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Rent1, a trans-effector of nonsense-mediated mRNA decay, is essential for mammalian embryonic viability

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The ability to detect and degrade transcripts that lack full coding potential is ubiquitous but non-essential in lower eukaryotes, leaving in question the evolutionary basis for complete maintenance of this function. One hypothesis holds that nonsense-mediated RNA decay (NMD) protects the organism by preventing the translation of truncated peptides with dominant negative or deleterious gain-of-function potential. All organisms studied to date that are competent for NMD express a structural homolog of Saccharomyces cerevisiae Upf1p. We have now explored the consequences of loss of NMD function in vertebrates through targeted disruption of the Rent1 gene in murine embryonic stem cells which encodes a mammalian ortholog of Upf1p. Mice heterozygous for the targeted allele showed no apparent phenotypic abnormalities but homozygosity was never observed, demonstrating that Rent1 is essential for embryonic viability. Homozygous targeted embryos show complete loss of NMD and are viable in the pre-implantation period, but resorb shortly after implantation. Furthermore, Rent1–/– blastocysts isolated at 3.5 days post-coitum undergo apoptosis in culture following a brief phase of cellular expansion. These data suggest that NMD is essential for mammalian cellular viability and support a critical role for the pathway in the regulated expression of selected physiologic transcripts.

INTRODUCTION

All eukaryotes studied to date possess the ability to recognize and degrade transcripts that harbor premature signals for translational termination. Nonsense and frameshift mutations which generate premature termination codons (PTCs) are estimated to be responsible for one-third of inherited genetic disorders and several forms of cancer. The ubiquitous nature of nonsense-mediated mRNA decay (NMD) may manifest the evolutionary pressure to protect organisms from aberrant proteins with dominant negative or gain-of-function activities that could be expressed from nonsense alleles if the corresponding transcripts were stable. Indeed, the importance of NMD in modulating the phenotypic severity of several diseases is well documented (1–5). Recent data suggest that the NMD pathway may also play a broad role in the regulated expression of selected physiological transcripts (6–9).

Three trans-acting factors, termed Upf1p, Upf2p and Upf3p, have been shown to be essential for NMD in Saccharomyces cerevisiae (10,11–13). All known functional domains of Upf1p are located in the central region of the protein which shares ~58% amino acid identity with its mammalian (rent1), Caenorhabditis elegans (SMG2) and Schizosaccharomyces pombe (Upf1p) homologs (10,14–17). Overexpression of a mutant form of rent1 in mammalian cells causes a relative stabilization of nonsense transcripts (18). Moreover, deletion of SMG2 in C. elegans (17,19) or Upf1p in S. pombe (our unpublished data), molecules as structurally divergent from Upf1p as rent1, results in complete stabilization of nonsense mRNAs. These data provide evidence that rent1 contributes to nonsense surveillance and perhaps to other genetically separable functions that have been attributed to Upf1p in budding yeast. Mutations in the cysteine/histidine-rich region impair the efficiency of translational termination (nonsense suppression) whereas mutations in the helicase domain inhibit the decay of nonsense transcripts (20,21).

Saccharomyces cerevisiae strains with complete loss of Upf1p function show a minor respiratory defect but are viable under laboratory conditions (15,22). NMD-deficient strains of C. elegans show minor morphologic abnormalities of genitalia and reduced brood sizes, but are otherwise viable (19,23). In order to assess the phenotypic consequences of loss of NMD function in mammals, we used homologous recombination to target disruption of expression of the Rent1 gene in murine embryonic stem (ES) cells. Remarkably, we show that rent1 is essential for the viability of murine embryos at the time of implantation. Homozygous-targeted pre-implantation embryos show a complete loss of NMD and cannot be maintained in

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of the targeted allele was observed at the expected frequency, and used to generate male chimeric mice. Germline transmission targeted ES cell lines were identified by Southern blotting (Fig. 1A). The blot was sequentially hybridized with probes specific for the loss of a Rent1 recombiantion. Correctly targeted neomycin-resistant ES cells were identified by EcoRI genomic DNA from heterozygous ES cells. The 3′ probe recognizes an endogenous 6.3 kb and a targeted 8.7 kb band in EcoRI-digested genomic DNA from heterozygous ES cells. The 3′ probe recognizes an endogenous Rent1-I-digested genomic DNA from heterozygous (E) and targeted (T) 5.5 kb band in EcoRI-digested genomic DNA from heterozygous cells. (B) Northern blot analysis of 13.5 d.p.c. embryos with indicate restriction fragments. The blot was sequentially hybridized with probes specific for Rent1, Neo' and G3PDH transcripts and levels were quantified. Two additional Rent1-containing transcripts were unique to the cell lines containing the targeted allele. Both isoforms also hybridized to the Neo' probe suggesting that Rent1–Neo' chimeric transcripts are being produced.

Figure 1. Targeted disruption of Rent1. (A) The Rent1 targeting vector was constructed by subcloning an 8.2 kb BamHI genomic Rent1 fragment into pBluescript/SKII+. A 1.9 kb region of Rent1 consisting of exons 4–6 was then replaced by a Neomycin resistance (Neo') expression cassette. This resulted in the loss of a BclI site and the introduction of an EcoRI site. The targeting vector was introduced into ES cells and allowed to undergo homologous recombination. Correctly targeted neomycin-resistant ES cells were identified by Southern blotting using Rent1 probes located 5′ and 3′ of the targeting vector. Shown are Southern blots of ES cell DNA. The 5′ probe recognizes an endogenous (E) 10.8 kb and a targeted (T) 5.5 kb band in EcoRI-digested genomic DNA from heterozygous ES cells. The 3′ probe recognizes an endogenous Rent1 gene. The mps nonsense allele of Gus encodes a transcript that is degraded by NMD.

RESULTS

Targeted disruption of Rent1 in murine ES cells

The Rent1 targeting vector was fashioned by replacing a 1.9 kb restriction fragment which spans exons 4–6 with a Neomycin-resistance (Neo') expression cassette (Fig. 1A). Correctly targeted ES cell lines were identified by Southern blotting (Fig. 1A) and used to generate male chimeric mice. Germline transmission of the targeted allele was observed at the expected frequency, with half of the agouti offspring demonstrating diagnostic restriction fragments.

Northern blot analysis of murine fetal fibroblast cell lines revealed that the steady-state level of endogenous full-length Rent1 message was reduced by 50% in heterozygous targeted cells (Fig. 1B). Two aberrantly sized Rent1 transcripts were unique to cell lines harboring the targeted allele and hybridized to a probe containing Neo' sequence. Sequencing of RT–PCR amplicons documented the presence of chimeric transcripts and also revealed the production of transcripts that skipped the Neo' cassette, joining exon 3 to exon 8 (data not shown). All transcripts derived from the targeted allele contain a termination codon just downstream of exon 3, precluding translation of any of the putative functional domains of rent1.

Absence of cellular or clinical phenotype in heterozygous targeted mice

Heterozygous targeted mice showed no apparent phenotypic abnormalities. They are fertile and have a normal lifespan. The Rent1 targeted allele was introduced into the gusmps+ mouse strain by breeding. This line carries a PTC in β-glucuronidase transcripts that have previously been shown to be substrates for NMD (24,25). Fetal fibroblast cell lines were established from 13.5 day post-coitum (d.p.c.) embryos produced by mating a gusmps/– mouse to a Rent1+/–, gusmps/– mouse. Northern blot analysis revealed that steady-state levels of the PTC-containing transcript were equally low in Rent1+/– and wild-type cells (Fig. 2), documenting that a half-normal level of rent1 is sufficient to fully support the NMD pathway.

Rent1 is essential for early embryonic viability

Heterozygous animals were mated to generate homozygous targeted offspring. Rent1+/– and Rent1–/– animals were produced in the expected 1:2 ratio. However, Rent1–/– mice were never observed (P < 0.0005) documenting that rent1 is essential for embryonic viability (Fig. 3A). Rent1 is expressed in unfertilized oocytes, ES cells and at least by 7.5 d.p.c. (Fig. 3B). Staged embryos (3.5–14.5 d.p.c.) derived from the crossing of Rent1–/– mice were genotyped to determine the timing of embryonic lethality. Pre-implantation blastocysts (3.5 d.p.c.) were harvested and maintained in culture medium for 2 days before DNA was isolated and analyzed by PCR for the presence of the targeted allele.
of the targeted allele. Resulting genotypes revealed that wild-type, heterozygous and homozygous targeted embryos were represented with the expected 1:2:1 ratio (Fig. 4). Homozygosity for the targeted allele was under-represented at 6.5 d.p.c. and was never observed at or after 7.5 d.p.c. (Fig. 4A). Histopathologic analysis revealed that 30% (6/20) of 5.5 d.p.c. embryos resulting from the mating of heterozygous targeted mice showed complete loss of architecture with predominance of pyknotic cells showing karyorrhexis and karyolysis (Fig. 4B). An empty decidua was seen at 28% (13/47) of implantation sites at 7.0–8.0 d.p.c. These data demonstrate that loss of Rent1 function is incompatible with embryonic development beyond the immediate implantation stage.

**Rent1 is required for NMD**

Overexpression of a form of rent1 harboring a single amino acid substitution in the helicase domain of the protein in mammalian cells resulted in a relative (~2-fold) stabilization of nonsense transcripts (18). Although this mutation is predicted to confer dominant negative activity, a gain-of-function mechanism for the modest accumulation of nonsense transcripts could not be excluded. Allele-specific oligonucleotide hybridization (ASO) analysis of amplified cDNA derived from 3.5 d.p.c. embryos with defined Rent1 genotypes was used to determine the relative abundance of wild-type and nonsense β-glucuronidase transcripts in gus<sup>neo<sup>+</sup></sup> embryos. Both Rent1<sup>+/+</sup> and Rent1<sup>+/−</sup> embryos showed a striking excess of wild-type Gus transcript that was not observed in the samples derived from the Rent1<sup>−/−</sup> embryos (Fig. 5). These data confirm that Rent1 is essential for NMD in mammalian cells.

**Rent1 may be essential for cellular viability**

In an attempt to establish a homozygous Rent1<sup>−/−</sup> ES cell line, blastocysts from heterozygous matings were harvested at 3.5 d.p.c. and maintained in culture medium with or without supplementation with leukemia inhibitory factor (LIF). Within 24 h the embryos had hatched from the zona pellucida and trophoblasts had attached to the plate. After 48 h in culture an inner cell mass was evident and size differences between colonies of various genotypes were not substantial. However, by 72 h the inner cell mass of Rent1<sup>−/−</sup> colonies showed progressive regression to the point where few cells remained after 5 days in culture (Fig. 6). Trophoblasts showed rapid regression beginning at ~96 h in culture. TUNEL analysis revealed occasional apoptotic cells within or overlying the inner cell mass of cultured Rent1<sup>+/+</sup> or Rent1<sup>+/−</sup> blastocysts. In contrast, Rent1<sup>−/−</sup> cultures showed a potent induction of apoptosis that was uniform throughout the regressing inner cell mass (Fig. 6). Our attempts to generate a homozygous targeted ES cell line through incubation of heterozygous ES cells in increased concentrations of neomycin were unsuccessful. A total of 95 clonal colonies exhibiting enhanced
resistance were screened by Southern analysis; Rent1<sup>−/−</sup> lines were never observed. Using the same methodology in other gene-targeting experiments, the frequency of homozygosity for the targeted allele has ranged between 24 and 88% (26,27). In conjunction with the observation that rent1 message is expressed in unfertilized oocytes, early embryos and cultured ES cells (Fig. 4B and data not shown), these data suggest that rent1 and NMD are critical to basic mammalian cellular viability. In this view, cellular lethality may initiate in homozygous targeted embryos once the pool of oocyte-derived rent1 transcripts is exhausted.

**DISCUSSION**

Despite the ubiquitous nature of NMD, its physiologic purpose and even selected aspects of its basic mechanism remain obscure. Several studies have shown NMD to be a potent modifier of the phenotypic consequences of nonsense mutations (6–10). This phenomenon has been well studied in *C.elegans* where heterozygous nonsense mutations in several genes are recessive when NMD is ongoing but confer a dominant phenotype when the pathway is inactivated (28). Recent studies in *S.cerevisiae* have provided new insight into the biological significance of NMD, documenting that loss of UPF gene function is associated with a significant change (generally increase) in the expression of ~8% of yeast genes (14). The vast majority of expression changes manifest alteration in transcriptional activity rather than message stability, suggesting that transcripts encoding transcription factors with broad influence may be among the relatively few physiologic substrates for NMD. Identification of these direct targets will ultimately allow appreciation of features that initiate transcript recruitment into a pathway that recognizes and degrades nonsense mRNAs. It has been hypothesized that short upstream open reading frames, decreased fidelity in the selection of translation initiation site or programmed frameshifting signals may allow the intermittent (perhaps regulated) detection of apparent premature termination signals by the nonsense surveillance machinery (29,30). Alternatively, the major role for NMD may relate to the clearing of a constant burden of PTC-containing transcripts that result from inefficient, alternative or faulty RNA processing events during transcription or splicing.

It is interesting to speculate why loss of Upf1p function and NMD is tolerated by lower eukaryotes, but the analogous function appears important for basic cellular viability in mammals. It is formally possible that rent1 has functions beyond those served by Upf1p, although the extreme evolutionary conservation of protein structure makes this less likely. A more plausible explanation is that lethality relates to either alteration in the expression level of a physiologic transcript essential for mammalian cellular metabolism or an increased burden of faulty transcripts in higher eukaryotes due to the increased number of genes, increased number of introns or increased complexity of RNA processing. Recently it has been proposed that pharmacologic manipulation of the efficiency of NMD may hold promise for the treatment of selected diseases caused by nonsense alleles (31–36). In one scenario, transcript stabilization would allow the generation of truncated proteins or readthrough products with sufficient function for phenotypic rescue. Such treatment strategies may be limited by the need to preserve selected essential functions of rent1 and the NMD pathway.

**MATERIALS AND METHODS**

**ES cell targeting and generation of chimeric mice**

A 15.5 kb murine *Rent1* genomic clone was isolated from a 129/SV mouse genomic library (Stratagene) using a radiolabeled human *Rent1* cDNA fragment as a probe. The targeting vector was constructed by cloning a 8.2 kb *BamHI* restriction fragment of this clone into pBluescript II/SK<sup>+</sup> (Stratagene). A central 1.9 kb *HindIII* restriction fragment spanning exons 4–6 was excised and replaced with a 1.1 kb Neo<sup>+</sup> expression cassette.

The targeting vector was linearized and electroporated into RW4 ES cells (Genome Systems). Neo<sup>+</sup> colonies were isolated and expanded. DNA was prepared from these colonies and screened for homologous recombination by Southern blotting. Correct 5′ end recombination was confirmed by probing...


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EcoRI-digested DNA with a radiolabeled 1 kb BamHI–SacI genomic Rent1 fragment adjacent to sequence at the 5’ end of the targeting vector. Correct 3’ end recombination was confirmed by probing BrII-digested DNA with a radiolabeled 1.8 kb BamHI–SacI genomic Rent1 fragment adjacent to sequence at the 3’ end of the targeting vector.

Correctly targeted ES cells were used to generate chimeric mice using standard procedures (Genome Systems). Male chimeric mice were mated to C57BL/6 females (Jackson Laboratories). Agouti offspring were screened for germine transmission of the targeted allele by southern blotting. DNA was prepared from tail snips (37) and analyzed as above. Targeted mice were mated onto a C57BL/6 background.

In an attempt to generate homozygous targeted ES cells, heterozygous lines were incubated in increasing concentrations of Neomycin, including 2 mg/ml (70 resistant colonies genotyped), 4 mg/ml (15 colonies) and 6 mg/ml (10 colonies).

**Generation of fetal fibroblast cell lines and northern blot analysis**

Mice heterozygous for the gus<sup>mpv</sup> mutation (B6.C-H2<sup>bml</sup>/ByBirgus<sup>mpv</sup>) were purchased from Jackson Laboratories and mated to targeted Rent1 heterozygotes to produce double heterozygotes. A Rent<sup>1+/–</sup>-gus<sup>mpv</sup> mouse was mated to a gus<sup>mpv</sup> mouse. At 13.5 d.p.c., the female was sacrificed and cells were prepared from embryos. After removing the head and internal organs, embryos were rinsed with Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL) and minced with a scalpel. Two milliliters of 1 mg collagenase/ml Hank’s balanced salt solution (HBSS) (Gibco BRL) was added and the mixture was incubated at 37°C for 60 min. The collagenase was inactivated by addition of 2 ml of DMEM, 20% fetal bovine serum (FBS) (HyClone). The tissue was passed through a 20 gauge needle several times prior to centrifugation for 15 min at 500 g. The cells were resuspended in 10 ml of high glucose DMEM (Gibco BRL), 20% FBS and plated in a 10 cm dish. Cultures were maintained at 37°C in 5% CO<sub>2</sub>.

DNA was isolated from cultured cells (37) and used for genotyping. Rent1 genotypes were determined by Southern blot analysis as described above. Gus genotypes were determined by acrylamide gel electrophoresis separation of PCR products as previously described (25).

Total RNA was isolated from cultured cells using Trizol Reagent (Gibco BRL) according to the manufacturer’s instructions. Poly(A) RNA was isolated from total RNA using Oligotex mRNA midi kit (Qiagen) according to the manufacturer’s instructions. RNA was quantified using a Beckman DU640 spectrophotometer.

For northern blot analysis, 2 μg of poly(A) RNA was electrophoresed through a 1% agarose/formaldehyde gel and transferred to GeneScreen Plus membrane (NEN). Northern blotting was performed using ExpressHyb Solution (Clontech) according to the manufacturer’s instructions. Probes were radiolabeled with [α-<sup>32</sup>P]ATP (NEN) using the Random Primed DNA Labeling kit (Boehringer Mannheim). The 213 bp cDNA probe used to detect the Rent<sup>1</sup> transcript was generated by PCR of mouse fibroblast total RNA using the Access RT–PCR System (Promega) with sense primer, 5′-CAGAGGGCATCTTGCAAA-3′, and antisense primer, 5′-AGTATTTCCCTGCCATTTG-3′. The probe used to detect the β-glucuronidase transcript was a 700 bp XhoI–HindIII fragment isolated from the pXbNaN plasmid containing mouse β-glucuronidase cDNA (obtained from M. Sands). The probe used to detect the Neo<sup>i</sup> transcript was generated by PCR using the sense primer, 5′-ATGATTTGAAAGATGATTTGCAG-3′, and the anti-sense primer, 5′-TCAGAGAAACCTCAGACGAGGC-3′. The probe used to detect the G3PDH transcript was the human G3PDH cDNA Control probe (Clontech). Signal intensities were quantified using a Packard Instant Imager.

Developmental expression analysis by RT–PCR and northern analysis utilized primers, probes and conditions as described above. Oocytes were isolated from superovulated female mice and cleared of visible cumulus cells by repeat passage through a narrow bore needle. The northern blot was purchased from Clontech.

**Embryo analysis and PCR genotyping**

Matings between heterozygous animals were timed and pregnant females were sacrificed at various days post-coitum. Embryos were dissected from the uterus and DNA was prepared (37). Rent1 genotypes were determined by PCR. The first round of 30 cycles of PCR utilized a common Rent1 sense primer, 5′-GCAAGCTCTTTCGATAGAGACCCAG-3′, a Rent1 antisense primer, 5′-GTCGGCCCTTCCAGGATG-3′, and a Neo<sup>i</sup> specific antisense primer, 5′-CCACACTGTCGACATTTGGGTG-3′. If a second round of PCR was required to visualize products, the nested primers employed were a Rent<sup>1</sup> sense primer, 5′-GTTGTAGTICGTTGAAACAAGCGGAGG-3′, a Rent1 antisense primer, 5′-CAGCAGCGTCTCCGCAAGAGGC-3′, and a Neo<sup>i</sup> specific sense primer, 5′-GAAATTTCCAGGCTTGTTGGTG-3′. PCR products were electrophoresed through 1% agarose, 2% NuSieve (FMC) gels and visualized by ethidium bromide staining.

Histopathologic analysis was performed on serial sections of uterine horns derived from staged pregnancies. Tissues were fixed in 2.0% paraformaldehyde and stained with hematoxylin and eosin (H&E).

**Pre-implantation embryo isolation and culture**

Timed matings of heterozygous animals were arranged and females were sacrificed at 3.5 d.p.c. Uterine horns were flushed with M2 medium (38) and blastocysts were rinsed with M2 medium and plated onto 1% gelatin-coated 96-well plates. Embryonic cultures were maintained in high glucose DMEM, 15% FBS in the presence or absence of LIF at 37°C in 5% CO<sub>2</sub>. DNA was prepared from the cells in the following manner. Cells were rinsed in HBSS, lysed in 20 μl of 200 mM KOH, heated to 65°C for 15 min then neutralized with an equal volume of 200 mM Tricine. Ten microliters of the DNA was used in a 50 μl PCR reaction using the three primers described above. If necessary, a nested PCR reaction was performed as above.

**ASO analysis**

ASO analysis was performed as previously described (39). PCR of Gus genomic DNA and cDNA utilized antisense primer, 5′-CAGCTGTGGCTGAATCACC-3′, and sense primer, 5′-GCAAAAGGACCTCAGACGAGG-3′. Different sense primers were used for genomic DNA (5′-CCTGTGTCACTTGCAAC-3′) and cDNA (5′-ACCCACACCAAAAGCCCTTTGG-3′) amplification. The ASOs for wild-type and mutant
sequence were 5′-GGCCCCGTACCGTGATG-3′ and 5′-GGCCCCGTACCGTGATG-3′, respectively. Final wash conditions for the wild-type probe were 2× SSPE, 0.1% SDS at 61°C. Final wash conditions for the mutant probe were 1.4× SSPE, 0.1% SDS at 60°C.

Apoptosis assay

Blastocysts were isolated at 3.5 d.p.c. as above and plated onto 1% gelatin-coated glass chamber slides (Nunc). Following 4 days in culture, cells were rinsed with HBSS and fixed for 10 min with 3.7% paraformaldehyde in PBS. The TUNEL assay was then performed on cells using the TdT-FragEL DNA Fragmentation Detection kit (Oncogene) according to the manufacturer’s instructions.

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