the mouse dihydrofolate reductase (Fig. 5C). In URT-based pulse-chase assays, the labeled test protein is quantified by measuring its levels relative to the levels of a stable reference at the same time point during a chase. In addition to being more accurate than pulse-chases without a built-in reference, URT also makes it possible to detect and measure the degradation of the test protein before the chase, i.e., during the pulse (17,91,92).

URT-based 35S-pulse-chases with C-terminally flag-tagged Glu455-Rec8f and its derivatives were performed in a transcription-translation-enabled rabbit reticulocyte extract, which contains the Arg/N-end rule pathway and has been extensively used to analyze this pathway (4,21,22). The indicated DHFR-UbR48-X455-Rec8f URT fusions (X=Glu; Val; ArgGlu) were labeled with 35S-Met/Cys in reticulocyte extract for 10 min at 30°C, followed by a chase, immunoprecipitation with a monoclonal anti-flag antibody, SDS-PAGE, autoradiography, and quantification (Fig. 5B-D). The logic of these assays involves a comparison between the degradation rates of a protein bearing a destabilizing N-terminal residue and an otherwise identical protein with an N-terminal residue such as Val, which is not recognized by the Arg/N-end rule pathway (Fig. 1A).

The Glu455-Rec8f fragment was short-lived in reticulocyte extract (t1/2 of 10-15 min) (Fig. 5B, D). Moreover, ~30% of pulse-labeled Glu455-Rec8f were degraded during the pulse, i.e., before the chase, in comparison to the otherwise identical Val455-Rec8f, which was also completely stable during the chase (Fig. 5B, D). We also constructed and examined Arg-Glu455-Rec8f.

This protein was a DNA-encoded equivalent of the posttranslationally Nt-arginylated Glu455-Rec8f fragment of Rec8. As would be expected, given the immediate (cotranslational) availability of N-terminal Arg in the DNA-encoded Arg-Glu455-Rec8f, this protein was destroyed by the Arg/N-end rule pathway even more rapidly than the already short-lived Glu455-Rec8f. Specifically, nearly 80% of Arg-Glu455-Rec8f was eliminated during the 10-min pulse (before the chase), in comparison to ~30% of Glu455-Rec8f, with the long-lived Val455-Rec8f control being a part of the reference set (Fig. 5B, D).

The results of pulse-chase analyses (Fig. 5B-D) were in agreement with other measurements, in which the synthesis-deubiquitylation-degradation of DHFR-UbR48-X-Rec8f fusions in reticulocyte extract was allowed to proceed for 1 hr, followed by detection of Glu455-Rec8f, of other test proteins, and of the DHFR-UbR48 reference protein by SDS-PAGE and immunoblotting with anti-flag antibody (Fig. 5E). In these assays, the samples were incubated in reticulocyte extract either without added dipeptides, or with 1 mM Arg-Ala (RA), bearing N-terminal Arg, a type-1 primary destabilizing residue, or with 1 mM Ala-Arg.
bearing N-terminal Ala, a residue that is not recognized by N-recognins of the Arg/N-end rule pathway (Figs. 1A and 5E).

Ubr1 and Ubr2, the two sequelogous (47% identical) and functionally overlapping 200-kDa N-recognins (E3 Ub ligases) of the mammalian Arg/N-end rule pathway, have several substrate-binding sites. These sites recognize (bind to) specific classes of N-degrons and specific internal (non-N-terminal) degrons. The two substrate-binding sites that recognize N-degrons are the type-1 site, which specifically binds to the N-terminal basic residues Arg, Lys or His, and the adjacent but distinct type-2 site, which specifically binds to the N-terminal bulky hydrophobic residues Leu, Phe, Tyr, Trp, and Ile (4,6,93,94). Dipeptides bearing, for example, type-1 N-terminal residues can competitively and selectively inhibit the binding of Ubr1/Ubr2 to a type-1 N-degron but not to a type-2 N-degron in a test protein (4,6,95-97).

In agreement with the rapid degradation of the Glu455-Rec8f fragment in 35S-pulse-chase assays (Fig. 5B-D), the synthesis-deubiquitylation-degradation of the 1DHFR-UbR48-Glu455-Rec8f fusion for 1 hr in reticulocyte extract either in the absence of added dipeptide, or in the presence of Ala-Arg (which does not bind to Ubr1/Ubr2) did not result in a detectable accumulation of the Glu455-Rec8f fragment, at its expected (roughly 15 kDa) position in SDS-PAGE-based immunoblots (Fig. 5E, lanes 8 and 10). In striking contrast, the same assay but in the presence of the Arg-Ala dipeptide (which competitively inhibits the recognition of type-1 N-degrons) resulted in a prominent protein band at the expected position of Glu455-Rec8f (Fig. 5E, lane 9 vs. lanes 8 and 10). Given the URT-based design of 1DHFR-UbR48-Glu455-Rec8f (Fig. 5C), that protein band was inferred to be the Glu455-Rec8f fragment that had been metabolically stabilized by the Arg-Ala dipeptide. In contrast, the larger Met206-Rec8f fragment, corresponding to a putative (and at most a minor) separase cleavage site in the full-length Rec8 protein, was metabolically stable irrespective of the presence or absence of the Arg-Ala dipeptide (Fig. 5E, lanes 5-7 vs. lanes 8-10). The same was true of full-length Rec8. It should also be mentioned that full-length Rec8 was not converted into a smaller fragment in reticulocyte extract, indicating (as would be expected) the absence of active separase in that extract (Fig. 5E, lanes 2-4).

DISCUSSION

The arginyltransferase (R-transferase) Ate1 is a component of the Arg/N-end rule pathway of protein degradation. Ate1 utilizes Arg-tRNA as a cosubstrate to arginylate N-terminal Asp, Glu or (oxidized) Cys of a targeted protein substrate. The resulting N-terminal Arg is recognized by E3 ubiquitin ligases (N-recognins) of the Arg/N-end rule pathway (Fig. 1A, Introduction, and references therein). In the present study, we constructed an Ate1−/− mouse strain in which the ablation of Ate1 was confined to germ cells. We used this and other experimental tools to characterize Glu455-Rec8, a specific C-terminal fragment of the kleisin-type, meiosis-specific Rec8 subunit of mouse cohesin. Rec8 is cleaved, late in meiosis I (see Introduction and references therein), by a nonprocessive protease called separase. The main C-terminal fragment of that cleavage, Glu455-Rec8, bears N-terminal Glu, a substrate of Ate1 (Fig. 1A, C). We have shown that the mouse Glu455-Rec8 fragment is a short-lived substrate of the Arg/N-end rule pathway and that the degradation of this fragment requires its Nt-arginylation by the Ate1 R-transferase (Figs. 5).

In S. cerevisiae, Sccl/Rad21/Mcd1 is the mitotic counterpart of the mammalian meiotic Rec8 subunit of cohesin. Similarly to the mouse Glu455-Rec8 fragment, the separase-generated C-terminal fragment of yeast Sccl is also a short-lived substrate of
the Arg/N-end rule pathway, and the failure to destroy this fragment in ubr1Δ cells (which lack the Arg/N-end rule pathway) results in chromosome instability (62). The separase-generated C-terminal fragment of yeast Scc1 retains, in part, the physical affinity of Scc1 for the rest of cohesin complex (62). The mouse Glu455-Rec8 fragment would also be likely to interact in vivo with the rest of meiotic cohesin. If so, the failure to arginylate the Glu455-Rec8 fragment in arginylation-lacking spermatocytes of Ate1−/− mice, and hence the failure to destroy this fragment (Figs. 1A and 5) would be expected to interfere with cohesin mechanics. This (at present hypothetical) interference would account, at least in part, for the observed arrest and apoptotic death of Ate1−/− spermatocytes at the end of meiosis I and the resulting strong decrease in the fertility of Ate1−/− males (Fig. 2C-L).

Immunoblotting analyses of the arginylation-dependent degradation of the endogenous Glu455-Rec8 fragment showed that this fragment was virtually absent, at steady state, in wild-type mouse testes but accumulated in Ate1−/− testes (Fig. 5A). Interestingly, the steady-state level of the full-length Rec8 protein was also significantly higher in Ate1−/− testes than in wild-type ones, at equal total protein loads (Fig. 5A). A plausible but unproven interpretation of this result is that the cleavage of the full-length mouse Rec8 by separase may be subject to a product-mediated inhibition of separase in Ate1−/− spermatocytes, if the product, Glu455-Rec8, is no longer eliminated by the Ate1-dependent arginylation branch of the Arg/N-end rule pathway (Figs. 1A and 5).

Although selective ablation of Ate1 in mouse germ cells nearly abrogates the fertility of male mice (Fig. 2C-E), it is unlikely that specific cases of human infertility can be caused by unconditionally null mutations of human Ate1, inasmuch as global mouse Ate1−/− mutants are late embryonic lethals (29). In addition, a post-natal ablation of mouse Ate1, in adult mice (using cre-lox technology and a ubiquitously expressed Cre recombinase), while compatible with mouse viability, causes a variety of abnormal phenotypes, including the loss of fat and hyperkinetic behavior (33). Nevertheless, partially active (hypomorphic) mutants of the human Ate1 R-transferase might underlie some, currently obscure, cases of human infertility.

The same disposition would obtain if the human Arg/N-end rule pathway would be partially inactivated “downstream” of the Ate1 R-transferase, at the level of pathway’s Ub ligases (Fig. 1A). For example, unconditional Ubr1−/− mice, lacking one of two major N-recognins, Ubr1 and Ubr2, are viable and fertile, while exhibiting some abnormal phenotypes (98). The analogous Ubr2−/− mice, lacking the second major N-recognin (it is structurally and functionally similar to Ubr1), are also viable (in some strain backgrounds) but exhibit male infertility (77). This infertility is similar to the low-fertility phenotype of Ate1−/− mice in the present study, as in both cases the infertility is caused by apoptotic death, in meiosis I, of either Ubr2−/− or Ate1−/− spermatocytes (Figs. 2C-D and 4C, D, F). A parsimonious interpretation of these results is that a failure to rapidly destroy the separase-generated Glu455-Rec8 fragment is a common mechanistic denominator of both Ate1−/− and Ubr2−/− infertility phenotypes.

No human Ubr2−/− mutants have been identified so far. In contrast, human patients with the previously characterized Johansen-Blizzard Syndrome (JBS) have been shown to be null Ubr1−/− mutants (99). It is unknown whether or not human JBS patients are fertile, in part because the overall phenotype of human JBS is more severe than analogous phenotype of Ubr1−/− mice. Abnormal phenotypes of human JBS (Ubr1−/−) patients include anatomical malformations, an
insufficiency and inflammation of the acinar pancreas, mental retardation, and deafness (4,99).

Although separase can cleave mouse Rec8 in vitro at more than one site, the cleavage between Arg-454 and Glu-455, resulting in the Glu\textsuperscript{455}-Rec8 fragment, is by far the predominant one (Fig. 1C) (76). Mammalian Rad21, the mitosis-specific counterpart of the meiotic Rec8 cohesin subunit, is also cleaved by separase, late in mitosis. Similarly to the cleavage of the meiosis-specific Rec8, the separase-mediated cleavage of the mammalian mitotic Rad21 subunit also yields the C-terminal fragment of Rad21 bearing N-terminal Glu (75). However, in contrast to the present results with the Glu\textsuperscript{455}-Rec8 fragment of meiotic Rec8 (Fig. 4B, C), our recent analyses of the N-terminal Glu-bearing mitotic Rad21 fragment using \textsuperscript{35}S-pulse-chases and the URT method (Fig. 5A) indicated that this fragment was long-lived (B.W. and A.V., unpublished data). Further analyses of these unexpected (and therefore particularly interesting) results about the apparently stable mitotic Glu-Rad21 fragment vis-à-vis the short-lived meiotic Glu\textsuperscript{455}-Rec8 fragment (Fig. 4) are under way.

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Conflict of interest

The authors declare no conflicts of interest with the contents of this article.

Author contributions

W.L. and the other coauthors of this paper designed the experiments. Y.J.L., C.L, Z.N.C., B.W., C.S.B., Z.H.S., Z.L.X., Y.L.S., W.X.L., L.N.W. and R.G.H. performed the experiments. W.L., A.V., R.G.H and W.D. wrote the paper. All authors discussed the results and commented on the manuscript.

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**The abbreviations used**

Ub, ubiquitin; R-transferase, arginyltransferase; N-degron, an N-terminal degradation signal recognized by the N-end rule pathway; N-recognin, an E3 ubiquitin ligase that can recognize at least some N-degrons.
FIGURE LEGENDS

FIGURE 1. The mammalian N-end rule pathway and the separase cleavage site in Rec8, a meiosis-specific cohesin subunit. See Introduction for references and descriptions of the pathway’s mechanistic aspects and biological functions. Amino acid residues are denoted by single-letter abbreviations. (A) The Arg/N-end rule pathway. It targets proteins for degradation through their specific unacetylated N-terminal residues. A yellow oval denotes the rest of a protein substrate. “R-transferase” is the Ate1 arginyltransferase. “Primary”, “secondary”, and “tertiary” refer to mechanistically distinct classes of destabilizing N-terminal residues. “Type 1” and “type 2” refer to two sets of primary destabilizing N-terminal residues, basic (Arg, Lys, His) and bulky hydrophobic (Leu, Phe, Trp, Tyr, Ile, and Met followed by a bulky hydrophobic residue (Φ)), respectively. These sets of N-terminal residues are recognized by two distinct substrate-binding sites of N-recognins, the pathway’s E3 ubiquitin ligases. (B) The Ac/N-end rule pathway. It targets proteins through their Nα-terminally acetylated (Nt-acetylated) residues. Red arrow on the left indicates the cotranslational removal of the N-terminal Met residue by Met-aminopeptidases (MetAPs). N-terminal Met is retained if a residue at position 2 is larger than Val. (C) Alignments of amino acid sequences near the main separase cleavage site in mammalian Rec8, between Arg$^{454}$ and Glu$^{455}$ of mouse Rec8. The consensus sequence of this cleavage site (its P4-P1 residues) in both mitotic (Rad21) and meiotic (Rec8) kleisin-type cohesin subunits is also shown. Conserved residues of mammalian Rec8 near the cleavage site are in yellow. The conserved P4, P3, P1 and P1′ residues of mammalian Rec8 at the cleavage site are in red, orange, green and blue, respectively.

FIGURE 2. Tnap-Ate1−/− mice, in which the ablation of Ate1 is confined to germ cells, are nearly infertile. (A) Immunohistochemical detection of the Ate1 R-transferase in sections of wild-type (Ate1$^{flox/flox}$) seminiferous tubules in adult mice, using affinity-purified anti-Ate1 antibody. Sub-panels on the right are enlargements of the areas demarcated by dashed rectangles on the left. Upper sub-panels: staining with primary (anti-Ate1) and secondary antibodies. Lower sub-panels: secondary antibody alone (control). Scale bars, 50 μm. (B) Dramatic decrease of the Ate1 R-transferase in the testes of Tnap-Ate1−/− mice, as determined by immunoblotting of testes extracts with anti-Ate1 antibody. (C) Decreased size of Tnap-Ate1−/− testes, in comparison to wild-type (Ate1$^{flox/flox}$) ones. (D) Pregnancy rates (%) of plugged wild-type females after matings with Tnap-Ate1−/− vs. wild-type males. (E) Average numbers of pups per all plugged wild-type testes after matings with Tnap-Ate1−/− vs. wild-type males. (F) Histological appearance (hematoxylin-eosin) of sections through epididymis of Tnap-Ate1−/− vs. wild-type males. Sub-panels on the right are enlargements of areas (indicated by dashed rectangles) on the left. Arrowheads indicate abnormal cells, absent in sections of wild-type epididymis. Scale bar, 20 μm. (G) Calculated total sperm number in epididymis of Tnap-Ate1−/− vs. wild-type males. (H) Ratio of testis mass to mouse body mass for Tnap-Ate1−/− vs. wild-type males. (I) Histological appearance (hematoxylin-eosin) of sectioned seminiferous tubules in Tnap-Ate1−/− vs. wild-type males. Scale bars, 50 μm. (J) Immunoblotting analyses, using antibodies to actin and to cyclin B1 (the latter a marker for metaphase) of extracts from Tnap-Ate1−/− vs. wild-type testes (see the main text). (K) Average diameters of seminiferous tubules in Tnap-Ate1−/− vs. wild-type testes. (L) Average numbers of cells per seminiferous tubule in Tnap-Ate1−/− vs. wild-type testes. Standard deviations are indicated in D, E, G, H, K and L (the corresponding assays were carried out in triplicate).
FIGURE 3. Knockout of the Ate1 R-transferase gene does not affect the progression of spermatocytes through meiosis I until their arrest at metaphase. (A) Similar frequencies of cells (Plzf+ cells) that could be stained with antibody to Plzf, a spermatogonia-specific marker, in testes of Tnap-Ate1−/− vs. wild-type males. Scale bars: 50 µm. (B) Symp3 (green) and Symp1 (red) proteins, the markers for synaptonemal complex, detected (using corresponding antibodies) on chromosomes of Tnap-Ate1−/− vs. wild-type spermatocytes at the pachytene stage of meiosis I. Scale bars, 5 µm. (C) Numbers of Plzf+ cells (presumptive spermatogonia) per section of a seminiferous tubule in Tnap-Ate1−/− vs. wild-type males (quantification of results in A). (D) Same as in B but for Symp3 and Mlh1 (the latter a marker for chromosome crossovers) in Tnap-Ate1−/− vs. wild-type males. Scale bars: 5 µm. (E) Numbers of prophase I (Sycp3-positive) cells per section of a seminiferous tubule in Tnap-Ate1−/− vs. wild-type males. (F) Percentages of spermatocytes at different prophase stages of meiosis I Tnap-Ate1−/− vs. wild-type males. (G) Sections of Tnap-Ate1−/− vs. wild-type testes were stained with hematoxylin/eosin. Sub-panels on the right are enlargements of the areas demarcated by dashed rectangles on the left. An arrow and arrowheads indicate anaphase and metaphase cells, respectively. Scale bars, 50 µm. (H) Percentages of spermatocytes at metaphase I of meiosis I (quantification of cell images in G). Standard deviations are indicated in C, E, F, and H (the corresponding assays were carried out in triplicate).

FIGURE 4. Increased apoptosis in seminiferous tubules of Tnap-Ate1−/− mice. (A) Spermatocytes in prophase I of meiosis I, stained with DAPI for DNA and with anti-Symp1 antibody in sections of seminiferous tubes in Tnap-Ate1−/− vs. wild-type testes. Scale bar, 50 µm. (B) Stages of meiosis I in spermatocytes of Tnap-Ate1−/− vs. wild-type testes, detected by staining chromosome spreads with anti-Symp3 antibody. Scale bar, 10 µm. (C) Percentages of seminiferous tubules containing apoptotic (TUNEL-positive) cells in Tnap-Ate1−/− vs. wild-type testes. (D) Representative TUNEL-assay patterns (stained with DAPI as well) in Tnap-Ate1−/− vs. wild-type testes. Sub-panels on the right are enlargements of areas (indicated by dashed squares) on the left. Scale bars, 50 µm. (E) Staining of testes sections from Tnap-Ate1−/− vs. wild-type testes with DAPI for DNA, and with antibody to α-tubulin for spindle bodies (see the main text). Scale bar, 50 µm. (F) Numbers of apoptotic (TUNEL-positive) cells per a section of seminiferous tubules in Tnap-Ate1−/− vs. wild-type testes. Standard deviations are indicated in C and F (the corresponding assays were carried out in triplicate).

FIGURE 5. The separase-generated C-terminal fragment of Rec8 as a short-lived substrate of the Arg/N-end rule pathway. (A) Immunoblotting of extracts from wild-type (lane 1) vs. Tnap-Ate1−/− (lane 2) mouse testes with antibody to a C-terminal region of Rec8. Note the presence of a prominent band, inferred to be the endogenous Glu455-Rec8 fragment, in Tnap-Ate1−/− but not wild-type testes. The lower panel shows the results of control immunoblots using antibody to the unrelated p97 protein. (B) Quantification of data in D. (C) The ubiquitin reference technique (URT; see the main text). (D) Lanes 1-4, 35S-pulse-chase of the C-terminally flag-tagged Glu455-Rec8f fragment of mouse Rec8, produced as the URT fusion fDHFR-UbR48-Glu455-Rec8f in reticulocyte extract (see the main text). The bands of Glu455-Rec8f and the reference protein fDHFR-UbR48 are indicated on the left. Lanes 5-8, same as in A but with the otherwise identical Val455-Rec8f, bearing N-terminal Val, which is not targeted by the Arg/N-end rule pathway. Lanes 9-12, same as in but with Arg-Glu455-Rec8f (see the main text). (E) In these assays, the synthesis-deubiquitylation-degradation of URT-based fDHFR-UbR48-X-Rec8f fusions in reticulocyte extract was allowed to proceed for 1 hr, followed by detection of Glu455-Rec8f, of
other test proteins, and of the \( ^{\text{f}}\text{DHFR-Ub}^{R48} \) reference protein by SDS-PAGE and immunoblotting with anti-flag antibody. Lane 1, control extract with the added vector plasmid (see Experimental Procedures). Lane 2, expression of the \( ^{\text{f}}\text{DHFR-Ub}^{R48} \)-Rec8\(^f\) fusion, which is processed by deubiquitylases in the extract to yield full-length Rec8\(^f\). Lane 3, same as lane 2, but in the presence of the Arg-Ala (RA) dipeptide at 1 mM. Lane 4, same as lane 3, but in the presence of the Ala-Arg (AR) dipeptide at 1 mM. Lanes 5-7, same as lanes 2-4, but expression of the \( ^{\text{f}}\text{DHFR-Ub}^{R48} \)-Met\(^{206}\)-Rec8\(^f\) fusion, which is processed in the extract to yield \( ^{\text{f}}\text{DHFR-Ub}^{R48} \) and Met\(^{206}\)-Rec8\(^f\) (see the main text). Lanes 8-10, same as lanes 5-7, but expression of the \( ^{\text{f}}\text{DHFR-Ub}^{R48} \)-Glu\(^{455}\)-Rec8\(^f\) fusion, which is processed in the extract to yield \( ^{\text{f}}\text{DHFR-Ub}^{R48} \) and the Glu\(^{455}\)-Rec8\(^f\) protein, the main product of the cleavage of full-length Rec8 by separase. Note the presence of a prominent protein band, inferred to be the 16 kDa flag-tagged Glu\(^{455}\)-Rec8\(^f\) protein, in the presence of the Arg-Ala dipeptide (lane 9) but neither in the absence of added dipeptide or in the presence of Ala-Arg (lanes 8 and 10; see also the main text). Also indicated, in panels D and E, are the molecular masses of key protein species, the C-terminally flag-tagged Rec8 fragment (16 kDa) and the N-terminally flag-tagged reference protein DHFR-Ub (33 kDa). An asterisk denotes a protein band that crossreacted with anti-flag antibody.
A. The Arg/N-end rule pathway

MetAPs, calpains, caspases, separases, other nonprocessive proteases

- Primary pathway
  - Hemin
  - Inhibition & proteolysis

- Secondary pathway
  - Nt-amidases
  - Internal degrons
  - Recognized by UBR E3s

B. The Ac/N-end rule pathway

- Ac-CoA
- Nt-acetylases
- AcM
- AcA
- AcS
- AcT
- AcV
- AcC

- MetAPs
- Very rarely Nt-acetylated
- Short peptides

C. P4-P1 consensus for Rec8 and Rad21: E X X R

- Mouse: 442 - R K T E A L S E I E V L R E A Q E P S - 460
- Rat: 392 - R K I E A L S E I E V L R E A Q E P S - 410
- Human: 392 - R K I E V P S E I E V P R E A L E P S - 410
- Chimpanzee: 442 - R K I E V P S E I E V P R E A L E P S - 460
- Cow: 430 - R K A E T P S D I E V L R E A Q E P S - 448

Fig. 1, Liu et al.
Fig. 2, Liu et al.
Fig. 5, Liu et al.
Degradation of the Separase-cleaved Rec8, a Meiotic Cohesin Subunit, by the N-end Rule Pathway
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