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An allelic series for studying the mouse *Thoc1* gene

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Abstract

Thoc1 encodes an essential component of the mammalian TREX protein complex. TREX is an evolutionary conserved complex that couples elongating RNA polymerase II with RNA processing factors. Depletion of *Thoc1* protein (pThoc1) compromises transcriptional elongation and nuclear export of some RNAs. Loss of *Thoc1* causes periimplantation embryonic lethality in the mouse. Early embryonic lethality precludes analysis of the physiological requirements for *Thoc1* in the developing embryo or adult. To circumvent this limitation, we have generated mice containing hypomorphic or conditional alleles of *Thoc1*. Mice homozygous for the conditional allele appear normal. Mice containing Cre recombined conditional alleles phenocopy the previously characterized *Thoc1* null allele. Mice homozygous for the hypomorphic allele are viable and born at a frequency that is not significantly different from the expected Mendelian ratio. However, these mice express less pThoc1 than wild type mice and exhibit a dwarf phenotype. The dwarf phenotype can be detected in mid-gestation embryos, suggesting that *Thoc1* is also required later in embryonic and postnatal development.

Keywords

gene expression; development; RNA processing; mRNP

Co-transcriptional loading of RNA processing and export factors onto nascent RNA supports efficient and regulated gene expression (Moore, 2005; Reed, 2003). Assembly of the TREX complex is one molecular mechanism utilized by cells to physically couple transcription with factors involved in RNA processing and export. TREX has been best characterized in *S. cerevisiae* where it is composed of the THO sub-complex containing four proteins (Hpr1p, Tho2p, Mft1p, and Thp2p) that are essential for normal transcriptional elongation and genome stability (Chavez et al., 2000). The THO sub-complex recruits two additional proteins, Sub2p and Yra1p, that are involved in nuclear RNA export and splicing (Luo et al., 2001; Strasser et al., 2002). Hpr1p genetically and physically interacts with RNA PolIII (Chang et al., 1999; Chavez et al., 2000; Fan et al., 1996; Strasser et al., 2002). Hpr1p is an essential component of TREX since its loss impairs both transcriptional elongation and nuclear RNA export (Chavez and Aguilera, 1997; Chavez et al., 2001; Fan et al., 1996; Huertas and Aguilera, 2003; Rondon et al., 2003; Strasser et al., 2002; Zenklusen et al., 2002). Despite the defects in gene expression associated with *HPRI* loss, it is not essential for yeast viability (Aguilera and Klein, 1990).

Metazoan structural homologues are apparent for the yeast TREX proteins Tho2p (Thoc2), Sub2p (UAP56), and Yra1p (Aly) (Luo et al., 2001; Strasser and Hurt, 2000; Stutz et al., 2000; West et al., 2000). Although lacking statistically significant similarity at the primary amino acid level, functional orthologues of yeast *HPRI* have been identified in both human and insect cells (alternatively known as *Thoc1*, Hpr1, or p84) (Li et al., 2005; Masuda et al.,

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2005; Rehwinkel *et al.*, 2004). Depletion of *Thoc1* protein (pThoc1) from human or insect cells compromises transcriptional elongation and nuclear RNA export of some genes. These observations suggest that metazoan species with long, intron-containing genes utilize an evolutionary conserved TREX complex to regulate gene expression. However, the subunit composition of yeast and metazoan TREX complexes are not identical, suggesting possible functional differences.

Thoc1 is expressed widely during mouse embryonic development and mice lacking *Thoc1* fail to develop past the late blastocyst stage (Wang *et al.*, 2006). Early embryonic lethality is associated with loss of cell viability, particularly among the pluripotent stem cells that make up the inner cell mass. *Thoc1* is also expressed widely in adult tissue, but early embryonic lethality of *Thoc1* null mice has precluded characterization of the physiological requirements for *Thoc1* during later stages of embryonic development or in the adult mouse. To address this limitation, we have generated hypomorphic and conditional alleles of the murine *Thoc1* gene.

The mutant *Thoc1* alleles were engineered by targeted homologous recombination in mouse ES cells. The targeting vector contained approximately 7 kilobase pairs (kbp) of *Thoc1* from intron 4 to intron 7 (Fig. 1A). A PGK-neo selection cassette flanked by *frt* sites was inserted within intron 5 in reverse orientation relative to *Thoc1*. A loxP site was situated within intron 5, 3' of the PGK-neo cassette. A second loxP site was inserted in intron 7. Eighty-two ES cell clones that resisted drugselection were screened by Southern blotting. Three of these clones gave the expected fragment size for the targeted allele using a 5' flanking probe (Fig. 1B). With a 3' flanking probe, two of these three gave bands of the size expected for a correctly targeted allele. The 3' recombination junction in the remaining clone was between the loxP sites, thus removing the 3' loxP site from the targeted allele. Correctly targeted ES cells of the 129/SvJae background were used to generate chimeric mice. The chimeric mice were crossed to C57BL/6J mice thus giving rise to a founder strain of mice carrying the *Thoc1* H allele on a mixed 129/SvJae X C57BL/6J genetic background (Fig. 1A). This strain was subsequently mated to a Flpe recombinase expressing mouse strain to remove the drug selection cassette. Successful excision of the neomycin selection cassette was demonstrated by Southern blotting that distinguishes the wild type, H, and F alleles (Fig. 1C). PCR assays have been designed for routine genotyping of the H, F, and the previously described *Thoc1* null alleles (Fig. 2A-B) (Wang *et al.*, 2006). Cre expression correctly excised exons 6 and 7 from the F allele to generate a new, presumed null allele (N) as indicated by the appearance of a PCR amplified fragment of expected size (Fig. 2C).

Mice heterozygous or homozygous for the H allele were born at a frequency not significantly different from the expected Mendelian ratio ($p > .05$ by Chi squared test) (Table 1). *Thoc1* H/+ mice were indistinguishable from wild type, but *Thoc1* H/H mice exhibited a dwarf phenotype with 100% penetrance (Fig. 3A). The weight of newborn *Thoc1* H/H mice was less than mice containing at least one wild type allele ($1.1\text{g} \pm 0.1$ versus 1.6 ± 0.1 , $P < 0.01$ by Student's T test). Reduced size persisted throughout adulthood (Fig. 3B). By four months of age, total body weight of H/H mice was approximately 80% of wild type and most individual organ weights (thymus, heart, lung, liver, kidney, spleen, and urogenital tract) were also proportionally less. The relatively small size of H/H mice was detectable as early as embryonic day 12.5 (E12.5) (Fig. 3C), suggesting the dwarf phenotype was not solely due to changes in the activity of growth factors and hormones that function during postnatal development. Viable neonates hemizygous for the *Thoc1* H allele (H/−) were not recovered (Table 1). However, hemizygous embryos were detected at E13.5, thus reaching a stage of embryonic development significantly more advanced than *Thoc1* null embryos (Wang *et al.*, 2006). As with H/H embryos, H/− embryos were considerably smaller than their wild type littermates (data not shown). To ascertain whether the phenotypes observed were due to reduced pThoc1 expression, we isolated tissue or embryonic fibroblasts (MEF) from mice of informative genotypes and assessed

pThoc1 levels by western blotting. In multiple tissues pThoc1 levels were significantly lower in *Thoc1* H/H mice than in wild type or H/+ mice (Fig. 3D–E). The relative decrease in pThoc1 levels ranged from more than 30-fold in brain tissue, to approximately 30% in lung tissue. Most H/H tissues and MEFs show a 2–4 fold decrease in pThoc1 levels relative to samples from wild type or heterozygous mice.

Mice homozygous, heterozygous, or hemizygous for the F allele were born at the expected Mendelian ratio (Table 1). Mice with these genotypes exhibited no phenotype distinguishable from wild type mice to at least nine months of age. These observations suggested that the F *Thoc1* allele functioned equivalently to the wild type allele. Cre mediated excision of exons 6–7 was accomplished by mating heterozygous *Thoc1* F mice to the *Meox2*-Cre strain (Tallquist and Soriano, 2000). Offspring heterozygous for the Cre excised N allele were then intermated, or mated with heterozygous *Thoc1* null mice. No viable neonates homozygous or hemizygous for the N allele were recovered. We also assayed the genotype of embryos at different stages of development resulting from intermating of *Thoc1* N/+ mice. Homozygous *Thoc1* N embryos were not recovered at any stage tested that was past E3.5. Homozygous *Thoc1* N E3.5 blastocysts were recovered at the expected Mendelian ratio and appeared normal. However, they were unable to form blastocyst outgrowths upon in vitro culture (Fig. 4A), phenocopying previously characterized homozygous *Thoc1* null blastocysts (Wang *et al.*, 2006). Treatment of MEFs hemizygous for the F allele with a recombinant adenovirus designed to express Cre significantly depleted pThoc1 levels relative to control MEFs containing a wild type allele (Fig. 4B). These observations indicated that Cre mediated excision of exons 6–7 precluded normal pThoc1 expression thus generating a functionally null allele.

We describe the construction and initial characterization of three new alleles of the murine *Thoc1* gene. The H allele is hypomorphic as mice homozygous or hemizygous for the allele display developmental phenotypes that are considerably less severe than those observed in *Thoc1* null mice. The hypomorphic phenotype is likely caused by diminished pThoc1 expressed from this allele since the protein coding sequence is unaltered. Intron 5 insertion of the drug selection cassette causes decreased expression from the H allele since its removal to generate the F allele restores normal pThoc1 expression (compare pThoc1 levels F/–, H/H, and F/+ MEFs in Figs. 3 and 4). *Thoc1* null mice demonstrate a requirement for Thoc1 early in embryonic development. The dwarf phenotype of the hypomorphic *Thoc1* mice, detectable as early as midgestation, indicate that *Thoc1* is also required for later stages of embryonic and postnatal development. Consistent with restoration of pThoc1 expression by removal of the drug selection cassette, the F allele functions normally since mice homozygous, heterozygous, or hemizygous for the allele do not display detectable phenotypes distinguishable from wild type. Cre mediated recombination of the F allele removes exons 6 and 7 deleting codons 126–173 and causing a reading frame shift that generates a premature stop codon. Consistent with Cre mediated generation of a premature stop codon, pThoc1 levels detected by a carboxy terminal specific antibody are significantly reduced in F/– MEFs exposed to Cre recombinase. The Cre recombined N allele phenocopies the previously characterized null allele since N/N embryos fail to develop beyond the blastocyst stage (Wang *et al.*, 2006). Due to the perimplantation embryonic lethality of *Thoc1* null mice, these two new alleles will be useful in studying *Thoc1* function during later stages of development and in adult mice.

Methods

Generation of mutant *Thoc1* alleles in the mouse

A BAC clone spanning the *Thoc1* gene was isolated from a 129/SV mouse strain genomic library and used as a template to PCR amplify the homology arms for construction of the targeting vector. The 2.1 kbp 5' homology arm spanning exon 5 was subcloned into the KpnI and BamHI sites of pPNT4 (Conrad *et al.*, 2003). The split 3' homology arm was subcloned in

two pieces, a 1.2 kbp fragment spanning exons 6 and 7 subcloned into the NotI and SalI sites and a 4.0 kbp piece of intron 7 subcloned into the SfiI and BlnI sites. All exons, flanking intron regions, frt sites, and loxP sites were verified by DNA sequencing. The targeting construct was electroporated into a 129/SV derived embryonic stem cell line and G418 resistant colonies screened by Southern blotting of XhoI+BglI restricted genomic DNA using 5' and 3' flanking probes. Correctly targeted ES cells were used to construct germ line transmitting chimeras by blastocyst injection. Transmission of the targeted alleles was confirmed by Southern blotting of genomic DNA extracted from tail biopsies.

Mice were mated with the FLPeR 129/SVJae strain to delete the PGK-neo drug selection cassette from the H *Thoc1* allele to generate the F allele (Farley *et al.*, 2000). Cre mediated recombination to generate the N allele was accomplished by mating *Thoc1* F/+ mice with the Meox2-Cre C57BL/6 mouse strain (Tallquist and Soriano, 2000). Both strains were obtained from the Jackson Laboratory (Bar Harbor, Maine). Southern blotting of BamHI+NcoI restricted genomic DNA using the 3' flanking probe was used to confirm the wild type, H, F, and N *Thoc1* alleles.

Genotyping and protein analyses

Routine genotyping of genomic DNA extracted from tail biopsy specimens or MEFs was performed by PCR assays. All PCR reactions were performed in Taq buffer (Fermentas Inc., Hanover, MD). PCR was initiated with a 3 minute denaturation cycle at 94°C, followed by 20–30 cycles of 30 seconds denaturation, 30 seconds annealing at 61°C, and 30 seconds extension at 72°C. After a final 7 minute extension at 72°C, PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide. Primers 5'-AAAGTACATGCTTTCAAGCAGGCA-3' and 5'-CAGAAAATGGAAACAGAATAGGCTT-3' amplified a 325bp PCR fragment from the N allele. Primers 5'-CAGTAAGTGCCTTGGTTTCCT-3' and 5'-GGAAATCAAACCCAGGTTCTCT-3' amplified a 390bp PCR fragment from the wild-type allele. Primers 5'-AGGTCGACGGTATCGATAAGCTTGATA-3' and 5'-CTGTTACTGATTTCAAGCATTTCCT-3' amplified a 350bp PCR fragment from the F allele. The primers 5'-CTCTTTTGGCCAGGCTTT-3' and 5'-CAGAAAATGGAAACAGAATAGGCTT-3' amplified a 390bp PCR fragment from the H allele and a 326bp PCR fragment from the wildtype allele.

Genotyping of preimplantation embryos was performed using a nested PCR assay. Individual embryos or cell outgrowths were lysed by incubation at 55°C for 4 hr in 20 µl of PCR lysis buffer [10mM Tris-HCl pH 8.8, 50mM KCl, 0.08% NP40, 0.2mM dNTP, 2.5 mM MgCl₂] containing proteinase K (60 µg/ml). After boiling, an aliquot of the lysates was subjected to nested PCR amplification using first round primers: 5'-AAAGTACATGCTTTCAAGCAGGCA-3' and 5'-CAGAAAATGGAAACAGAATAGGCTT-3' (N allele); 5'-CAGTAAGTGCCTTGGTTTCCT-3' and 5'-GGAAATCAAACCCAGGTTCTCT-3' (wild type). Second round primers were: 5'-TGTAAGTTATGAGGTATAGGTAGGAATGC-3' and 5'-TCTGAAATTGAATACAGACAGAATAGTT-3' (N allele); 5'-TAATTCTAATACTGGTGGTTGCCA-3' and 5'-CTTTGTTGTGACAGGTTCTCC-3' (wild type). The N allele generates a 250bp PCR fragment while the wild type allele produces a 229bp fragment.

Isolation of protein extracts and western blot analysis of pThoc1 was performed as previously described (Li *et al.*, 2005).

Embryo and cell culture

Timed pregnancies in superovulated *Thoc1* heterozygous females were used to generate preimplantation embryos (E3.5) for analysis as previously described (Wang *et al.*, 2006). Embryos were cultured in M16 medium without leukemia inhibitory factor. Mouse embryo fibroblasts (MEFs) were isolated from E13.5 embryos by standard procedures. Cells are cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.5% penicillin and streptomycin and grown at 37°C in a humidified atmosphere of 5% CO₂. Cells were cultured according to the standard 3T3 protocol.

Adenovirus

The Ad-Cre adenoviral stock was purchased from the Gene Transfer Vector Core of the University of Iowa. The early promoter of cytomegalovirus drives expression of the Cre gene in the recombinant adenovirus. Infections are performed at a multiplicity of infection of 100 infectious units per cell for two consecutive passages.

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Figure 1. Construction of mutant *Thoc1* alleles in the mouse

A) A representation of the exon/intron structure of the targeted region of the *Thoc1* gene (not to scale), the targeting vector, and the expected structure of the successfully targeted alleles. Exons are numbered and shown as solid boxes. The position of XhoI (X), BglII (B), NcoI (N), and BamHI (Ba) restriction enzyme sites, the flanking hybridization probes, and the expected sizes of fragments detected by Southern blotting are indicated. The positions of the loxP and frt sites as well as the PGK-neo drug selection cassette, in reverse orientation to the *Thoc1* gene, are also indicated. B) Southern blot analysis of XhoI+BglII restricted genomic DNA from the three successfully targeted ES cell lines. The left panel shows Southern blotting results using the 5' flanking probe while the right panel displays the results with the 3' flanking probe.

Sizes of the bands are indicated based on comparison to molecular weight markers run on the agarose gel. The band marked with an * indicates a successfully targeted clone where the 3' recombination junction occurs between the loxP sites of the 3' homology arm. C) Southern blot analysis of BamHI+NcoI restricted genomic DNA from representative mice of the indicated genotypes using the same 3' flanking probe used in B. As shown by the indicated expected band positions in A, this analysis distinguishes the wild type (wt), H, and Flp recombined (F) *Thoc1* alleles.

Figure 2. PCR assays for genotyping the H, F, and N *Thoc1* alleles

A) A representative ethidium bromide stained agarose gel resolving PCR fragments amplified from genomic DNA of mice with the indicated genotypes. The bands amplified from the wild type (wt) or H alleles can be resolved on the gel and serve as an assay for routine genotyping. The pixels of the image have been inverted for improved legibility. B) Representative ethidium bromide stained agarose gels resolving PCR fragments amplified from MEF genomic DNA with the indicated genotypes. The PCR reactions utilize primers specific for the wild type (wt), F, or previously characterized null alleles of *Thoc1*. C) A representative agarose gel resolving PCR fragments specific for the *Thoc1* N, generated by Cre mediated excision of the F allele. The MEFs were treated with a Cre expressing recombinant adenovirus (+) or mock treated (-) 72 hours prior to genomic DNA isolation.

Figure 3. The *Thoc1* H allele is hypomorphic

A) Representative three-week-old mice of the indicated genotype demonstrating a dwarf phenotype in mice homozygous for the H allele. B) Male mice of the indicated genotype were weighed at the indicated ages in weeks. The data points represent the mean weight and standard deviation from at least three mice for each datapoint. C) Representative E12.5 mice of the indicated genotype photographed under dark field microscopy indicating a detectable size difference prior to birth. D) A representative western blot indicating pThoc1 levels in various tissues isolated from adult mice of the indicated genotypes. β -actin serves as a protein loading control. Relative signal intensities are shown at bottom after normalization against the loading

control. E) MEFs isolated from mice of the indicated genotype were analyzed for pThoc1 levels by western blotting as above. Each lane represents an independent MEF isolate.

Figure 4. The F allele is conditionally null for *Thoc1*

A) Blastocysts isolated at E3.5 from intermating of mice heterozygous for the N allele were cultured in vitro for 0 or 48 hours. Representative blastocysts were photographed under phase contrast microscopy. Wild type blastocysts generate typical outgrowths consisting of flat trophoblast cells and rounded cells of the inner cell mass. Homozygous *Thoc1* N blastocysts typically fail to hatch from the zona pellucidae and do not generate viable blastocyst outgrowths. B) Individual MEF isolates of the indicated genotypes are treated with the Cre expressing adenovirus (+) or mock treated (-) and total proteins extracted 72 hours after treatment. A representative western blot showing pThoc1 levels is shown. The bottom panel shows the same blot immunostained for β-actin to verify protein loading. Relative signal intensities are shown at bottom after normalization against the loading control. Since the

efficiency of adenoviral infection under the conditions used is less than 100%, residual pThoc1 detected in hemizygous MEFs likely reflects pThoc1 expression from cells that have escaped infection.

Table 1Viable neonates from intermating of *Thoc1* mutant mice

Mating	Genotype	Number	Expected
H/+ X H/+	+/+	51	42.5
	H/+	83	85
	H/H	36	42.5
H/+ X +/-	+/+	29	21
	+/-	25	21
	H/+	30	21
	H/-	0*	21
F/+ X F/+	+/+	14	15.25
	F/+	30	30.5
	F/F	17	15.25
F/+ X +/-	+/+	6	7.75
	+/-	4	7.75
	F/+	12	7.75
	F/-	9	7.75

Embryos from *Thoc1*^{+/-} heterozygote intercrosses were collected on day 14 post-partum (P14) and genotyped by PCR.

* Statistically significant difference from expectation by Chi squared test ($p < .001$).