Generation of Mice With a Conditional Allele for Trim33

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Summary: Trim33 (Tif1γ, ectodermin, moonshine), a member of the TIF1 family of transcriptional coactivators and corepressors, is a large nuclear protein that contains an N-terminal tripartite (Trim) domain composed of a RING domain, two B-box domains, and a coiled coil domain. It has been suggested that Trim33 (Ectodermin) mediates ectodermal induction in the Xenopus by functioning as a Smad4 ubiquitin ligase, while in the zebrafish Trim33 (moonshine) has been reported to act as a R-Smad binding protein in induction of erythroid differentiation. Since the developmental role of Trim33 in mammals is currently unknown, we generated mice carrying the conditional Trim33 (Trim33FX) allele by flanking exons 2–4 encoding the functionally critical N-terminal tripartite domain by loxP sites. We confirmed the null genotype by using the Ella-Cre transgenic approach to create mice that lack exons 2–4. Embryos deficient in Trim33 die during early somitogenesis, demonstrating that Trim33 plays an important nonredundant role in mammalian embryonic development. genesis 46:329–333, 2008. © 2008 Wiley-Liss, Inc.

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Trim33 (Tif1γ) is one of 70 tripartite motif-containing Trim proteins (Yan et al., 2004). Together with Trim24 (Tif1α) and Trim28 (Tif1β), it forms a transcription intermediary factor-1 (Tif-1) subfamily of transcriptional regulators (Venturini et al., 1999). Although Tif-1 proteins all share several characteristic functional domains, for example, amino terminal Trim domains, carboxy terminal PHD finger, and Bromo domains, it is thought that their biological functions are quite divergent. For instance, Trim28, but neither Trim24 nor Trim33, has been shown to interact with members of the KRAB zinc finger proteins (Friedman et al., 1996; Kim et al., 1996). Moreover, Trim28 is an intrinsic component of the histone deacetylase N-CoR1/HDAC3 complex (Underhill et al., 2000), whereas Trim24 does not bind nuclear hormone receptors, but it has been shown to function as a coactivator of the retinoid acid receptor (Fraser et al., 1998). Whereas mouse embryos deficient in Trim28 die soon after implantation, Trim24 is not essential for embryogenesis, but was found to be a potent liver-specific tumor suppressor (Cammas et al., 2000; Khetchouman et al., 2007). Much less is known about the biological role of Trim33, particularly in mammals. This is largely because of the fact that knockout mice have not yet been developed. However, it was recently shown that in Xenopus Trim33 functions as an E3 ubiquitin-protein ligase promoting Smad4 degradation via the ubiquitin proteasome pathway (Dupont et al., 2005). In the zebrafish, Trim35 was shown to be required for erythroid lineage-specific control of hematopoietic gene expression (Ransom et al., 2004). Subsequently, it was shown that R-Smad/Trim33 interaction is required for Tgf-β-dependent erythroid differentiation. Interestingly, it seems that in this context Trim33 does not target Smad4 for degradation, but rather competes with it by binding activated R-Smads (He et al., 2006).

To address the role of Trim33 in mammalian embryogenesis, we generated mice harboring the conditional knockout allele for Trim33. Our strategy was to flank exons 2–4 encoding the functionally critical RING and B1 and B2 Box domains by loxP sites. The short (1.6 kb) and long (8.2 kb) arms of the targeting vector were PCR amplified from a Bac genomic DNA. The loxpPNeoloxP cassette was inserted into intron 1 and a single loxP site was inserted into intron 4 (see Fig. 1). As a negative selection marker we used the diphtheria toxin (DT) gene. The linearized targeting vector was electroporated into TVB2 embryonic stem cells as described (Yang and Kaartinen, 2007). Fifteen out of 261 G418-resistant colonies and long (8.2 kb) arms of the targeting vector were PCR amplified from a Bac genomic DNA. The loxpPNeoloxP cassette was inserted into intron 1 and a single loxP site was inserted into intron 4 (see Fig. 1). As a negative selection marker we used the diphtheria toxin (DT) gene. The linearized targeting vector was electroporated into TVB2 embryonic stem cells as described (Yang and Kaartinen, 2007). Fifteen out of 261 G418-resistant colonies were targeted to the correct locus; however, only three of them contained the 5′ loxP site. All three correctly targeted ES clones were able to produce highly

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chimeric male mice, which in turn were potent germ line transmitters. To remove the Neo selection marker and to generate a presumed knockout allele for Trim33, we crossed the Trim33FXNeo/FXNeo mice (homozygotes for the targeting vector) with EIIa-Cre transgenic mice (Xu et al., 2001). In these mice, Cre is expressed under the control of the adenoviral EIIa promoter that targets expression of the Cre recombinase to the early mouse embryo (Lakso et al., 1996). The EIIa-Cre transgene creates both partial and complete recombinations and...
of embryos harvested at E8.5 using primers for exons 4 and 7 demonstrates that the mRNA encoded by exon 2–4 is able to produce a stable mRNA product, while wild-type and homozygote controls show an expected 423-bp fragment from the heterozygote samples gives rise to both 733-bp and 336-bp products, respectively. To further validate the Trim33KO/KO allele, we used exon 1- and exon 6-specific primers. The wild-type and homozygote Trim33KO/KO alleles produced the expected 733-bp and 366-bp products, respectively, while the heterozygote samples gave rise to both the wild-type and mutant alleles produce a 684-bp amplification product (second panel from bottom).

Therefore Ella-Cre transgenic mice can be used both as a deleter mouse to generate knockout alleles and to remove selection markers (e.g., loxP-pGKNeo-loxP) when a triple loxP-strategy is used (Holzenberger et al., 2000; Xu et al., 2001). The obtained mosaic male mice progeny were subsequently crossed with wild-type female mice to obtain the floxed (Trim33KO) and null (Trim33KO) alleles (see Fig. 2). Homozygote Trim33KO/KO mice were viable and fertile, and they did not display any recognizable phenotypes.

To confirm that the Trim33KO allele encoded the true null allele and to provide initial information about the biological role of Trim33 during embryogenesis, we intercrossed the heterozygote Trim33KO/WT mice to obtain homozygote Trim33KO/KO mice (Fig. 3A). Genotype analyses of newborn offspring revealed that all the homozygote mutant pups died during gestation (Table 1). To examine the time frame during which embryonic lethality occurred, we harvested embryos at different time points and discovered that at E9 the mutant embryos displayed a dramatic developmental delay when compared with controls (Table 1 and Fig. 3H–J). Nevertheless, they had formed a body axis, displayed head folds and the neural tube, and showed 5–6 somite pairs. We were unable to discover any living mutant embryos after E9.5. At E8.0–8.5 (3–6 somite pairs in controls), the Trim33 mutant embryos were aligned at the base of the yolk sac, and while they had formed the anterior–posterior body axis and identifiable anterior structures, for example, head folds (arrows in Fig. 3D, E, and G), it was difficult to identify any other embryonal structures. RT-PCR using primers with target sequences in exons 4 and 7 did not produce any detectable amplification product, while the heterozygote and wild-type samples showed the expected 423-bp fragment (Fig. 3B). This is concordant with the lack of sequences encoded by exon 4. To further validate the Trim33KO/KO allele, we used exon 1- and exon 6-specific primers. The wild-type and homozygote Trim33KO/KO alleles produced the expected 733-bp and 366-bp products, respectively, while the heterozygote samples gave rise to both PCR fragments (Fig. 3B). This is concordant with the lack of sequences encoded by exon 4. To further validate the Trim33KO/KO allele, we used exon 1- and exon 6-specific primers. The wild-type and homozygote Trim33KO/KO alleles produced the expected 733-bp and 366-bp products, respectively, while the heterozygote samples gave rise to both PCR fragments (Fig. 3B).
lacks sequences encoded by exons 2–4, and that it is highly likely that the phenotype observed in homozygote samples results from a loss of Trim33 function, particularly since most of the possible splicings, for example, exon 1 to 4, will lead to a frameshift and premature translational stop. These mutant mRNAs would produce only the very N-terminal peptide encoded by Trim33 exon 1. Only the splicing from exon 1 to 9 or exon 1 to 13 would maintain the open reading frame. In these unlikely hypothetical cases the protein product would lack the tripartite motif, but would contain the C-terminal PHD and Bromo domains. Based on the current knowledge, it is impossible to say whether these aberrant proteins lacking the functionally important tripartite motif would possess any biological activity.

A recent publication of Dupont et al. showed that in Xenopus embryos Xtrim33 (Ectodermin) plays a critical role in controlling TGF-β responses during gastrulation (Dupont et al., 2005). Specifically, these authors demonstrated that Xtrim33 is required for specification of the ectoderm and for restricting the mesoderm-inducing activity of TGF-βs. Our finding that Trim33 null embryos die during somitogenesis demonstrates that Trim33 is also required for early embryogenesis in mammals. If the role of Trim33 is to negatively regulate Tgf-b signaling during embryogenesis, one would expect that Trim33 null mutants would display phenotypes that are consistent with amplified Tgf-b signaling. The observed external Trim33KO/KO phenotype is very similar to that seen in transgenic mouse embryos that demonstrate ectopic expression of the Tgf-b type II receptor (Zwijsen et al., 1999). Another study recently demonstrated that abrogation of Drap1, a negative regulator of Nodal, leads to aberrant Nodal signaling and early embryonic lethality (Iratni et al., 2002). Interestingly, similar to Trim33KO/KO mutants, embryos that are heterozygous for Nodal and lack Drap1 undergo gastrulation, but die at E9.5. Subsequent studies will show whether the observed phenotype results from defects in ectodermal specification or some other catastrophic mechanistic failure during early embryogenesis. Moreover, mice carrying the floxed Trim33 allele (Trim33FX) as described herein can be used to investigate the role of this gene later in development during organogenesis, as well as during postnatal life.

METHODS

Generation of Mice Carrying the Floxed and Knockout Trim33 Alleles

RP2468C16 Bac DNA was used as a template to PCR amplify both short and long arms as ClaI-XhoI and KpnI-Sall fragments, respectively. High-fidelity Supermix (Qiagen, Valencia, CA) polymerase was used for amplification, and the generated arms were sequenced to verify that no PCR-generated mutations were introduced. A single loxp site was inserted into intron 4 by replacing a small 553-bp SacI-PstI fragment with a loxp sequence flanked by SacI and PstI restriction sites. Subsequently, a loxPNeoLOXp cassette was inserted as a XhoI-NotI fragment into the pKODT plasmid. A long arm containing the single loxp sequence in intron 4 was inserted as a KpnI-Sall fragment into loxPNeoLOXp/pKODT, and finally a short arm was added into the construct as a ClaI-XhoI fragment. The targeting vector was electroporated into TVB2 mouse ES cells, and recombinant ES cell clones were selected with G418 as described (Kaartinen et al., 2004, 1995). Mouse chimeras were generated by injecting correctly targeted ES clones into C57BL/6J mouse blastocysts. The floxed Trim33 allele (Trim33FX) and the knockout Trim33 allele (Trim33KO) were generated by crossing female mice homozygous for the targeting vector with Ella-Cre transgenic male mice (Xu et al., 2001), which were obtained from Jackson Laboratories (Bar Harbor, ME).

PCR Screening, Clone Verification, and Genotyping

ES cell DNAs were first screened for correct targeting by PCR using a forward primer (a) 5’ CACCTCCTCCTCTCTCC 3’ and a reverse primer (b) 5’ GAGCGACACCACTTGCATGT 3’ or the reverse primer (d) 5’ GGAGGGAAAAATCTGGGCTGAA 3’. Subsequently, the presence of the single loxp site in intron 4 was verified by PCR using a forward primer (c) 5’ CATGTGCTTCCACCTCCTCCTCTCC 3’ and a reverse primer (d) 5’ GGCAGAGAACATCTGGGCTGAA 3’. Trim33KO mice were genotyped using forward and reverse primers (a) 5’ GCACCTTGATGAGATCTTCCTCCTCGTCC 3’ and (b) 5’ GACGATACCTGGAACGCCT 3’, respectively, while Trim33KO mice were genotyped using forward and reverse primers (a) 5’ GCACCTTGATGAGATCTTCCCTCCC 3’ and (b) 5’ GACGACATGCACTGTGCTGTA 3’, respectively.

Timed Matings and Embryos Analyses

Mice were mated during the dark period of the controlled light cycle. Female mice acquiring vaginal plugs were designated as day 0. At the time interval indicated in respective figures (E8 to E9), females were euthanized by CO2 and embryos were extracted in PBS (Invitrogen) followed by further analyses. All studies and procedures performed on mice were carried out at the Animal Care Facility of the Saban Research Institute and were approved by the CHLA Animal Care and Use Committee (IACUC). The mice were maintained in mixed genetic backgrounds.

RT-PCR

Total RNA was isolated from E8 embryos using the RNeasy mini kit (Qiagen), and cDNAs were synthesized by the Omniscript reverse transcription kit (Qiagen) according to the manufacturers’ protocols. Subsequently, the cDNAs were analyzed by PCR for Trim33 expression using the following primer pairs. β-actin was used as a quality and loading control.
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LITERATURE CITED


