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Generation of Mice with a Conditional Allele for *Trim33*

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Abstract

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* (*Trim33*^{FX}) *allele* by flanking exons 2–4 encoding most of the functionally critical N-terminal tripartite domain by loxP sites. We confirmed the null genotype by using the *EIIa-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 non-redundant role in mammalian embryonic development.

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). While Tif-1 proteins all share several characteristic functional domains, e.g., 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), while 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; Khetchoumian *et al.*, 2007). Much less is known about the biological role of Trim33, particularly in mammals. This is largely due to 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, Trim33 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).

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In order 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 *loxPNeoloxP* cassette was inserted into intron 1 and a single loxP site was inserted into intron 4 (Fig. 1). As a negative selection marker we used the *diphteria 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 3 of them contained the 3′ *loxP* site. All three correctly targeted ES clones were able to produce highly 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 $Trim33^{FXNeo/FXNeo}$ 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 therefore *EIIa-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* ($Trim33^{FX}$) *and* null ($Trim33^{KO}$) alleles (Fig. 2). Homozygote $Trim33^{FXFX}$ mice were viable and fertile, and they did not display any recognizable phenotypes.

To confirm that the $Trim33^{KO}$ allele encoded the true null allele and to provide initial information about the biological role of *Trim33* during embryogenesis, we inter-crossed the heterozygote $Trim33^{KO/WT}$ mice to obtain homozygote $Trim33^{KO/KO}$ 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 to 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, e.g., 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 $Trim33^{KO/KO}$ allele, we used exon 1- and exon 6-specific primers. The wild-type and homozygote $Trim33^{KO/KO}$ alleles produced the expected 733-bp and 366-bp products, respectively, while the heterozygote samples gave rise to both PCR fragments (Fig. 3B). RT-PCR analysis using primers specific for 3′ exons 15 and 20 produced the expected 684-bp fragment from wildtype, heterozygote ($Trim33^{KO/WT}$) and homozygote ($Trim33^{KO/KO}$) samples suggesting that the mutated allele lacking exons 2–4 is able to produce a stable mRNA (Fig. 3B). To conclude, our RT-PCR analyses demonstrated that the mRNA encoded by $Trim33^{KO/KO}$ allele 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, e.g., 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 posses any biological activity.

A recent publication of Dupont and coworkers 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 either during somitogenesis demonstrates that *Trim33* is also required for early embryogenesis in mammals. If the role of Trim33 is to negatively regulate Tgf-β signaling during embryogenesis, one would expect that *Trim33* null mutants would display phenotypes that are consistent with amplified Tgf-β signaling. The observed external $Trim33^{KO/KO}$ phenotype is very similar to that seen in transgenic mouse embryos that demonstrate ectopic expression of the Tgf-β 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 $Trim33^{KOKO}$ 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 spefication or some other catastrophic mechanistic failure during early embryogenesis. Moreover, mice carrying the floxed *Trim33* allele *(*$Trim33^{FX}$*)* as described herein can be used to investigate the role of this gene later in development during organogenesis, as well as during post-natal 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 *Cla*I-*Xho*I and *Kpn*I-*Sal*I fragments, respectively. High-fidelity Supermix (Qiagen) 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 *Sac*I-*Pst*I fragment with a *loxP* sequence flanked by *Sac*I and *Pst*I restriction sites. Subsequently a *loxPNeoloxP* cassette was inserted as a *Xho*I-*Not*I fragment into the *pKODT* plasmid. A long arm containing the single *loxP* sequence in intron 4 was inserted as a *Kpn*I-*Sal*I fragment into *loxPNeoloxP/pKODT*, and finally a short arm was added into the construct as a *Cla*I-*Xho*I 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; Kaartinen *et al.*, 1995). Mouse chimeras were generated by injecting correctly targeted ES clones into C57BL/6J mouse blastocysts. The floxed *Trim33* allele ($Trim33^{FX}$) and the knockout *Trim33* allele ($Trim33^{KO}$) were generated by crossing female mice homozygous for the targeting vector with *EIIa-Cre* transgenic male mice(Xu *et al.*, 2001), which were obtained from Jackson Laboratories (Maine).

PCR screening, clone verification and genotyping

ES cell DNAs were first screened for correct targeting by PCR using a forward primer (a′) 5′ CACGACACAAAGAACTGTAG 3′ and a reverse primer (b′) 5′ CAAGCAAAACCAAATTAAGG 3′. Subsequently, the presence of the single *loxP* site in intron 4 was verified by PCR using a forward primer (c) 5′ CATTGTGCTTCACCTCCTCCTCTTCG 3′ and a reverse primer (d) 5′ GGGAGGGAAAATCTGGCTGAA 3′. $Trim33^{FX}$ mice were genotyped using forward and reverse primers (a) 5′ GCACCTTGATGAGATCTTCCTCCTCC 3′ and (b) 5′ GACGACATACTGGACACCGTA 3′, respectively, while $Trim33^{KO}$ mice were genotyped using forward and reverse primers (a) 5′ GCACCTTGATGAGATCTTCCTCCTCC 3′ and (d) 5′ GGAGGGAAAATCTGGCTGAA 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 CO_2 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.

Trim33-ex4-S	5′GAGTCTGTTGGAACATCTGGTCAGCG3′
Trim33-ex7-AS	5′GGCCTGTGATATCATTCTGCTGCTGT3′
Trim33-ex1-S	5′GGTGTGTCAGCAGAGCTTGCA3′
Trim33-ex6-AS	5′GATAAGGGTGAAGATGGCCACT3′
Trim33-ex15-S	5′ACCTCATGCACAGGTCGGCAAGGAT3′
Trim33-ex20-AS	5′GCTCAAACTCTGGCAAAGGAGTGAAG3′
β-actin-S	5′GTGGGCCGGTCTAGGCACCAA3′
β-actin-AS	5′CGGTTGCCTTAGGGTTCAGG3′

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References

Cammas F, Mark M, Dolle P, Dierich A, Chambon P, Losson R. Mice lacking the transcriptional corepressor TIF1beta are defective in early postimplantation development. Development 2000;127:2955–2963. [PubMed: 10851139]

Dupont S, Zacchigna L, Cordenonsi M, Soligo S, Adorno M, Rugge M, Piccolo S. Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell 2005;121:87–99. [PubMed: 15820681]

Fraser RA, Heard DJ, Adam S, Lavigne AC, Le DB, Tora L, Losson R, Rochette-Egly C, Chambon P. The putative cofactor TIF1alpha is a protein kinase that is hyperphosphorylated upon interaction with liganded nuclear receptors. J Biol Chem 1998;273:16199–16204. [PubMed: 9632676]

Friedman JR, Fredericks WJ, Jensen DE, Speicher DW, Huang XP, Neilson EG, Rauscher FJ III. KAP-1, a novel corepressor for the highly conserved KRAB repression domain. Genes Dev 1996;10:2067–2078. [PubMed: 8769649]

He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MA, Massague J. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. Cell 2006;125:929–941. [PubMed: 16751102]

Holzenberger M, Lenzner C, Leneuve P, Zaoui R, Hamard G, Vaulont S, Bouc YL. Cre-mediated germline mosaicism: a method allowing rapid generation of several alleles of a target gene. Nucleic Acids Res 2000;28:E92. [PubMed: 11058142]

Iratni R, Yan YT, Chen C, Ding J, Zhang Y, Price SM, Reinberg D, Shen MM. Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. Science 2002;298:1996–1999. [PubMed: 12471260]

Kaartinen V, Dudas M, Nagy A, Sridurongrit S, Lu MM, Epstein JA. Cardiac outflow tract defects in mice lacking ALK2 in neural crest cells. Development 2004;131:3481–3490. [PubMed: 15226263]

Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. Nat Genet 1995;11:415–421. [PubMed: 7493022]

Khetchoumian K, Teletin M, Tisserand J, Mark M, Herquel B, Ignat M, Zucman-Rossi J, Cammas F, Lerouge T, Thibault C, Metzger D, Chambon P, Losson R. Loss of Trim24 (Tif1alpha) gene function confers oncogenic activity to retinoic acid receptor alpha. Nat Genet 2007;39:1500–1506. [PubMed: 18026104]

Kim SS, Chen YM, O'Leary E, Witzgall R, Vidal M, Bonventre JV. A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. Proc Natl Acad Sci USA 1996;93:15299–15304. [PubMed: 8986806]

Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, Westphal H. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci U S A 1996;93:5860–5865. [PubMed: 8650183]

Ransom DG, Bahary N, Niss K, Traver D, Burns C, Trede NS, Paffett-Lugassy N, Saganic WJ, Lim CA, Hersey C, Zhou Y, Barut BA, Lin S, Kingsley PD, Palis J, Orkin SH, Zon LI. The zebrafish moonshine gene encodes transcriptional intermediary factor 1gamma, an essential regulator of hematopoiesis. PLoS Biol 2004;2:E237. [PubMed: 15314655]

Underhill C, Qutob MS, Yee SP, Torchia J. A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. J Biol Chem 2000;275:40463–40470. [PubMed: 11013263]

Venturini L, You J, Stadler M, Galien R, Lallemand V, Koken MH, Mattei MG, Ganser A, Chambon P, Losson R, de TH. TIF1gamma, a novel member of the transcriptional intermediary factor 1 family. Oncogene 1999;18:1209–1217. [PubMed: 10022127]

Xu X, Li C, Garrett-Beal L, Larson D, Wynshaw-Boris A, Deng CX. Direct removal in the mouse of a floxed neo gene from a three-loxP conditional knockout allele by two novel approaches. Genesis 2001;30:1–6. [PubMed: 11353511]

Yan KP, Dolle P, Mark M, Lerouge T, Wendling O, Chambon P, Losson R. Molecular cloning, genomic structure, and expression analysis of the mouse transcriptional intermediary factor 1 gamma gene. Gene 2004;334:3–13.:3–13. [PubMed: 15256250]

Yang LT, Kaartinen V. Tgfb1 expressed in the Tgfb3 locus partially rescues the cleft palate phenotype of Tgfb3 null mutants. Dev Biol 2007;312:384–395. [PubMed: 17967447]

Zwijsen A, Goumans MJ, Lawson KA, Van Rooijen MA, Mummery CL. Ectopic expression of the transforming growth factor beta type II receptor disrupts mesoderm organisation during mouse gastrulation. Dev Dyn 1999;214:141–151. [PubMed: 10030593]

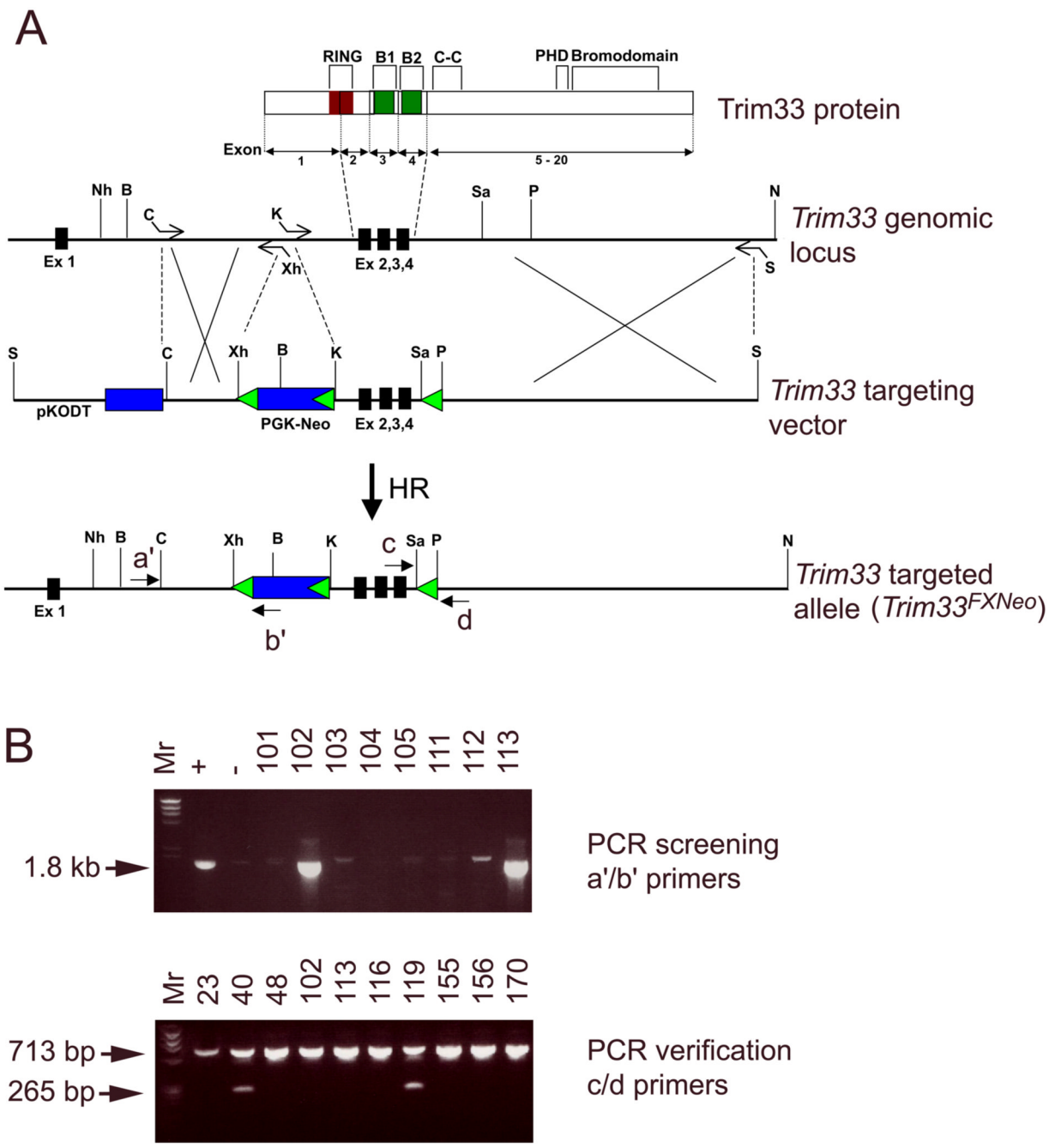

Figure 1. *Trim33* targeting vector and screening of ES colonies

A, a schematic presentation of the Trim33 protein domains, *Trim33* genomic locus depicting a segment from exon1 to intron 4, the *Trim33* targeting vector and the *Trim33* targeted allele. B, An example of a PCR screen (upper panel); positive clones #102 and #113 can be easily identified. PCR analysis using c/d primers and template DNA from clones that suggested the correct targeting demonstrated that only clones number #40, #119 and #259 (not shown) had retained the 3′ loxP site (lower panel). The wild type allele produces a 713-bp amplification product. Since the strategy to insert the 3′ loxP site into intron 4 involved the replacement of a 553-bp SacI-PstI fragment with a loxP site, the mutant allele gives rise only to a 265-bp amplification product.

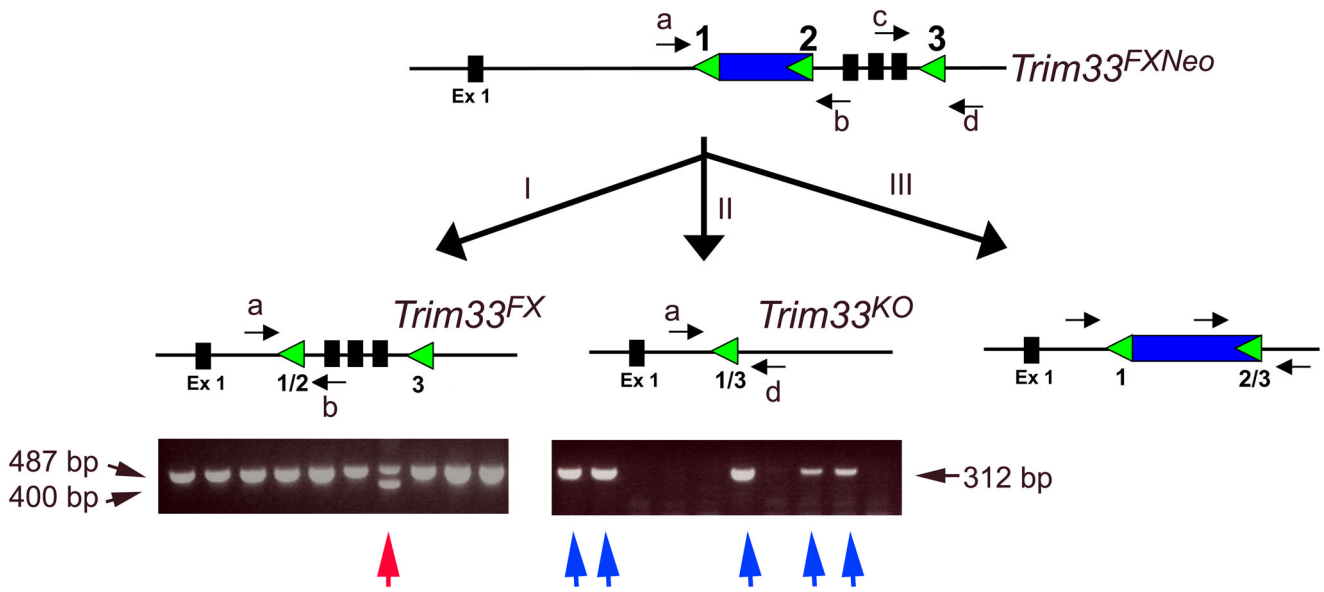

Figure 2. Generation of mice carrying the floxed (*Trim33*^{FX}) and knockout (*Trim33*^{KO}) *Trim33* alleles

Transgenic *EIIa-Cre* mice were crossed with mice homozygous for the targeting vector. The obtained mosaic males were further crossed with wild-type females to obtain *Trim33*$^{FX/WT}$ mice (type I recombination; red arrow) and *Trim33*$^{KO/WT}$ mice (type II recombination; blue arrows).

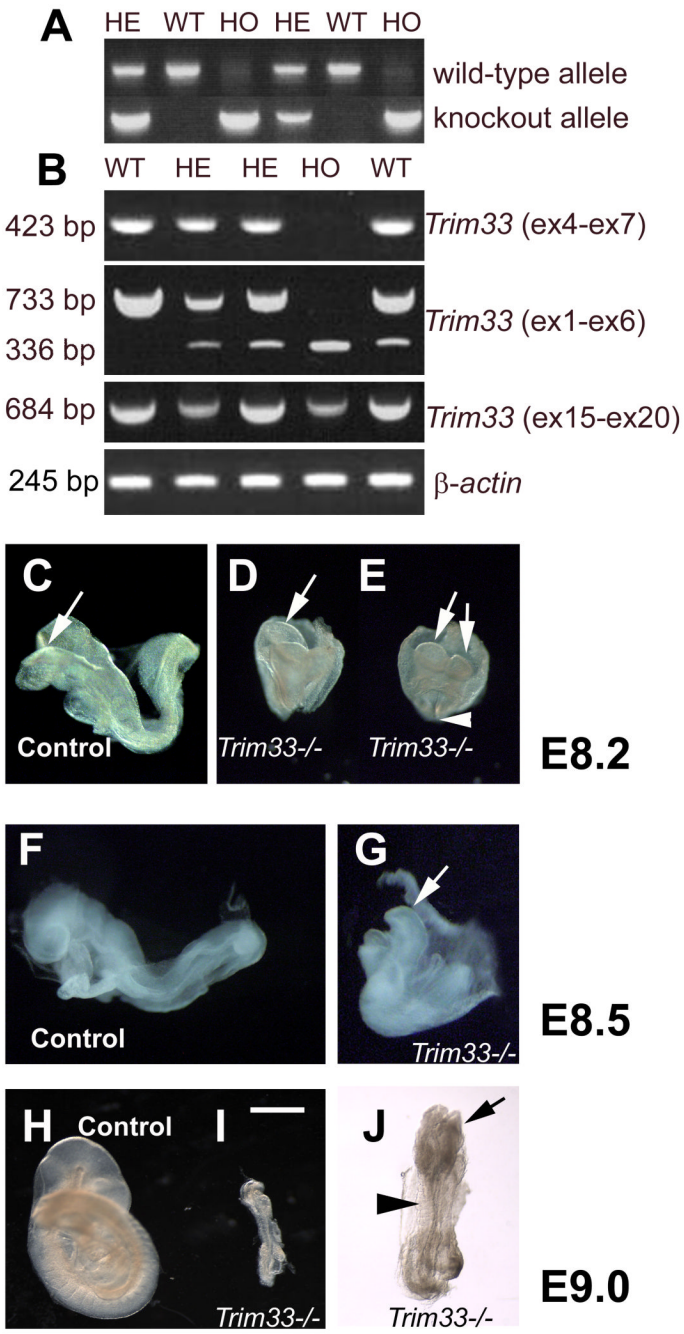

Figure 3. Development of embryos deficient in *Trim33* is arrested at the early somitogenic stage

A, PCR genotyping of embryos from the crossing between *Trim33*$^{KO/WT}$ males and females at E8.5. HE=heterozyggotes, WT=wildtype, HO=homozygotes. B, RT-PCR analysis of embryos harvested at E8.5 using primers for exons 4 and 7 demonstrates that *Trim33*$^{KO/KO}$ embryos do not display any detectable mRNA product, while wild-type and heterozygote controls show an expected 423-bp PCR product (B, upper panel). Corresponding analysis using primers for exons 1 and 6 show that the wild-type sample produces the expected 733-bp PCR product, the homozygote mutant sample produces the expected 336-bp product, while the heterozygote samples gives rise to both 733-bp and 336-bp products (second panel from the top). Corresponding analysis using primers for exons 15 and 20 shows that both the wild-type

and mutant alleles produce a 684-bp amplification product (second panel from bottom). β-actin specific primers produced a 245-bp amplification product with comparable intensity from all samples (bottom panel). At E8.2–8.5, *Trim33* null mutants (D, E, G) demonstrate retarded development when compared to controls (C,F) . D and E depict both lateral and frontal images of the same embryo, respectively. I, Development of the *Trim33* −/− embryos is arrested at the early somitogenic stage (E9.0). J, A high power picture of a *Trim33* −/− embryo shown in (I). H, Control littermate. Arrows in C, D, E, G and J point to head folds, white arrowhead in E points to the primitive streak, and black arrowhead in J points to the somites.

Table 1

Genotype analysis of offspring from *Trim33*+/− intercross.

Developmental stage	Somite pairs	*Trim33*−/−	*Trim33*+/−	Wild-type
Post-natal		0	12	2
E9.5		1	8	0
E9.0		2	4	4
E8.25 – E8.5	5–6	6	4	2
E8.0	3	4	7	4