DEVELOPMENTAL BIOLOGY

An RNF12-USP26 amplification loop drives germ cell specification and is disrupted by disease-associated mutations

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The E3 ubiquitin ligase RNF12 plays essential roles during development, and the gene encoding it, RLIM, is mutated in the X-linked human developmental disorder Tonne-Kalscheuer syndrome (TOKAS). Substrates of RNF12 include transcriptional regulators such as the pluripotency-associated transcriptional repressor REX1. Using global quantitative proteomics in male mouse embryonic stem cells, we identified the deubiquitylase USP26 as a putative downstream target of RNF12 activity. RNF12 relieved REX1-mediated repression of Usp26, leading to an increase in USP26 abundance and the formation of RNF12-USP26 complexes. Interaction with USP26 prevented RNF12 autoubiquitylation and proteosomal degradation, thereby establishing a transcriptional feed-forward loop that amplified RNF12-dependent derepression of REX1 targets. We showed that the RNF12-USP26 axis operated specifically in mouse testes and was required for the expression of gametogenesis genes and for germ cell differentiation in vitro. Furthermore, this RNF12-USP26 axis was disrupted by RLIM and USP26 variants found in TOKAS and infertility patients, respectively. This work reveals synergy within the ubiquitylation cycle that controls a key developmental process in gametogenesis and that is disrupted in human genetic disorders.

INTRODUCTION
Ubiquitylation is a posttranslational modification that serves as a cellular control system by altering the activity, function, subcellular localization, and/or stability of protein targets (1). Depending on linkage topology, ubiquitylation can target proteins for proteosomal degradation, control signaling activity, or promote complex assembly (2). A key role of protein ubiquitylation is the regulation of developmental cell fate decisions (3–5), and hence, ubiquitylation components are frequently disrupted in developmental disorders. This is exemplified by the RING E3 ubiquitin ligase RNF12, encoded by the gene RLIM, which is mutated in the X-linked developmental disorder Tonne-Kalscheuer syndrome (TOKAS) (6–8). TOKAS affects hemizygous males and is characterized by intellectual disability and associated craniofacial abnormalities (6–8), with syndromic features including urogenital abnormalities and hypogenitalism (6–8). These data suggest key functions for RNF12 in neurological and genital development that are disrupted in TOKAS. Further reports suggest a role for RNF12 in fertility, because heterozygous female carriers of RNF12-TOKAS variants exhibit fertility defects (6) and RNF12-deficient male mice display impaired spermiogenesis (9).

RNF12 variants associated with TOKAS display impaired catalytic E3 ubiquitin ligase activity, leading to defects in substrate ubiquitylation (6, 10). Thus, a major molecular function of RNF12 that is disrupted by TOKAS variants is the regulation of gene expression by ubiquitylation and proteosomal degradation of transcriptional regulators. RNF12 has been shown to ubiquitylate the transcriptional regulators SMAD7 (11), REX1 (12), and CLIM (13), among others. As a result, RNF12 plays a key role in coordinating developmental gene expression, with functions including gene dosage compensation by imprinted X-chromosome inactivation (XCI) (14–16) and transcriptional repression of neurodevelopmental genes (17). However, beyond these notable examples, RNF12 regulation and function have not been systematically investigated at the molecular level, which may shed light on further key developmental functions that are disrupted in disease.

Here, we deployed quantitative proteomics and CRISPR (clustered regularly interspaced short palindromic repeat)–Cas9 gene editing in embryonic stem cells to systematically elucidate key molecular functions of RNF12. A major feature of RNF12-dependent proteome dynamics was the induction of the X-chromosome-encoded deubiquitylase ubiquitin specific peptidase 26 (USP26). Mechanistically, the RNF12-mediated ubiquitylation and resulting degradation of the transcriptional repressor REX1 derepressed Usp26, which drove a biochemical interaction between USP26 and RNF12. RNF12-USP26 complexing inhibited RNF12 autoubiquitylation, thereby stabilizing RNF12 to establish a feed-forward loop that amplified RNF12-dependent developmental gene expression. This RNF12-USP26 amplification loop specifically operated in testes and promoted efficient expression of genes that were associated with gametogenesis and germ cell differentiation in vitro, including Dazl (18), Usp9y (19), and Dppa3 (20). Last, we showed that the RNF12-USP26 axis was disrupted by RLIM variants from patients with TOKAS, whereas RNF12 stabilization and downstream transcriptional regulation were disrupted by USP26 gene variants identified from patients with azoospermia. Together, our results uncovered molecular synergy within the ubiquitylation cycle that controls germ cell development and is disrupted in diverse human genetic disorders.

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**RESULTS**

**RNF12 activity drives expression of USP26 by promoting the degradation of REX1**

To identify previously unappreciated molecular functions of the RNF12 signaling pathway, we performed a comprehensive mapping of RNF12-dependent proteome dynamics in male mouse embryonic stem cells (mESCs) using quantitative proteomics. Rlim−/y mESCs were reconstituted with either empty vector or wild-type (WT) mouse RNF12 (mRNF12) and relative protein abundances determined using quantitative mass spectrometry (Fig. 1A). Proof of principle for this approach was demonstrated by identification of a key RNF12 substrate, the transcriptional repressor REX1 (also known as ZFP42) (Fig. 1A), the abundance of which was suppressed by the expression of WT mRNF12 as predicted. The cohort of proteins that were significantly induced by WT mRNF12 included the X-chromosome-encoded deubiquitylase USP26 (Fig. 1A), which was reported in a previous study of RNF12 proteomics (fig. S1) (21). USP26 and RNF12 exhibit substantial functional overlap, including regulation of SMAD7 ubiquitylation (22) and pluripotency (23). USP26 is implicated in male fertility (24–28), another reported function of RNF12 (9). Thus, we hypothesized that USP26 plays a functional role in the RNF12 signaling network.

![Image](image-url)
To confirm that USP26 protein abundance was increased by endogenous RNF12 E3 ubiquitin ligase activity, we used control RNF12 WT knock-in (WT-KI) mESCs and RNF12 knock-in mESCs encoding a mutant form with impaired catalytic activity (W567Y-KI) (17). Consistent with disrupted RNF12 E3 ubiquitin ligase activity, RNF12 W567Y-KI mESCs displayed accumulation of the RNF12 substrate REX1 (Fig. 1B). USP26 protein was present in control RNF12 WT-KI mESCs, but abundance was substantially reduced in RNF12 W567Y-KI mESCs (Fig. 1B), supporting the mass spectrometry results. These data also confirm that USP26 protein was dynamically increased by RNF12 E3 ubiquitin ligase activity. Because RNF12 ubiquitylates REX1 and other developmental transcriptional regulators, we hypothesized that RNF12 E3 ubiquitin ligase activity increases the expression of Usp26. Usp26 mRNA was abundant in RNF12 WT-KI mESCs and reduced in RNF12 W567Y-KI mESCs (Fig. 1C). Therefore, RNF12 E3 ubiquitin ligase activity increased Usp26 mRNA, most likely by promoting Usp26 gene transcription.

We next addressed the mechanism by which RNF12 drives Usp26 expression. The REX1 transcriptional repressor is a major RNF12 substrate in mESCs (Fig. 1A) and plays a critical role in the initiation of imprinted XCI (12, 17, 21, 29). During imprinted XCI, RNF12-dependent ubiquitylation and degradation of REX1 relieve transcriptional repression of the long noncoding RNA Xist (12, 21). Thus, we tested whether RNF12 also promotes Usp26 expression by relieving REX1-mediated transcriptional repression. To this end, we took advantage of a series of CRISPR-Cas9 knockout mESC lines (17) comprising WT (Rlim+/y), RNF12 knockout (Rlim−/y), and RNF12;REX1 double knockout (Rlim−/y; Zfp42−/−) (Fig. 1D). As expected, RNF12 knockout led to increased REX1 protein in these cells, which was reversed by REX1 disruption (Fig. 1D). Usp26 protein was reduced by RNF12 knockout and restored in RNF12;REX1 double-knockout mESCs (Fig. 1D), suggesting that REX1 is the key RNF12 substrate in the regulation of Usp26. Usp26 mRNA followed a similar pattern (Fig. 1E), suggesting that REX1 may directly inhibit Usp26 gene transcription. In that regard, RNF12;REX1 double-knockout mESCs produced more Usp26 mRNA than did WT mESCs (Fig. 1E), consistent with REX1-mediated transcriptional repression of Usp26. Restoration of REX1 in RNF12;REX1 double-knockout mESCs reduced Usp26 protein (Fig. 1F) and Usp26 mRNA (Fig. 1G), providing evidence that REX1 is epistatic to RNF12 for Usp26 transcription. Together, these results confirm that degradation of the REX1 transcriptional repressor is a primary mechanism by which RNF12 drives Usp26 gene expression.

RNF12 and USP26 form a complex in mESCs

Our data indicate that transcriptional induction of Usp26 is a major function of the RNF12-REX1 pathway. We therefore hypothesized that USP26 plays a role in regulating RNF12-dependent ubiquitin signaling. Because mouse USP26 (mUSP26) and human USP26 (hUSP26) are poorly conserved (37% identity and 55% similarity at the amino acid level), we first explored whether mUSP26 and hUSP26 colocalize and/or interact with mRNF12. In Rlim−/y mESCs reconstituted with hemagglutinin (HA)–tagged mRNF12, FLAG-tagged mUSP26 localized to the cytoplasm with a proportion localized to the nucleus, whereas FLAG-hUSP26 and HA–mRNF12 were predominantly localized to the nucleus (Fig. 2A). Endogenous USP26 displayed a similar subcellular localization as transgenically expressed tagged mUSP26 (Fig. 2B). The specificity of endogenous USP26 immunofluorescence was confirmed using Usp26Δ/Δ mESCs generated by CRISPR-Cas9 genome editing (Fig. 2B and fig. S2, A to C). Gel filtration indicated that endogenous RNF12 and USP26 from mESCs coeluted at a predicted molecular weight of ~200 to 500 kDa (Fig. 2C) and, furthermore, cofractionated with chromatin (Fig. 2D). These data suggest that RNF12 and USP26 may form a complex in mESCs. Coimmunoprecipitation revealed that FLAG-hUSP26 specifically interacted with HA–mRNF12 (Fig. 2E). Similarly, endogenous USP26 coimmunoprecipitated with mRNF12 in knock-in mESCs in which N-terminally HA-tagged mRNF12 was expressed from the endogenous locus (Fig. 2F). These data confirm that RNF12 and USP26 colocalize and form a complex in mESCs.

To define the RNF12 motif(s) responsible for interaction with USP26, we performed coimmunoprecipitation analyses using hUSP26 and various mRNF12 deletion constructs lacking specific functional regions (Fig. 2G). The N-terminal region, the catalytic RING domain, and the nuclear export signal (NES) were dispensable for hUSP26 interaction (Fig. 2H). Deletion of the mRNF12 nuclear localization signal (NLS) or mutation of the NLS phosphorylation sites (4xS/A) disrupted the interaction with hUSP26 (Fig. 2G), presumably because these mutants were mislocalized to the cytosol and therefore segregated from nuclear hUSP26 (Fig. 3S) (17, 30). However, the mRNF12 basic region (BR) deletion mutant, which colocalized to the nucleus with hUSP26 (Fig. 3S), failed to interact with hUSP26 (Fig. 2H). We therefore conclude that USP26 formed a complex with RNF12 by interacting with the BR.

RNF12 autoubiquitylation leads to rapid proteasomal degradation

We next sought to determine the function of RNF12-USP26 complex formation. An important facet of the ubiquitin system with important regulatory consequences is “self-destruction,” in which E3 ubiquitin ligases autoubiquitylate (31). Therefore, we explored the possibility that USP26 recruitment counteracts RNF12 autoubiquitylation and proteasomal degradation. RNF12 has been reported to be autoubiquitylated (13), and previous mass spectrometry studies (32–34) suggest that RNF12 can be ubiquitylated on all six possible acceptor lysine residues in vivo (Fig. 3A; www.phosphosite.org). WT mRNF12 was ubiquitylated in mESCs, as determined by tandem ubiquitin binding element (TUBE) enrichment of ubiquitylated proteins followed by immunoblotting (Fig. 3B). Although mRNF12 ubiquitylation was not affected by mutation of four of the six potential acceptor lysine residues to arginine (K71/526/544/558R = 4xR; Fig. 3B), ubiquitylation was lost when all potential acceptor lysine residues were mutated (all K-R; Fig. 3B). Mutation of only the two N-terminal Lys residues (K9/71R = N-term 2xK-R) or the four C-terminal Lys residues (K526/544/558/561R = 4xK-R) partially disrupted mRNF12 ubiquitylation, with greater disruption observed after mutation of 4xC-terminal Lys residues (Fig. 3B). mRNF12 ubiquitylation was abolished in the catalytically inactive mutant (W567Y; Fig. 3B), consistent with previous findings that RNF12 undergoes autoubiquitylation (13).

We investigated the ubiquitin chain linkage topology associated with RNF12 autoubiquitylation and found that RNF12 autoubiquitylation generated K48-linked ubiquitin chains, which are primarily associated with targeting for proteasomal degradation (35), as determined by probing HA–mRNF12 immunoprecipitates with a K48-specific antibody (Fig. 3C) or using MUD1 ubiquitin binding elements that are selective for K48-linked ubiquitin chains (Fig. 3D) (36). Endogenous RNF12 from mESCs was also modified by...
Fig. 2. USP26 colocalizes with RNF12 and interacts with the RNF12 basic region (BR). (A) Immunofluorescence localization of FLAG-tagged human USP26 (FLAG-hUSP26) and FLAG-tagged mouse USP26 (FLAG-mUSP26) coexpressed with HA-mRNF12 in Rlim−/y mESCs. Nuclei were stained with Hoechst. Scale bar, 20 μm. Data are representative of n = 3 independent experiments. (B) Immunofluorescence localization of endogenous USP26 in Usp26+/y and Usp26Δ/y mESCs and RNF12 in Rlim+/y and Rlim−/y mESCs. Nuclei were stained with Hoechst. Scale bar, 20 μm. Data are representative of n = 2 independent experiments. (C) Immunoblotting (IB) for USP26 and RNF12 in size exclusion chromatography fractions of Rlim+/y mESC extracts. Data are representative of n = 3 independent experiments. (D) Immunoblotting for USP26 and RNF12 in Rlim+/y mESCs fractionated into soluble cytoplasmic and nucleoplasmic (TUBB3) and chromatin-associated (phospho-Ser10 histone H3, pH3) fractions. ERK1/2 is a loading control. Data are representative of n = 3 independent experiments. (E) Immunoblotting for FLAG and HA in HA immunoprecipitates (IP) from Rlim−/y mESCs expressing the indicated combinations of empty vector (−), FLAG-hUSP26, and HA-mRNF12. Data are representative of n = 3 independent experiments. (F) Immunoblotting for USP26, HA, and ERK1/2 in immunoprecipitates from endogenous HA-tagged RNF12 knock-in (HA-RNF12 KI) mESCs. Immunoprecipitation with IgG is a negative control. Data are representative of n = 3 independent experiments. (G) mRNF12 deletion mutants used for mapping the interaction with hUSP26. In the 4xSA mutant, Ser residues at positions 212, 214, 227, and 229 were mutated to Ala. (H) Immunoblotting for FLAG and HA in HA immunoprecipitates from Rlim−/y mESCs expressing empty vector (control) or FLAG-hUSP26 with the indicated HA-tagged mRNF12 deletion mutants. Data are representative of n = 3 independent experiments.
K48-linked ubiquitin chains, as confirmed by MUD1 ubiquitin binding elements (Fig. 3E). Furthermore, linkage-specific antibodies suggested that mRN12 was modified by K48-linked ubiquitin but was not substantially modified by K11- or K63-linked chains (fig. S4). However, note that RNF12 may be modified by trace K11- or K63-linked chains or other types of ubiquitin linkage. As expected, RNF12 autoubiquitylation led to proteasomal degradation, because WT mRN12 was degraded more rapidly than a nonubiquitylatable mRN12 mutant (all K-R) or an mRN12 catalytic mutant (W576Y; Fig. 3F). mRN12 all K-R retained comparable catalytic E3 ubiquitin ligase activity to WT mRN12, as determined by the ability to promote REX1 degradation (Fig. 3G). In summary, these data demonstrate the importance of RNF12 autoubiquitylation in controlling protein stability and suggest a potential function for USP26 recruitment in modulating this process.

**USP26 inhibits RNF12 autoubiquitylation and suppresses RNF12 proteasomal degradation**

Our results suggested that RNF12 specifically complexes with USP26, which we hypothesize may modulate RNF12 autoubiquitylation. To directly test this prediction, we incubated purified recombinant mRN12 and mUSP26 and performed an in vitro ubiquitylation reaction. Autoubiquitylation of mRN12 was observed in this assay, as evidenced by the appearance of mRN12 ubiquitylated bands (Fig. 4A). The addition of mUSP26 or the broad-specificity deubiquitylase hUSP2 before initiating the autoubiquitylation reaction prevented mRN12 autoubiquitylation (Fig. 4A), suggesting that USP26 may catalyze deubiquitylation of autoubiquitylated RNF12. However, in contrast to hUSP2, addition of mUSP26 after completion of the mRN12 autoubiquitylation reaction failed to remove mature ubiquitin chains (Fig. 4A). These results indicate that although USP26 inhibits RNF12 autoubiquitylation, USP26 appears to act, at least in part, either by removing nascent RNF12-linked ubiquitin chains or by interfering with RNF12 autoubiquitylation.
Fig. 4. Interaction with USP26 inhibits RNF12 autoubiquitylation and suppresses proteosomal degradation. (A) mRNF12 in vitro autoubiquitylation in the presence of GST-mUSP26, GST-hUSP2, or GST added before or after mRNF12 autoubiquitylation. RNF12 and GST proteins were detected by immunoblotting (IB), and autoubiquitylated RNF12 (RNF12-UB\(^\text{B}\)) was quantified. Data are represented as means ± SEM. Statistical significance compared to control was determined by paired t test; confidence level, 95%. (B) TUBE-mediated ubiquitin pulldown from Rlim\(^{-}\) mESCs expressing the indicated combinations of empty vector or mRNF12 WT and FLAG-mRNF12 WT or H569A/C572A. FLAG and ubiquitin were detected by immunoblotting. ERK1/2 is a loading control. (C) FLAG immunoprecipitates (IP) from Rlim\(^{-}\) mESCs expressing the indicated combinations of empty vector (control), FLAG-mRNF12 WT and HA-mRNF12 WT (1-600), Δ1-206 (ΔN), Δ206-226 (ΔNLS), Δ502-513 (ΔNES), Δ546-587 (ΔRING), Δ326-423 (ΔBR), or Δ212/214/227/229A (4xSA). FLAG, HA, and ERK1/2 were detected by immunoblotting. (D) Rlim\(^{-}\) mESCs expressing empty vector (control) or FLAG-hUSP26 and HA-mRNF12 WT and treated with cycloheximide (CHX) for the indicated times. FLAG, HA, and ERK1/2 were detected by immunoblotting and quantified. Data are represented as means ± SEM. Statistical significance compared to control was determined by paired t test; confidence level, 95%. (E) Rlim\(^{-}\) mESCs expressing HA-mRNF12 plus empty vector (control) or FLAG-hUSP2, hUSP26, hUSP29, or mUSP38 and treated with CHX for the indicated times. FLAG-HA, -RNF12, and ERK1/2 were detected by immunoblotting and quantified. Data are represented as means ± SEM. Statistical significance compared to control was determined by paired t test; confidence level, 95%. (F) HA immunoprecipitates from Rlim\(^{-}\) mESCs expressing the indicated combinations of empty vector (−), FLAG-hUSP29, and HA-mRNF12. FLAG, HA, and ERK1/2 were detected by immunoblotting. For all panels, data are representative of n = 3 independent experiments.

We next explored the mechanisms by which USP26 might interfere with RNF12 autoubiquitylation. Because RING E3 ubiquitin ligases frequently self-associate and autoubiquitylate in trans, we hypothesized that USP26 recruitment to RNF12 may disrupt this process. We previously showed that hRNF12 self-associates (10), which prompted us to determine whether RNF12 autoubiquitylates in trans. FLAG-mRNF12 WT was autoubiquitylated when expressed in RNF12 knockout (Rlim\(^{-}\)) mESCs with or without untagged mRNF12 (Fig. 4B). When a FLAG-mRNF12 catalytic mutant (H569A/C572A) was expressed alone, no autoubiquitylation was detected (Fig. 4B), but when untagged WT mRNF12 was expressed with FLAG-mRNF12 H569A/C572A, the FLAG-mRNF12 H569A/C572A catalytic mutant was ubiquitylated (Fig. 4B). RNF12 ubiquitylation was largely dependent on its own catalytic activity, confirming that RNF12 can autoubiquitylate in trans, but we cannot rule out the possibility that RNF12 is also capable of autoubiquitylation in cis. Given that USP26 inhibited RNF12 autoubiquitylation and that RNF12 self-association facilitated its autoubiquitylation, we investigated whether USP26 might interfere with RNF12 self-association. We therefore defined the regions of RNF12 that were required for self-association by performing coimmunoprecipitation experiments with FLAG-tagged full-length mRNF12 and various HA-tagged deletion mutants (Fig. 4C). mRNF12 self-association was mediated by the BR, which was also required for the interaction with hUSP26 (Fig. 2H). This suggests that the interaction...
with USP26 may sterically hinder RNF12 self-association, thereby preventing autoubiquitylation. This is in addition to the potential for direct reversal of RNF12 autoubiquitylation by the intrinsic de-ubiquitylase activity of USP26.

Because RNF12 autoubiquitylation consists of K48-linked degradative ubiquitin chains that drive proteasomal degradation, we investigated whether inhibition of RNF12 autoubiquitylation by USP26 promoted RNF12 stabilization in cells. To this end, we reconstituted RNF12 signaling in Rlimfl/y mESCs, which lack endogenous RNF12 and produce little USP26 (Fig. 1D), by selectively expressing HA-mRNF12 and/or FLAG-hUSP26. We confirmed that HA-mRNF12 had a relatively short half-life in mESCs due to autoubiquitylation and proteasomal degradation (Fig. 4D). However, expression of FLAG-hUSP26 stabilized HA-mRNF12 over a 4-hour time course (Fig. 4D). This depended on proteasomal degradation, because mRNF12 was stabilized by hUSP26 or by the proteasome inhibitor MG132 (Fig. S5A). RNF12 stabilization was specific to hUSP26 and its close relative hUSP29 (Fig. 4E), because expression of the more distantly related mUSP38 or hUSP2 did not stabilize RNF12 in mESCs (Fig. 4E). Furthermore, similar to hUSP26, hUSP29 was also capable of interacting with mRNF12 (Fig. 4F), consistent with its effect on RNF12 stability. In these experiments, hUSP26 was expressed at amounts similar to endogenous mUSP26 (Fig. S5B; compare USP26 staining for endogenous and transgenically expressed mUSP26 and FLAG staining for mUSP26 and hUSP26; note that FLAG-hUSP26 is not detected by mUSP26 antibody), and hUSP26, hUSP29, and mUSP38 all colocalized with mRNF12 in the nucleus (Fig. S5C). However, although hUSP2 is a broad-specificity deubiquitylase that deubiquitylates RNF12 in vitro (Fig. 4A), hUSP2 and mRNF12 did not colocalize in the nucleus (Fig. S5C), which explains why hUSP2 did not stabilize mRNF12 in cells (Fig. 4E). These data indicate that USP26 and the closely related USP29 specifically suppress RNF12 autoubiquitylation and proteasomal degradation. Collectively, our results thus far reveal that Usp26 is transcriptionally induced by RNF12-dependent degradation of REX1, leading to the formation of an RNF12-USP26 complex that stabilizes RNF12.

The RNF12-USP26 signaling axis operates specifically in the testes

We next investigated the physiological function of the RNF12-USP26 signaling axis. RNF12 is required for normal sperm development (9), a process in which USP26 has also been implicated (24–28). Furthermore, USP26 variants have been associated with azosper- mia in various fertility disorders, including Sertoli cell–only syndrome (24, 25, 37). To explore the possibility that RNF12-USP26 signaling may contribute to sperm development, we first sought to confirm the tissue distribution of RNF12 and USP26. As shown previously in mouse tissues, RNF12 protein was most abundant in adult brain, lung, and testes (17), whereas USP26 was largely restricted to mESCs and testes in a selection of somatic tissues (Fig. 5A) (24). USP26 was observed in primary spermatocytes and spermatids and spermatogonia, the precursors of spermatocytes and spermatids, in the testes of control adult RlimY/Y mice. USP26 was reduced in primary spermatocytes and spermatids of adult RlimY/Y mice but unchanged in spermatogonia (Fig. 5B). This suggests that USP26 abundance does not depend on the RNF12-REX1 axis in spermatogenic stem cells but does depend on RNF12-REX1 in their progeny. These data confirm that the RNF12-USP26 signaling axis operates specifically in the mouse testes, raising the possibility that it may play an important function in this context.

RNF12-REX1-USP26 signaling regulates the expression of gametogenesis genes

To identify potential molecular functions of the RNF12-USP26 axis that may be relevant to the biology of male reproduction and fertility, we analyzed our previously reported RNA sequencing (RNA-seq) dataset for RNF12-dependent mRNAs (17). Among mRNAs that were induced by WT mRNF12 in mESCs (Fig. 5C), Gene Ontology (GO) term analysis identified enrichment of transcripts relating to metabolism, development, and differentiation (see data file S1 for a full list). However, the RNF12-dependent transcriptional signature in mESCs was also significantly enriched for genes associated with reproduction and gametogenesis (Fig. 5C, data file S1, and table S1). This included Usp26 itself, Dazl, which encodes an RNA binding protein that is essential for gametogenesis in both males and females (18), Usp9y, a Y-linked gene encoding a deubiquitylase associated with male infertility (19), and Dppa3, a primordial germ cell–specific protein (20) (Fig. 5C). These data suggest that the RNF12-USP26 axis may function to promote the expression of genes that are required for gametogenesis and germ cell development, consistent with a functional role for the RNF12-USP26 axis in the testes.

These data prompted us to explore the mechanism by which RNF12 controls the expression of genes associated with gametogenesis. Initially, we sought to confirm that Dazl, Usp9y, and Dppa3 were induced by RNF12 E3 ubiquitin ligase activity. We again used control RNF12 WT–K1 and W567Y-K1 mESCs (17), in which endogenous RNF12 substrates, including REX1, accumulate (Fig. 1B). As expected, Dazl, Usp9y, and Dppa3 were expressed in control RNF12 WT–K1 mESCs but reduced in RNF12 W567Y-K1 mESCs (Fig. 5D), confirming that RNF12 E3 ubiquitin ligase activity is critical for the expression of these genes. We then determined whether REX1 was the relevant RNF12 substrate driving the expression of key gametogenesis genes. Similar to Usp26 (Fig. 1E), Dazl, Usp9y, and Dppa3 mRNAs were suppressed by loss of RNF12 (RlimΔ/Δ) but restored in RNF12;REX1 double-knockout mESCs (RlimΔ/Δ;Zfp42Δ/Δ) (Fig. 5E). Furthermore, REX1 chromatin immunoprecipitation DNA sequencing (ChIP-seq) data indicated that REX1 was directly recruited to the Usp26 promoter (Fig. 5F) and the promoters of other RNF12-REX1–dependent gametogenesis genes (fig. S6). Together, our results indicate that RNF12, by ubiquitylating and thereby promoting degradation of the transcriptional repressor REX1, promotes a gene expression program that is associated with gametogenesis.

We next set out to address whether endogenous USP26 also promotes expression of gametogenesis genes. Because USP26 is produced in only a small percentage of mESCs when cultured in leukemia inhibitory factor–fetal calf serum (LIF-FCS) (fig. S5B), we used mESCs cultured under 2i conditions, in the presence of inhibitors of the kinases mitogen-activated protein kinase kinase 1 (MEK1) and MEK2 (MEK1/2) and glycogen synthase kinase 3, which maintain them in the naïve “ground state.” To explore the function of endogenous USP26 in gametogenesis gene expression, we used Usp26α/β mESCs (fig. S2, A to C) and cultured the cells under 2i conditions. In this context, reduction of USP26 (Usp26α/β) (Fig. 5G; note the residual USP26 expression in Usp26α/β mESCs) or loss of RNF12...
Fig. 5. The RNF12-REX1-USP26 signaling axis operates in testes and promotes expression of germ cell–specific genes. (A) RNF12, USP26, DAZL, and DPPA3 were detected in the indicated mouse tissues by immunoblotting (IB). Actin is a loading control. Data are representative of n = 3 independent experiments. (B) USP26 immunohistochemistry of mouse testes sections from Rlim+/y and Rlim−/− littermates. Scale bar, 100 μm. Data are representative of n = 2 animals of each genotype. (C) RNA sequencing (RNA-seq) data from Rlim−/− mESCs expressing empty vector (control) or mRNF12 WT (17). RNAs were ranked according to fold change increase in expression. The magnified view of the boxed area shows RNAs for which expression increased >2-fold in Rlim−/− mESCs expressing mRNF12, with RNAs implicated in reproduction or germ cell development highlighted. (D and E) Quantification of Usp9y, Dazl, and Dppa3 mRNAs in RNF12 WT-KI and W576Y-KI mESCs (D) and in Rlim−/−, Rlim+/y, and Rlim−/−; Zfp42−/− mESCs (E) by qRT-PCR. Data are represented as means ± SEM. n = 3 independent experiments. Statistical significance was determined by paired t test; confidence level, 95%. (F) REX1 and control chromatin immunoprecipitation followed by DNA sequencing peaks for the Usp26 gene. REX1–enriched regions near the transcriptional start site are highlighted. RefSeq, NCBI Reference Sequence database. (G) Usp26+/y and Usp26−/− mESCs were cultured under 2i conditions. USP26 and ERK1/2 (loading control) were detected by immunoblotting, and Dazl and Dppa3 mRNAs were quantified by qRT-PCR. Data are represented as means ± SEM. n = 3 independent experiments. Statistical significance was determined by paired t test; confidence level, 95%. (H) Quantification of the indicated mRNAs in Rlim+/y and Rlim−/− mESCs by qRT-PCR. Data are represented as means ± SEM. n = 3 independent experiments. Statistical significance was determined by paired t test; confidence level, 95%.
(Rlim<sup>−/+y</sup>) (Fig. 5H) led to reduction in Dazl and Dppa3 mRNAs. Therefore, these data indicate that endogenous USP26 is required for efficient expression of RNF12-dependent gametogenesis genes.

**RNF12 signaling promotes differentiation of mouse primordial germ cell–like cells**

Our results thus far indicated that the RNF12-USP26 axis specifically operates in the testes and promotes the expression of genes associated with gametogenesis. Thus, we sought to address the function of the RNF12 pathway in germ cell development in vitro. To this end, we used an established protocol for differentiation of primordial germ cell–like cells (PGCLCs) from mESCs (38) in which 2i mESCs are differentiated first to epiblast-like cells (EpiLCs) and then to PGCLCs (Fig. 6A). mESCs efficiently differentiated to PGCLCs by this method, as assessed by the induction of PGCLC markers Dazl, Dppa3, Blimp1, and Prdm14 (Fig. 6B). Usp26 expression was also induced during PGCLC differentiation (Fig. 6B). RNF12 knockout reduced the efficiency of PGCLC differentiation (Fig. 6C), whereas RNF12;REX1 double knockout restored the induction of PGCLC markers (Fig. 6D). Usp26 expression was largely dependent on RNF12 in these experiments (Fig. 6, C and D), confirming that the RNF12-USP26 axis is operational in the context of PGCLC differentiation. In summary, our data indicate that the RNF12 pathway plays a crucial role in promoting germ cell differentiation in vitro.

**Disrupted RNF12-USP26 signaling contributes to TOKAS and azoospermia**

Last, we investigated whether the RNF12-USP26 axis is dysregulated in human disease. Variants in RLM that disrupt RNF12 E3 ubiquitin ligase activity cause TOKAS, a developmental disorder characterized by intellectual disability and syndromic anomalies including urogenital defects with hypogonadism in affected males and fertility defects in heterozygous carrier females (6). To determine the impact of a TOKAS-associated RNF12 variant on the regulation and function of the RNF12-USP26 axis, we took advantage of knock-in mESCs harboring the mouse equivalent of the human RNF12 R599C variant identified from a TOKAS patient kindred (RNF12 R575C-KI) (10). Compared to control RNF12 knock-in mESCs (RNF12 WT-KI), RNF12 R575C-KI mESCs displayed impaired RNF12 E3 ubiquitin ligase activity and REX1 accumulation (Fig. 7A) (10). Accordingly, RNF12 R575C-KI mESCs displayed reduced USP26 (Fig. 7A), suggesting that RNF12 TOKAS variants may interfere with Usp26 gene expression. Usp26 mRNA was diminished...
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azoospermia variants result in amino acid substitutions largely the RNF12-USP26 axis and gametogenesis gene expression. Patients with azoospermia may also lead to deregulation of the RNF12-USP26 axis is likely impaired in patients harboring a disease-causing variant of RNF12. USP26 variants identified from azoospermia, USP catalytic domain (Fig. 7C), with one variant implicated in the USP catalytic domain (Fig. 7C), with one variant implicated in the

USP26 variants (Fig. 7C) to investigate the effects of those amino acid substitutions on USP26 and RNF12 function. In most cases, hUSP26 variants exhibited reduced protein abundance when clustered around a nuclear localization signal and within the USP catalytic domain (Fig. 7C), with one variant implicated in the disruption of catalytic activity (39). Thus, we prioritized a panel of RNF12, REX1, and USP26 in control RNF12 WT knock-in (WT-KI) and RNF12 TOKAS mutant knock-in (R57SC-KI) mESCs were detected by immunoblotting (IB) and quantified. ERK1/2 is a loading control. Asterisk indicates a nonspecific band. Data are represented as means ± SEM. Statistical significance was determined by paired t test; confidence level, 95%. (B) Quantification of the indicated mRNAs in RNF12 WT-KI and R57SC-KI mESCs by qRT-PCR. Data are represented as means ± SEM. Statistical significance was determined by paired t test; confidence level, 95%. (C) hUSP26 showing variants associated with azoospermia. Prioritized frequently reported variants used for subsequent experiments are highlighted in red. (D) Immunoblotting for FLAG in Rlim−/y mESCs expressing empty vector or increasing amounts of FLAG-hUSP26 WT or the indicated azoospermia variants. Data are represented as means ± SEM. Statistical significance was determined by paired t test; confidence level, 95%. (E) Representative immunofluorescence images of HA-mRNF12 and FLAG-hUSP26 in Rlim−/y mESCs expressing HA-mRNF12 and FLAG-hUSP26 WT or indicated azoospermia variants. Nuclei were stained with Hoechst. Scale bar, 10 μm. (F) Rlim−/y mESCs expressing HA-mRNF12 and either empty vector or FLAG-hUSP26 WT or L364F were treated with cycloheximide (CHX) for the indicated times. FLAG, HA, and ERK1/2 were detected by immunoblotting. Quantification shows 2-hour samples only. Data are represented as means ± SEM. Statistical significance was determined by paired t test; confidence level, 95%. (G) Quantification of the indicated mRNAs in Rlim−/y mESCs expressing HA-mRNF12 and either empty vector or FLAG-hUSP26 WT or L364F by qRT-PCR. Data are represented as means ± SEM. Statistical significance was determined by paired t test; confidence level, 95%. For all panels, n = 3 independent experiments.

in RNF12 R57SC-KI mESCs compared to control RNF12 WT-KI mESCs (Fig 7B). RNF12 R57SC-KI mESCs also displayed reduced Usp9y, Dazl, and Dppa3 mRNAs (Fig. 7B). Our data therefore demonstrate that an RNF12 TOKAS variant disrupts expression of Usp26 and other gametogenesis genes, suggesting that the RNF12-USP26 axis is likely impaired in patients harboring a disease-causing variant of RNF12.

USP26 gene variants have been implicated in the azoospermia, such as that associated with Sertoli cell–only syndrome (24, 25, 37). We therefore hypothesized that USP26 variants identified from patients with azoospermia may also lead to deregulation of the RNF12-USP26 axis and gametogenesis gene expression. USP26 azoospermia variants result in amino acid substitutions largely...
expressed in mESCs (Fig. 7D). However, all hUSP26 variants tested, including those surrounding the NLS, localized correctly to the nucleus (Fig. 7E). These data suggest that reduced protein abundance may be a pathogenic mechanism in fertility patients harboring USP26 variants.

Our finding that USP26 gene variants associated with fertility defects reduced protein expression in mESCs suggests that these variants may affect RNF12 deubiquitylation and stabilization. We addressed this possibility using USP26 L364F, a variant within the USP catalytic domain (Fig. 7C) that was very poorly expressed in mESCs (Fig. 7D). As shown previously, mRNF12 was rapidly degraded as a result of autoubiquitylation but was significantly stabilized by expression of WT hUSP26 compared to control (Fig. 7F). When expressed in equivalent amount to WT hUSP26, hUSP26 L364F also stabilized RNF12 to some extent, but the variant’s stabilization of mRNF12 was not statistically significant compared to control cells not expressing hUSP26 (Fig. 7F), indicating that USP26 azoospermia variants may interfere with USP26 function in preventing RNF12 autoubiquitylation and proteasomal degradation. We found that WT hUSP26 was a relatively long-lived protein, whereas the hUSP26 L364F variant was rapidly degraded (Fig. 7F), suggesting that impaired stability may explain the reduced protein amounts observed for USP26 azoospermia variants.

Last, we explored the impact of the azoospermia-associated hUSP26 L364F variant on RNF12-dependent regulation of gametogenesis gene expression. Expression of WT hUSP26 resulted in increased expression of Usp26, Usp9y, Dazl, and Dppa3 (Fig. 7G), whereas induction of these genes by the azoospermia-associated USP26 L364F variant was not significantly different from control samples (Fig. 7G). Together, our data indicate that the RNF12-USP26 axis promotes gametogenesis gene expression, and this is functionally disrupted by RLIM and USP26 variants associated with TOKAS and azoospermia, respectively.

**DISCUSSION**

Ubiquitylation is critical for regulating many developmental processes, and hence, ubiquitylation components are mutationally disrupted in human developmental disorders. Here, we uncovered a ubiquitylation axis involving the RING-type E3 ubiquitin ligase RNF12 and the deubiquitylase USP26 that controls gametogenesis gene expression. RNF12 E3 ubiquitin ligase activity promoted Usp26 gene transcription and an increase in USP26 protein, which stimulated complex formation between RNF12 and USP26, resulting in RNF12 stabilization. This system created a feed-forward amplification loop that drove RNF12-dependent signaling and downstream transcription. Furthermore, we provided evidence that the amplification loop is of critical importance in human disease, because it is disrupted by RLIM and USP26 variants that cause human genetic disorders. Thus, our results provide detailed molecular insight into the complex interplay within the ubiquitin system in the regulation of developmental processes and how this is dysregulated in disease (Fig. 8). Of particular interest is the fact that an E3 ubiquitin ligase used transcriptional induction of a deubiquitylase to amplify its own function, which is an unprecedented molecular mechanism for activation of a ubiquitin signaling pathway. However, this fits with previous understanding of how cell fate decisions are executed and reinforced during development, which frequently involves amplification and negative feedback loops within the signaling and transcriptional machinery to confer robust cellular decision-making (40).

A key question that remains to be answered is the molecular mechanism by which USP26 stabilizes RNF12. Our data indicate that USP26 is recruited to RNF12, which may directly catalyze deubiquitylation and stabilization. However, the fact that USP26 engages RNF12 within the BR that is involved both in catalysis and self-association for autoubiquitylation suggests that USP26 might function by a more complex mechanism whereby it disrupts RNF12 self-association—and potentially also RNF12 catalytic activity—to prevent autoubiquitylation. This is supported by our demonstration that recombinant USP26 inhibited RNF12 autoubiquitylation in vitro only when added before the autoubiquitylation reaction, suggesting that USP26 may operate independently of RNF12 catalytic activity, at least in part. Alternatively, USP26 may be able to remove only simple ubiquitin modifications as they are added to RNF12 and not the complex ubiquitin chains observed after the RNF12 autoubiquitylation reaction. This question can be resolved by the development of USP26 catalytically defective mutants and recombinant USP26 with verified deubiquitylase activity, which has thus far proven elusive.

Last, we showed that the RNF12-USP26 axis operated specifically in the testes and was disrupted in human genetic disorders associated with genital abnormalities and/or infertility. In this regard, USP26 variants are found in patients with azoosperma, and RLIM variants cause TOKAS, which is characterized by syndromic features including urogenital abnormalities in affected males and fertility problems in carrier females. However, the specific role of the RNF12-USP26 axis in gametogenesis and fertility in vivo is yet to be determined. For example, it is not yet clear whether affected male
patients with TOKAS exhibit fertility defects, although recent research indicates that male RNF12 knockout (Rlim−/−) mice have a defect in spermiogenesis (9). Similarly, the role of USP26 in gametogenesis has not yet been defined, and there are conflicting data about whether USP26 is required for male fertility in mouse models (24–28). In this case, it appears likely that USP29, which is also present in emerging USP26 is required for male fertility in mouse models (28).

**MATERIALS AND METHODS**

**Cell culture**

Male mESCs (CCE line) were originally from the laboratory of Janet Rossant, SickKids Research Institute, Toronto. These are authenticated by pluripotent embryonic stem cell marker expression analysis and confirmed mycoplasma negative. mESCs were cultured in ES-DMEM (Dulbecco’s Modified Eagle Medium) medium [DMEM base, 5% knockout serum replacement (KSR) (v/v), 2 mM glutamine, 0.1 mM minimum essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, penicillin (50 U/ml), streptomycin (50 µg/ml), 10% FCS (v/v), and 0.1 mM β-mercaptoethanol; supplemented with leukemia inhibitory factor (LIF) (20 ng/ml; Medical Research Council Protein Phosphorylation and Ubiquitin Unit Reagents and Services (MRC-PPU R&S)) on plates coated with 0.1% gelatin at 5% CO2 and 37°C.

**PGCLC differentiation**

mESCs were cultured in 2i medium consisting of N2B27 medium with 1% (v/v) B27 supplement, 0.5% (v/v) N2 supplement, 2 mM glutamine (all from Thermo Fisher Scientific), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and penicillin and streptomycin in 1:1 DMEM F12:neurobasal medium (both from Thermo Fisher Scientific) supplemented with a cocktail of inhibitors (0.4 µM PD0325901 and 3 µM CHIR99021, Axon) and LIF (20 ng/ml; MRC-PPU R&S). EpilPC differentiation was induced by plating 1 × 10^5 2i mESCs per well in a 0.1% gelatin-coated 12-well plate. EpiLCs were kept in 2i medium consisting of N2B27 medium supplemented with activin A (20 ng/ml) and basic fibroblast growth factor (12 ng/ml) (both from PeproTech) and 1% KSR (Thermo Fisher Scientific) at 5% CO2 and 37°C for 2 days. EpiLCs were differentiated into PGCLCs by plating 2 × 10^5 EpiLCs per well in a low–cell binding U-bottom 96-well plate in serum-free medium [GK15 medium consisting of GMEM (Glasgow Minimal Essential Medium; Thermo Fisher Scientific) with 15% KSR, 0.1 mM non-essential amino acids (NEAA), 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 2 mM l-glutamine] in the presence of the cytokines bone morphogenetic protein 4 (BMP4) (500 ng/ml; R&D Systems), LIF (1000 U/ml; Merck Millipore), stem cell factor (100 ng/ml; R&D Systems), and epidermal growth factor (50 ng/ml; R&D Systems). After 3 days in culture, PGCLC clusters were harvested for RNA extraction or immunofluorescence.

**Transfection and plasmids**

mESCs were transfected using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer’s instructions. pCAGGS puro plasmids (table S2) were generated by the MRC-PPU R&S and verified by DNA sequencing (MRC-PPU DNA Sequencing Service) using Applied Biosystems BigDye version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. All cDNA clones can be found at the MRC-PPU R&S website (http://mrcppureagents.dundee.ac.uk/).

**Pharmacological inhibition**

Small-molecule inhibitors and compounds used are listed in table S2. MG132 and cycloheximide treatments were at a final concentration of 10 and 350 µM, respectively.

**CRISPR-Cas9 genome editing**

Genome editing was conducted using CRISPR-Cas9 system (41). Rlim−/−, RNF12 WT-KI and R575C-KI (10), RNF2 W576Y-KI, and Rlim−/−; Zfp42−/− double-knockout (17) mESCs were described previously using guide RNA sequences summarized in table S2. For knock-in experiments, a third vector containing the donor DNA sequence followed by an internal ribosomal entry site–enhanced green fluorescent protein cassette was coexpressed (table S2). CRISPR-Cas9 plasmid DNAs were transfected into mESCs using Lipofectamine (LTX) according to the manufacturer’s instructions and cultured for 24 hours. Transfected mESCs were selected with puromycin (3 µg/ml) for 48 hours and then subjected into single-cell sorting using a fluorescence-activated cell sorting instrument into gelatinized 96-well cell culture plates. Candidate clones were analyzed by immunoblotting and genomic DNA sequencing to confirm gene editing. Sequencing was carried out by immunoblotting and genomic DNA sequencing service. Endogenous N-terminal HA-tagged RNF12 WT-KI (HA-RNF12 WT-KI) mESCs were generated from RNF12 WT-KI (RNF12 WT-KI) (17) using single-stranded oligodeoxyribonucleotide (ssODN) CRISPR. One microgram of DU 69536 plasmid (guide RNA sequence: GAGAATCTGAGTTCCATCT) and 1 µl of 100 µM HA-RNF12 WT-KI ssODN donor (TAAAGGTACGC AATGCAATAATGCGTCTGTTTTCAATTTG – GTCTTTTTGCTTTTTAGATAATTTTCCCATTGACCAAGATGCTACCCATACGACGTcCCAGATTACGCTGAGAATCTGACGTACTCAATCTACAGTACAAGAAATAGCTACAGTCTCGAGCTCAGCGCAAGAAGTGGACAGCTGC GCTTTGACGATGCGGAGAGG) were transfected into mESCs using Lipofectamine (LTX).

**Immunofluorescence**

For mESCs cultured in LIF-FCS, 2 × 10^5 cells/cm^2 were plated into 12-well plates containing a single gelatin-coated coverslip per well. For transfected mESCs, this procedure was conducted 24 hours after transfection. Cells were left to attach for 24 hours, medium was removed, and cells were washed twice with phosphate-buffered saline (PBS). Cells were then fixed to coverslips using 1% paraformaldehyde (PFA), diluted in PBS, and incubated for 20 min at room temperature in the dark. For PGCLCs, cell clusters were dissociated using trypsin-EDTA (Gibco) and washed twice with PBS. Cells were then fixed to coverslips in suspension using 4% (w/v) PFA, diluted in PBS, and incubated for 20 min at room temperature in the dark. After 15 min, fixation cells were transferred to poly-L-lysine (Sigma-Aldrich)–treated coverslips and centrifuged for the remaining 5 min into the coverslip.

Fixed cells were then washed three times with PBS and permeabilized in 0.5% Triton X-100 (w/v) diluted in PBS for 5 min at room temperature. Permeabilized cells were blocked using 1% (w/v) fish gelatin diluted in PBS for at least 30 min at room temperature inside a humid chamber. Cells were then stained with primary antibody.
oxycholate, 10 mM \(-\text{glycerophosphate}, 10 \text{mM sodium pyrophosphate}, 150 \text{mM NaCl}, 1 \text{mM EDTA}, 1\% \text{(v/v) NP-40, 0.5}\% \text{(w/v) sodium deoxycholate, 10 mM } \beta\text{-glycero-}

Table S2). These experiments were performed as described previously (9).

Protein extraction
mESCs were harvested using lysis buffer [20 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 10 mM \(\beta\)-glycero-phosphate, 10 mM sodium pyrophosphate, 1 mM NaF, 2 mM Na\(_3\)VO\(_4\), and cOmplete Protease Inhibitor Cocktail Tablets (0.1 U/ml); Roche]. Mouse organs were harvested from 19-week-old C57BL/6J mice, snap-frozen in liquid nitrogen, re-suspended in lysis buffer, and lysed using a Polytron PT 1200 E homogeniser (Kinematica AG). Mouse studies were previously approved by the University of Dundee ethical review committee and further sub- jected to approved study plans by the Named Veterinary Surgeon and Compliance Officer (N. Dennison) and performed under a U.K. Home Office project license in accordance with the Animal Scientific Procedures Act (1986). Protein concentration of protein extracts was deter- mined using the bicinechonic acid (BCA) Protein Assay Kit (Pierce) according to the manufacturer’s directions. Protein concentration was calculated using a standard bovine serum albumin protein curve.

Immunoprecipitation
For RNF12 immunoprecipitation, 20 \(\mu\)l of protein G agarose beads (MRC-PU R&S) were washed three times in lysis buffer and incubated with 1 mg of mESC lysate and 2 \(\mu\)g of RNF12 antibody (table S2) overnight at 4°C. For FLAG- or HA-tag pulldowns, 10 \(\mu\)l of pre-coupled FLAG- M2 agarose, 20 \(\mu\)l of HA-separaseo beads (MRC-PU R&S), or 10 \(\mu\)l of Pierce anti-HA magnetic beads or immunoglobulin G (IgG) coupled to protein G magnetic beads (Thermo Fisher Scientific) was washed three times with lysis buffer and incubated with 1 mg of mESC lysate overnight at 4°C. Modified haloalkane dehalogenase (HALO)–tagged TUBE (HALO-TUBE) beads and HALO- MUD1 beads were produced as described (42). One milligram of mESC lysate was mixed with 40 \(\mu\)l of HALO-TUBE or HALO-MUD1 beads and incubated overnight at 4°C on a rotating wheel. In all cases, beads were washed three times with lysis buffer containing 500 mM NaCl. At each step, beads were centrifuged at 2000 rpm for 2 min or separated using a magnetic stand, and the supernatant was discarded. Last, proteins bound to the beads were eluted by the addition of LDS sample buffer and boiling the mixture for 5 min at 95°C.

Size exclusion chromatography
Size exclusion chromatography (SEC) running buffer [50 mM tris-HCl (pH 7.5), 10% glycerol (v/v), 150 mM NaCl, and 1 mM dithiothreitol (DTT)] was freshly prepared and degassed by passing through a 0.45-\(\mu\)m polyvinylidine difluoride (PVDF) filter (Millipore). Twenty-four hours before use, an AKTA pure protein purification instrument was equilibrated using SEC running buffer. A single confluent 15-cm plate of mESCs was harvested in SEC cell collection buffer (1 mM EGTA and 1 mM EDTA in PBS). Detached cells were collect- ed and centrifuged at 1000 rpm for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 5 volumes of lysis buffer [20 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40 (v/v), 0.5% sodium deoxycholate (w/v), 10 mM \(\beta\)-glycero-phosphate, 10 mM sodium pyrophosphate, 1 mM NaF, 2 mM Na\(_3\)VO\(_4\), and cOmplete Protease Inhibitor Cocktail Tablets (0.1 U/ml); Roche] and incubated for 15 min on ice. After lysis, mixture was subjected to centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was then collected and passed through a 0.45-\(\mu\)m filter. Last, clarified sample was mixed with Gel Filtration Standards (molecular weight range, 670 to 1.35 kDa; #1511901, Bio-Rad) and injected into an AKTA pure protein purification instrument loaded with a Superose 6 column through which a standard SEC protocol was run and 30 fractions were collected per sample.

Chromatin fractionation
For fractionation of soluble and chromatin-associated proteins, mESCs were lysed in CSK buffer [0.5% Triton X-100, 10 mM Hepes (pH 7.4), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl\(_2\), 1 mM EGTA, 5 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1 mM sodium pyrophosphate, 1 mM \(\beta\)-glycero-phosphate, and cOmplete Protease Inhibitor Cocktail Tablets (0.1 U/ml); Roche] and incubated on ice for 5 min. Samples were centrifuged at 13,000 rpm for 5 min, and the supernatant (soluble fraction) was saved. The pellet was washed three times with CSK buffer and resuspended in NaCl buffer [0.1% Triton X-100, 50 mM tris (pH 7.4), 250 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1 mM sodium pyrophosphate, 1 mM \(\beta\)-glycero-phosphate, and cOmplete Protease Inhibitor Cocktail Tablets (0.1 U/ml); Roche] with Benzonase (1:500; Sigma-Aldrich, E1014-5KU). Samples were incubated on ice for 30 min and resuspended every 10 min. Samples were then centrifuged at 13,000 rpm for 15 min, and the supernatant (chromatin fraction) was saved. Soluble fraction was centrifuged again before analysis to remove any chromatin contamination.

Immunoblotting
NuPAGE 4 to 12% SDS–polyacrylamide gel electrophoresis gels were transferred onto PVDF membrane and incubated with primary antibody (table S2) diluted in 5% (w/v) nonfat milk buffer in TBS-T overnight at 4°C. After incubation, membranes were washed three times with TBS-T buffer [20 mM tris-HCl (pH 7.5) and 150 mM NaCl supplemented with 0.2% (v/v) Tween 20 (Sigma-Aldrich)] and incubated for 1 hour with secondary horseradish peroxidase (HRP)–conjugated antibodies (table S2) at room temperature. Last, membranes were washed three times with TBS-T and subjected to chemiluminescence detection with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) using a Gel Doc XR+ System (Bio-Rad). Acquired images were analyzed and quantified using Image Lab software (Bio-Rad).

RNF12 in vitro autoubiquitylation assays
Two versions of the mRNF12 autoubiquitylation assay were per- formed. RNF12 (140 nM) was incubated with 500 nM glutathione S-transferase (GST)–mUSP26, GST–hUSP2, or GST for 1 hour at
4°C. The reaction was started by adding a mix containing 0.1 μM UBE1, 0.05 μM UBE2D1, 2 μM FLAG-ubiquitin, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) (pH 7.5) and 5 mM adenosine triphosphate (ATP) (both from Sigma-Aldrich), 50 mM tris-HCl (pH 7.5), and 5 mM MgCl₂ and then incubated for 1 hour at 30°C. Either RNF12 or ATP was omitted in control samples. Reactions were stopped by adding SDS sample buffer and boiled for 5 min. This version is labeled “before” in Fig. 4A. Alternatively, 140 nM RNF12, 0.1 μM UBE1, 0.05 μM UBE2D1, 2 μM FLAG-ubiquitin, 0.5 mM TCEP (pH 7.5) and 5 mM ATP (both from Sigma-Aldrich), 50 mM tris-HCl (pH 7.5), and 5 mM MgCl₂ were mixed and incubated for 1 hour at 30°C. Either RNF12 or ATP was omitted in control samples. After incubation, ATP was depleted with apyrase (4.5 U/ml; New England Biolabs) for 10 min at room temperature. Last, 500 nM GST-mUSP26, GST-hUSP2, or GST was added, and the reaction was incubated for a further 1 hour at 4°C. Reactions were stopped by adding SDS sample buffer and boiled for 5 min. This version is labeled “after” in Fig. 4A. Samples were loaded on 4 to 12% bis-tris gradient gels (Thermo Fisher Scientific) and analyzed by immunoblotting.

RNA extraction and quantitative reverse transcription–polymerase chain reaction
mESCs were seeded in six-well plates and left to grow for 24 to 48 hours until confluent. RNA was extracted using the E.Z.N.A. MicroElute Total RNA Kit (Omega Bio-Tek) according to the manufacturer’s instructions. RNA samples were subjected to reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. Quantitative polymerase chain reaction (qPCR) primer sequences were identified from the PrimerBank database (https://pga.mgh.harvard.edu/primerbank) or designed using Primer3 software with a melting temperature between 58° and 62°C. For each primer, the integrity and specificity were confirmed in silico by National Center for Biotechnology Information Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast). All primers are 20 to 24 bases long with an overlap of 7 bases at the intron/exon boundary producing an amplicon of 100 to 300 bases. Primers were supplied by Invitrogen or Thermo Fisher Scientific according to availability. qPCR primer sequences are listed in table S2. qPCR reactions were carried out using SsoFast EvaGreen Supermix (Bio-Rad) or TB Green Premix Ex Taq (Takara). Each sample consisted of a 10-μl reaction containing 1 μl of cDNA with 400 nM forward and reverse primers, 5 μl of SYBR Green, and nuclelease-free water. Each sample was prepared in technical duplicate. qPCR and absorbance detection were carried out in a CFX384 real-time PCR system (Bio-Rad). The ΔΔCt method, also known as the Pfaffl method (43), was used to calculate relative RNA abundance with Gapdh expression as a loading control. Data were analyzed in Excel software and plotted in GraphPad Prism software v8.00.

Protein preparation for quantitative proteomic profiling
mESCs were washed in PBS and then lysed in 8.5 M urea and 50 mM ammonium bicarbonate (pH 8.0) supplemented with protease inhibitors. Lysate was sonicated using Biosonicator operated at 50% power for 30 s on/off each on ice water bath for 5 min. The lysates were then centrifuged at 14,000 rpm for 10 min at 4°C, and supernatants were collected. Protein concentration of the lysate was determined by BCA protein assay. Proteins were reduced with 5 mM DTT at 55°C for 30 min and cooled to room temperature. Reduced lysates were then alkylated with 10 mM iodoacetamide at room temperature for 30 min in the dark. The alkylation reaction was quenched by the addition of another 5 mM DTT. After 20 min of incubation at room temperature, the lysate was digested using endoprotease Lys-C with the weight ratio of 1:200 (enzyme:lysate) at 37°C for 4 hours. The samples were further diluted to 1.5 M urea with 50 mM ammonium bicarbonate (pH 8.0), and the sequencing-grade trypsin was added with the weight ratio of 1:50 (enzyme:lysate) and incubated overnight at 37°C. The digest was acidified to pH 3.0 by addition of trifluoroacetic acid (TFA) to 0.2% and gently mix at room temperature for 15 min; the resulting precipitates were removed by centrifugation at 7100 relative centrifugal force (RCF) for 15 min. The acidified lysate was then desalted using a C18 SPE cartridge (Waters), and the eluate was aliquoted into 100 μg and dried by vacuum centrifugation. To check the digest, 1 μg of each sample was analyzed by mass spectrometry before tandem mass tag (TMT) labeling.

One hundred micrograms of peptide from each sample was re-suspended into 100 μM triethylammonium bicarbonate buffer (pH 8.5). Then, 0.8 μg of TMT tag (Thermo Fisher Scientific) dissolved in 41 μl of anhydrous acetonitrile was transferred to the peptide sample and incubated for 60 min at room temperature. The TMT labeling reaction was quenched with 5% hydroxyamine. One microgram of each labeled sample was analyzed by mass spectrometry to assess the labeling efficiency before pooling. After checking the labeling efficiency, the TMT-labeled peptides were mixed together and dried by vacuum centrifugation. After dryness, the mixture of TMT-labeled peptides was dissolved into 0.2% TFA and then desalted using a C18 SPE cartridge. The desalted peptides were subjected to orthogonal basic pH reverse phase fractionation, collected in a 96-well plate, and consolidated for a total of 20 fractions for vacuum dryness.

LC-MS/MS, data processing, and analysis
Each fraction was dissolved in 0.1% formic acid (FA) and quantified by NanoDrop. One microgram of peptide was loaded on C18 trap column at a flow rate of 5 μl/min. Peptide separations were performed over EASY-Spray column (C18, 2 μm, 75 mm by 50 cm) with an integrated nano electrospray emitter at a flow rate of 300 nI/min. The liquid chromatography (LC) separations were performed with a Thermo Dionex Ultimate 3000 RSLC Nano LC instrument. Peptides were separated with a 180-min segmented gradient as follows: 7 to 25% buffer B [80% acetonitrile (ACN)/0.1% FA] for 125 min, 25 to 35% buffer B for 30 min, 35 to 99% buffer B for 5 min, followed by a 5-min 99% wash and 15-min equilibration with buffer A (0.1% FA).

Data acquisition on the Orbitrap Fusion Tribrid platform with instrument control software version 3.0 was carried out using a data-dependent method with multithread synchronous precursor selection MS3 scanning for TMT-9plex tags. The mass spectrometer was operated in data-dependent most intense precursors Top Speed mode with 3 s per cycle. The survey scan was acquired from mass/charge ratio (m/z) 375 to 1500 with a resolution of 120,000 resolving power with an automatic gain control (AGC) target of 400,000. The maximum injection time for full scan was set to 60 ms. For the tandem mass spectrometry (MS/MS) analysis, monoisotopic precursor selection was set to peptide. AGC target was set to 50,000 with the maximum injection time 120 ms. Charge states unknown and 1 or higher than 7 were excluded. The MS/MS analyses were performed.
by 1.2 m/z isolation with the quadrupole, normalized higher collisional dissociation (HCD) collision energy of 37%, and analysis of fragment ions in the Orbitrap using 15,000 resolving power with auto normal range scan starting from m/z 110. Dynamic exclusion was set to 60 s. For the MS3 scan, the MS3 precursor population from MS2 scan ranging from m/z 300 to 100 was isolated using the SPS waveform and then fragmented by HCD. The HCD normalized collision energy was set to 65. The MS3 scan was acquired from m/z 100 to 500 with a resolution of 50,000 and an AGC target of 50,000. The maximum injection time for full scan was set to 86 ms.

Data from the Orbitrap Fusion were processed using Proteome Discoverer software (version 2.2). MS2 spectra were searched using Mascot against a UniProt Mouse database appended to a list of common contaminants (10,090 total sequences). The searching parameters were specified as trypsin enzyme, two missed cleavages allowed, minimum peptide length of 6, precursor mass tolerance of 20 ppm, and a fragment mass tolerance of 0.05 daltons. Oxidation of methionine and TMT at lysine and peptide N termini was set as variable modifications. Carbamidomethylation of cysteine was set as a fixed modification. Peptide spectral match error rates were determined using the target-decoy strategy coupled to Percolator modeling of positive and false matches. Data were filtered at the peptide spectral match level to control for false discoveries using a q value cutoff of 0.01, as determined by Percolator. For quantification, the signal-to-noise values higher than 10 for unique and razor peptides were summed within each TMT channel, and each channel was normalized with total peptide amount. Quantitation was further performed by adjusting the calculated P values according to Benjamini-Hochberg. The significance regulated proteins with P values less than 0.05 were further manually investigated with the SDs of biological replicates.

For comparison of this RNF12 quantitative proteomic dataset with that of Gontan et al. (21), proteins with >1.5-fold increase and t test significance P < 0.05 in RNF12-expressing Rlim−/− mESCs compared to control Rlim−/− mESCs (this dataset) were intersected with proteins with >1.5-fold increase in Rlim−/− mESCs (21). Significance P value was not considered as a threshold for data from (21) to include the positive control REX1. Data were intersected using Venny (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

RNA-seq and GO term analysis
RNA-seq data for Rlim−/− mESCs expressing either empty vector or RNF12 WT were described previously (17) [see Gene Expression Omnibus (GEO) accession GSE149554]. Reads were aligned using STAR (Spliced Transcripts Alignment to a Reference) software. Differential gene expression was estimated using DESeq2 package, and further statistical analysis and plot generation were performed with the SARTools R package. GO analysis was carried out using the GO stat R package.

ChIP-seq analysis
Chip-seq data analyzed were from (12), GEO accession number GSM892958. The quality of sequencing was analyzed using FastQC software. The sequences were aligned to the mm10 reference genome using Bowtie 2 with standard settings (44). PCR duplicates were removed with Picard (http://broadinstitute.github.io/picard/). The reads from each group of samples were merged to call peaks using MACS2 software (45). Peak finding was performed by applying broad peak calling parameters. For basic annotation of identified peaks, the HOMER function annotatePeaks.pl was used (46).

Data analysis
Data were presented as means ± SEM with individual points representing a single biological replicate. In qPCR experiments, two technical replicates were run per sample and averaged. Immunofluorescence images were processed using ImageJ (Image) and Photoshop CS5.1 (Adobe) software. The percentage of cells expressing a certain protein was calculated as the ratio between cells positive for protein expression (containing antibody fluorescence) and the total number of cells (containing DNA fluorescence provided by Hoechst stain). Parameters were quantified using Fiji (Image) software. Graphs were created using GraphPad Prism software. In all cases, statistical significance was determined through analysis of variance followed by Tukey's post hoc test or Student's t test using GraphPad Prism software, and significant differences were considered when P < 0.05.

SUPPLEMENTARY MATERIALS
www.science.org/doi/10.1122/scisignal.abbm5995
Figs. S1 to S6
Tables S1 and S2
Data file S1

View/request a protocol for this paper from Bio-protocol.

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