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Mice lacking the transcriptional corepressor TIF1β are defective in early postimplantation development

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SUMMARY

TIF1β, a member of the transcriptional intermediary factor 1 family, has been reported to function as a corepressor for the large class of KRAB domain-containing zinc finger proteins of the Krüppel type. To address the biological function of TIF1β, we have generated TIF1β-deficient mice by gene disruption. TIF1β protein was detected in wild-type but not *TIF1β*$^{-/-}$ blastocysts. Homozygous mutant embryos, which developed normally until the blastocyst stage and underwent uterine implantation, were arrested in their development at the early egg-cylinder stage at about embryonic day (E) 5.5 and were completely resorbed by E8.5. Taken together, these results provide genetic evidence that TIF1β is a developmental regulatory protein that exerts function(s) essential for early postimplantation development.

Key words: TIF1 gene family, Heterochromatin-mediated silencing, KRAB domain, Gene disruption, Early lethality, Gastrulation, Mouse

INTRODUCTION

Transcriptional repression plays a central role in a variety of developmental and differentiation processes from yeast to mammals (reviewed in Gray and Levine, 1996; Ip and Hemavathy, 1997; Ogbourne and Antalis, 1998). Accordingly, numerous important developmental regulatory genes have been identified that encode sequence-specific transcriptional repressors (Carroll, 1990; Schoenherr and Anderson, 1995; Gray and Levine, 1996; Zazopoulos et al., 1997; Fisher and Caudy, 1998 and refs therein). These regulatory proteins typically contain a DNA-binding domain (DBD) and one or more repression domains, which can exert their repressing effects through interactions with transcriptional intermediary factors (TIFs), whose ultimate function is to remodel chromatin structure (Kornberg and Lorch, 1999), to inhibit (pre)initiation complex formation (Orphanides et al., 1996), or to associate target genes with specialized nuclear compartments that confer transcriptional repression (Cocktell and Gasser, 1999 and refs therein).

The KRAB (Krüppel-associated box) domain, which is present in about one third of the 300-700 human zinc-finger proteins (ZFPs) of the Krüppel type (Bellefroid et al., 1991), is one of the most widely distributed transcriptional repression domains yet identified in mammals (Margolin et al., 1994; Witzgall et al., 1994). When fused to a heterologous DBD, this regulatory domain of approx. 75 amino acids silences both basal and activated transcription in transfected cells, in a dose-dependent manner and over large distances (Pengue et al., 1994; Deuschle et al., 1995; Moosmann et al., 1997). Insight into the molecular mechanism(s) underlying this silencing activity came from the recent identification of a nuclear protein, TIF1β, also named KAP-1 (Friedman et al., 1996) or KRIP-1 (Kim et al., 1996), which exhibits all the hallmarks of being a corepressor for the large family of KRAB-ZFPs; TIF1β was demonstrated to interact through a KRAB box (see Fig. 1B) with several different KRAB domains but not KRAB mutants deficient in repression, to enhance KRAB-mediated repression and to repress transcription when directly tethered to DNA (Friedman et al., 1996; Kim et al., 1996; Moosmann et al., 1996; Agata et al., 1999; Nielsen et al., 1999).

On the basis of sequence homologies, TIF1β was also identified as a member of the transcriptional intermediary factor 1 (TIF1) family (Le Douarin et al., 1996). In addition to TIF1β, the family includes TIF1α, a putative nuclear receptor cofactor (Le Douarin et al., 1995, 1996; Zhong et al., 1999), and TIF1γ, whose function is unknown (Venturini et al., 1999). These three proteins are defined by the presence of two conserved amino acid regions: an N-terminal RBCC (RING finger, B boxes, coiled coil) motif, which may be involved in intermolecular interactions that influence the targeting to (and/or assembly of) subnuclear structures (Saurin et al., 1996; Boddy et al., 1997), and a C-terminal region containing a PHD finger and a bromodomain (see Fig. 1B). These latter two motifs are often associated and are present in a number of transcriptional cofactors acting at the chromatin level (Aasland et al., 1995; Jeanmougin et al., 1997 and refs therein). Interestingly, the bromodomain interacts with lysine-acetylated peptides derived from histones H3 and H4, suggesting a chromatin-targeting function for this highly conserved domain (Dhalluin et al., 1999; Winston and Allis, 1999).

Supporting the notion that TIF1β may exert its corepressor

function by a chromatin-mediated mechanism, TIF1β is known (1) to be associated with members of the heterochromatin protein 1 (HP1) family (Nielsen et al., 1999), a class of nonhistone proteins with a well-established function in heterochromatin-mediated silencing in *Drosophila* (Eissenberg et al., 1995; Elgin, 1996 and refs therein), (2) to phosphorylate HP1 proteins, to which it binds directly through a HP1 box (see Fig. 1B) (Le Douarin et al., 1996; Nielsen et al., 1999; Ryan et al., 1999) and (3) to possess an HP1-dependent and Trichostatin A (TSA; a specific inhibitor of histone deacetylase)-sensitive repression function (Nielsen et al., 1999). Thus, TIF1β may mediate the repression function of the KRAB domain by an epigenetic mechanism, which involves HP1 interaction and histone deacetylation, to induce formation of (and/or juxtaposition with) heterochromatin (Nielsen et al., 1999; Ryan et al., 1999).

To elucidate the physiological functions of TIF1β, we generated TIF1β-deficient mice. Null mutants develop normally to the blastocyst stage and implant, but fail to gastrulate, indicating that TIF1β is essential for early postimplantation mouse development.

MATERIALS AND METHODS

Construction of the targeting vector

To generate the *TIF1β* targeting vector, a 7-kb genomic fragment from the mouse *TIF1β* locus (F. Cammas, J.-M. Garnier, P. Chambon and R. Losson, unpublished) was used (see Fig. 1A). A 395-bp *Eco*RI-*Xho*I *TIF1β* fragment containing the 3′ 377 bp of intron 3 and the 5′ 18 bp of exon 4 was inserted between *Eco*RI and *Xho*I sites in pKSM3 (modified from pBluescript SK$^+$, and containing a *Cla*I-*Aat*II-*Xho*I-*Eco*RI-*Not*I-*Cla*I polylinker) to generate pTIF1B3. pTIF1B3 was digested with *Hin*dIII removing 43 bp in intron 3, which were replaced with a floxed *PGK-Neo* cassette (PGK-NeoLS5), to generate pTIF1B8. To create the 5′ homologous arm, a 3-kb *Eco*RI *TIF1β* genomic fragment containing exons 1 to 3 and the 5′ sequences of intron 3 (see Fig. 1A), was subcloned into the *Eco*RI site of pTIF1B8, yielding pTIF1B11. A 4.5-kb *Xho*I fragment 3′ downstream to position 18 of exon 4 (see Fig. 1A), was inserted into the *Xho*I site of pKSM3, to generate pTIF1B4. A double-stranded oligonucleotide containing a *loxP* site (underlined), a *Bam*HI site immediately downstream of the *loxP* site, and *Hin*dIII overhangs was produced by annealing two synthetic oligonucleotides WV144 (5′-AGCTATCATAACTTCGTATAATGTATGCTATACGAAGTTATGGATCCG-3′) and WV255 (5′-AGCTCGGATCCATAACTTCGTATAGCATACATTATACGAAGTTATGAT-3′), and was cloned into intron 14 *Hin*dIII site (*Hin*dIII disrupted) of pTIF1B4, yielding pTIF1B7. The *loxP*-containing *Xho*I fragment of pTIF1B7 was then inserted into the *Xho*I site of pTIF1B11 to generate the final targeting vector (pTIF1B13, also called pTIF1β$^{(L^{NL}:L)}$ in Fig. 1A).

ES cell selection

The targeting vector was linearized with *Cla*I, purified on sucrose gradient, and electroporated into 129/Sv H1 ES cells (Dierich and Dollé, 1997) as described (Lufkin et al., 1991). After selection with G418 (150 μg/ml), neomycin-resistant clones were expanded, their genomic DNA was prepared, digested with *Bam*HI and analyzed by Southern blotting with the 3′ probe (a 1.2-kb DNA fragment obtained by PCR amplification with the oligonucleotides XN134: 5′-GGTTGTATCTAGAACCACAT-3′ and XF51: 5′-AAGGCTTCGGAGGGGTATAG-3′). Positive clones for homologous recombination were further analyzed after *Eco*RV digestion with the 5′ probe (a 1.5-kb *Eco*RI genomic fragment) and with a *neomycin* probe.

Generation of mutant mice

ES cells bearing an *L3* targeted allele (AO180 and AO122) were injected into C57BL/6 blastocysts. Chimeric mice were backcrossed with C57BL/6 mice, and germ-line transmission of the targeted allele was determined in the agouti offspring by PCR analysis using a sense primer located in intron 3 upstream to the 5′ *Hin*dIII site (primer YD208, 5′-GGAATGGTTGTTCATTGGTG-3′), and an antisense primer located downstream of the 5′ l*ox*P site in the *PGK-Neo* cassette (primer RR189, 5′-AAGCGCATGCTCCAGACTGC-3′). These primers generated a 180-bp DNA fragment from the targeted allele. Mice heterozygous for the targeted *TIF1β* gene (*TIF1β*$^{+/L3}$) were crossed with *cytomegalovirus-Cre* (*CMV-Cre*) transgenic mice (Dupé et al., 1997). Tail DNA of the offspring was analyzed by Southern blotting after *Eco*RV digestion and hybridization with the 5′ probe. Mice positive for the presence of the deleted *TIF1β* allele (*L*–) were crossed with C57BL/6 mice to derive *TIF1β*$^{+/L-}$ heterozygotes devoid of the *Cre* transgene and selected by genomic PCR using a mixture of three primers: sense primer (YD208), antisense primer (VR211, 5′-ACCTTGGCCCATTTATTGATAAAG-3′) located in intron 3 downstream to the 3′ *Hin*dIII site, and antisense primer (TV210, 5′-GCGAGCACGAATCAAGGTCAG-3′) located in exon 16 (see Fig. 2A). Mice heterozygous for the *TIF1β* mutation were intercrossed to generate homozygotes. For the timing of embryo collection, the morning of the day of the vaginal plug was taken as E0.5. Genotypes were determined by Southern blotting and/or PCR on DNA extracted from tails (newborn and adult mice), from the yolk sac (E8.5 to E17.5 embryos), from the entire embryo (E3.5 to E8.0 embryos), or from paraffin-embedded sections as described in Zeitlin et al. (1995).

Histological analysis

Decidua were collected in 10 mM phosphate-buffered saline (PBS), pH 7.2, then fixed in Bouin's fluid for 14 hours, dehydrated and embedded in paraffin. Serial sections were cut at 6 μm and stained with Hematoxylin and Eosin.

In situ hybridization

ISH with ^{35}S-labelled riboprobes was performed as described in Niederreither and Dollé (1998). The probes used included *Brachyury* (a kind gift from D. Herrmann, Freiburg University, Germany) and *TIF1β* (region +1168 to +1695 of the *TIF1β* cDNA; Le Douarin et al., 1996).

Western blot analysis

Proteins from ES whole-cell extracts, prepared by three cycles of freeze-thaw in PBS containing 10% glycerol and a protease inhibitor cocktail, were separated on 8% gel by SDS-PAGE and electrotransferred onto nitrocellulose filters. The filters were incubated with specific antibodies followed by a peroxidase-conjugated anti-mouse IgG secondary antibody and developed using an ECL detection kit (Amersham Pharmacia Biotech.). Antibodies used include anti-TIF1β mAb (1Tb3), raised against recombinant *E. coli*-expressed mouse TIF1β (Nielsen et al., 1999), anti-TIF1β mAb (3Tb1H3), directed against TIF1β (amino acids 20-38) and anti-mRXRα polyclonal antibody, RPRXα(A) (Rochette-Egly et al., 1994).

Whole-mount immunofluorescence

Preimplantation embryos from heterozygote intercrosses were flushed from uteri at E3.5, washed in PBS, fixed in freshly prepared 2% paraformaldehyde in PBS for 4 minutes, permeabilized with PBS containing 0.1% Triton X-100 twice for 5 minutes, and incubated with the anti-TIF1β mAb (1Tb3) overnight at room temperature. An irrelevant antibody (anti-Flag antibody 2FIIB4) was used as a negative control. Embryos were washed twice for 5 minutes in PBS-0.1% Triton X-100, followed by incubation for 1 hour at room temperature with Cy3 indocarbocyanine-conjugated goat anti-mouse IgG. They were stained for DNA with Hoechst 33258. The stained embryos were

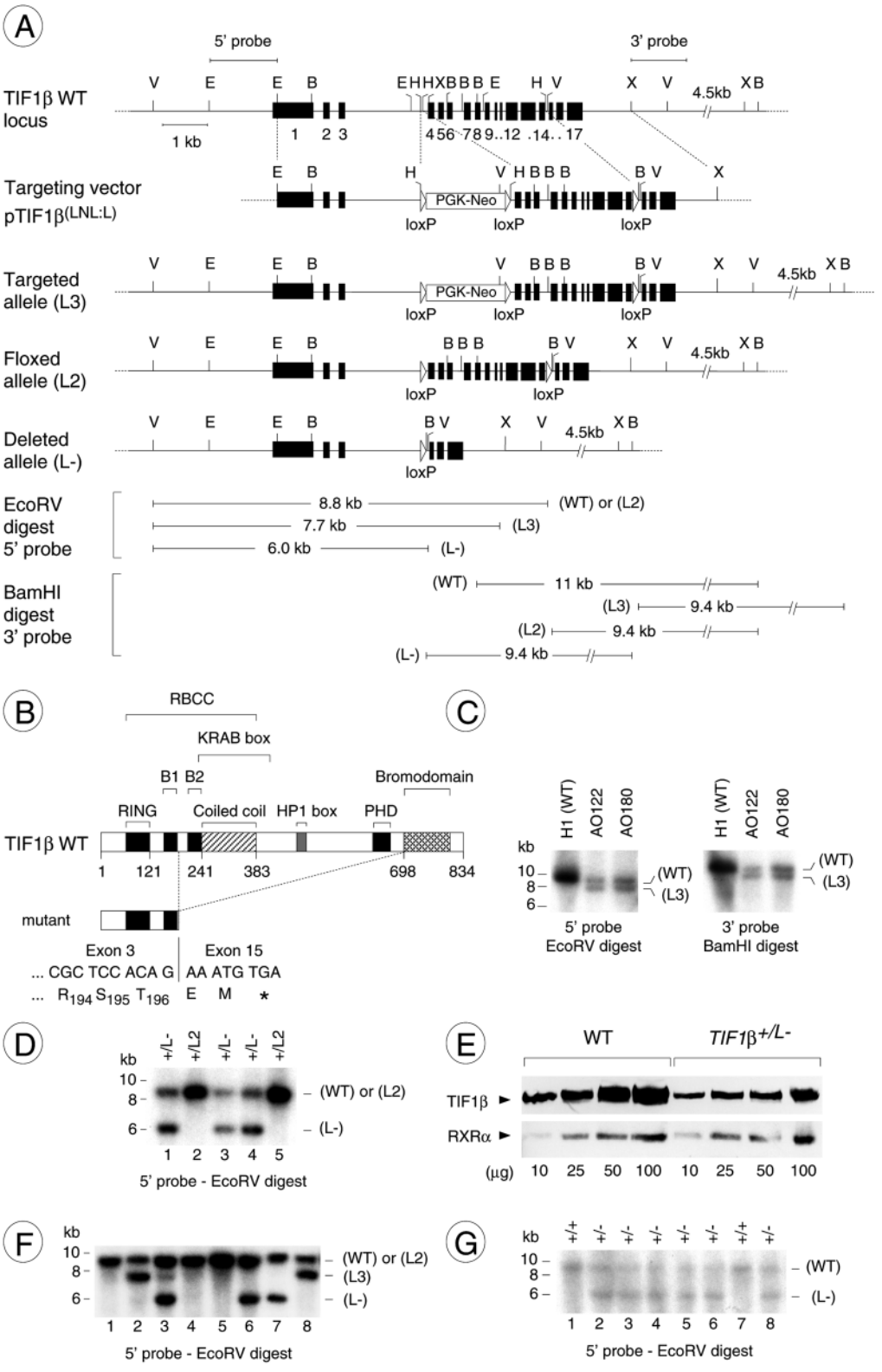

Fig. 1. Targeted disruption of *TIF1β* using the Cre-loxP system. (A) Diagram showing the genomic map of *TIF1β*, the targeting construct, and the targeted allele before (*L3*) and after (*L2* and *L*–) Cre-mediated excision of the selection cassette. Exons are represented as numbered boxes, introns as connecting lines. The three *loxP* sites (open triangles) and the *PGK-Neo* cassette are indicated. The 5′ and 3′ probes are 1.5 kb *Eco*RI-*Eco*RI fragment and 1.2 kb PCR fragment, respectively. The size of the DNA fragments expected with the 5′ probe upon digestion with *Eco*RV and with the 3′ probe upon digestion with BamHI are indicated. Relevant restriction sites: V, *Eco*RV; E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; X, *Xho*I. (B) Schematic representation of wild type (WT) and mutant TIF1β proteins. The structural and functional domains are indicated (Le Douarin et al., 1996, 1998; Nielsen et al., 1999; Ryan et al., 1999). Numbers refer to amino acid positions (Le Douarin et al., 1996). The predicted product of the mutated *TIF1β* cDNA would correspond to a C-terminally truncated protein consisting of the first 196 amino acids of TIF1β. (C) Southern blot analysis of DNAs derived from wild-type (H1) and targeted (AO122 and AO180) ES cells. Genomic DNA was digested with *Eco*RV or *Bam*HI, as indicated, blotted and hybridized with the 5′ probe (left side) or the 3′ probe (right side). (D) Southern blot analysis of DNAs from a series of ES cell clones derived from the AO180 *TIF1β*$^{+/L3}$ ES cell line transfected with the Cre-encoding expression plasmid PIC-Cre. Cre expression led to removal of the selection cassette, generating floxed (*L2*) and deleted (*L*–) alleles (see A). (E) TIF1β protein levels in wild type and *TIF1β*$^{+/L-}$ ES cells. Increasing amounts of whole-cell extracts (10 to 100 μg protein) were analyzed by western blotting using the anti-TIF1β monoclonal antibody 1TB3, directed against the TIF1β carboxy terminus (amino acids 123-834), or the anti-RXRα polyclonal antibody RPRXα(A) (used as a control for the amount of protein extract loaded). (F) Southern blot analysis of representative tail DNA samples derived from 1-week-old offspring of crosses between heterozygous *TIF1β*$^{+/L3}$ mice and *CMV-Cre* transgenic mice. Incomplete excision of the *loxP*-flanked DNA sequences was observed in lane 3. (G) Southern blot analysis of tail DNA derived from 1-week-old offspring of heterozygous *TIF1β*$^{+/-}$ intercrosses.

mounted and photographed with a Leica TCS4D fluorescence confocal microscope.

RESULTS

Targeted disruption of the mouse TIF1β gene

A 7-kb genomic fragment containing the entire mouse *TIF1β* coding sequence (F. Cammas, J.-M. Garnier, P. Chambon and R. Losson, unpublished) was used to generate a targeting vector pTIF1β$^{(LNL:L)}$, in which a *PGK-Neo* selection cassette flanked by two *loxP* sites was introduced into intron 3 and a *loxP* site inserted into intron 14 (Fig. 1A; see Materials and Methods for details). Upon homologous recombination and subsequent Cre-mediated excision, exons 4 to 14 of *TIF1β* together with the selection marker cassette are expected to be deleted, thereby causing a frameshift mutation with an immediate stop of translation in exon 15 (Fig. 1B). The predicted product of this mutated gene would correspond to a C-terminally truncated TIF1β protein lacking all functional domains beyond the B1 motif (Fig. 1B).

The targeting vector pTIF1β$^{(LNL:L)}$ was electroporated into 129/Sv H1 ES cells, and 273 G418-resistant clones were isolated, of which five (including AO122 and AO180) were found to be positive for homologous recombination by Southern blot analysis using 'outside' 5′ and 3′ probes (Fig. 1A,C). All of them carried a single-copy integration at the *TIF1β* locus as revealed by Southern blot analysis with a *Neo* probe (data not shown). One of these targeted ES cell lines (AO180) was transiently transfected with a Cre-encoding expression plasmid (PIC-Cre) to test whether a Cre-mediated excision of the targeted allele (*L3*) could be achieved. Clones, in which a complete excision of the *loxP*-flanked DNA sequences generated a deleted allele (*L–*; Fig. 1A), were isolated (Fig. 1D). The effect of this deletion on *TIF1β* gene expression was then assessed by western blot analysis, using whole cell extracts from wild type and *TIF1β*$^{+/L-}$ ES cells. A single reacting species of the expected size for the TIF1β protein was detected with a monoclonal antibody against the carboxy-terminal end of TIF1β (see Materials and Methods, and Fig. 1E). The reactivity of this protein was decreased by half in the heterozygous ES cells, showing a dosage effect (Fig. 1E). No lower molecular mass species, which might correspond to a shortened translation product from the deleted allele, was detected with a monoclonal antibody raised against the amino terminus of TIF1β (see Materials and Methods; not shown). These results confirm that, as expected, the *TIF1β*$^{L-}$ allele is a null allele.

The two independent *TIF1β*$^{+/L3}$ ES cell lines, AO122 and AO180, were injected into C57BL/6 blastocysts to produce

Table 1. Genotype analysis of TIF1β$^{+/-}$ intercross progeny

	Genotype				
Stage	+/+	+/–	–/–	Resorption	Total
Newborn	24	42	0	-	66
E17.5-8.5	14	35	0	16	65
E8.0	6	5	3*	2	16
E7.5	4	11	4*	1	20
E3.5	13	27	12	-	52

*Embryos are either severely growth-retarded or being resorbed.

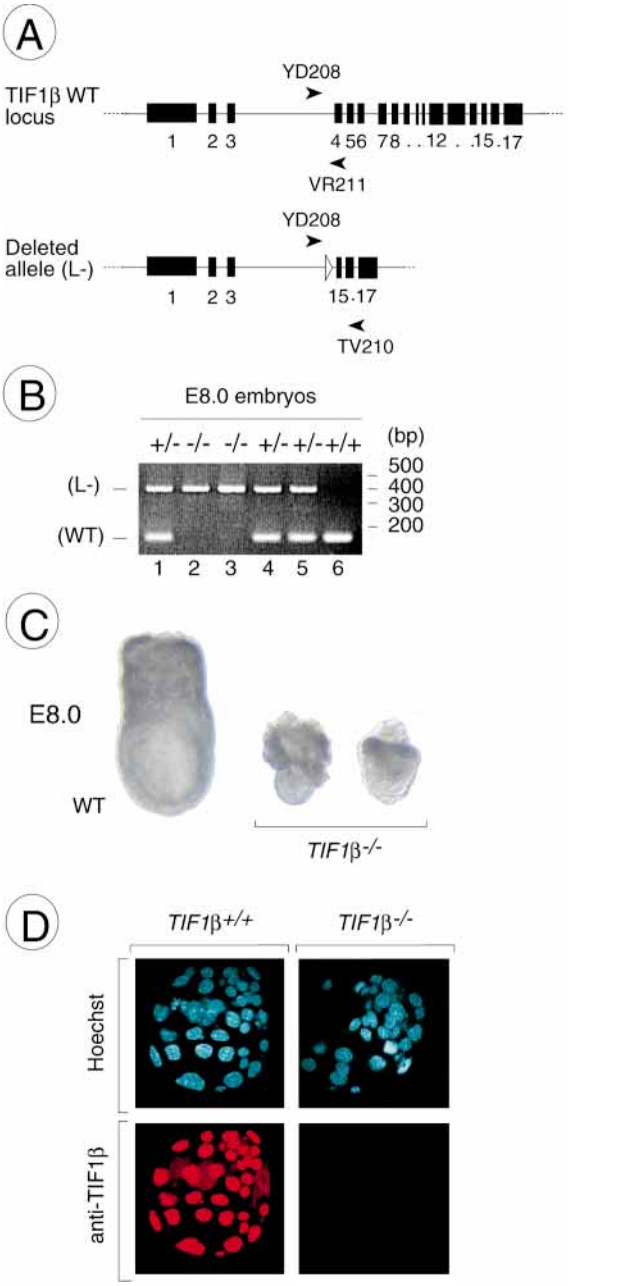

Fig. 2. Analysis of E8.0 wild type and *TIF1β*$^{-/-}$ mutant embryos, and TIF1β immunodetection in E3.5 wild type and mutant embryos. (A) PCR strategy for amplification of wild type (WT) and deleted (*L–*) *TIF1β* alleles. DNA samples were subjected to PCR amplification using a mixture of three primers (see Materials and Methods for sequences). PCR amplification of the wild-type *TIF1β* allele by sense and antisense primers YD208 and VR211 produces a 152-bp DNA fragment (B, lower band), while PCR amplification of the deleted allele by sense and antisense primers YD208 and TV210 produces a 390-bp DNA fragment (B, upper band). (B) Representative genotypic analysis of E8.0 embryos from a *TIF1β*$^{+/-}$ intercross. (C) Dissected wild type (left) and *TIF1β*$^{-/-}$ mutant (right) littermates at E8.0. (D) *TIF1β* detection in wild type (left) and *TIF1β*$^{-/-}$ E3.5 blastocysts. Upper panels show the Hoechst DNA staining, and lower panels correspond to immunodetection with anti-TIF1β antibody.

chimeric mice, and both contributed to the germ line. Mice heterozygous for the targeted *TIF1β* gene (*TIF1β*$^{+/L3}$) were crossed with *cytomegalovirus-Cre* transgenic mice (*CMV-Cre*) expressing the Cre recombinase under the control of the human *cytomegalovirus* promoter (Dupé et al., 1997). Tail DNA of the offspring was analyzed by Southern blot and genomic PCR to detect Cre-mediated excision (Fig. 1F, and data not shown). This cross led to the generation of mosaic animals, in which cells containing either nonexcised (*L3*) or excised (*L*–) DNA coexisted (see Fig. 1F, lane 3). These animals were crossed with wild-type C57BL/6 mice, and *TIF1β*$^{+/L-}$ mice devoid of the *CMV-Cre* transgene were selected, thereafter designated as *TIF1β*$^{+/-}$.

TIF1β is essential for early postimplantation development

Mice heterozygous for the *TIF1β* mutation were viable and fertile. Genotype analysis of progeny from heterozygote intercrosses revealed that 36% were wild type, 64% heterozygous, and none homozygous (Fig. 1G and Table 1), indicating that the *TIF1β* null mutation was recessive embryonic lethal.

To determine the time of embryonic lethality, embryos from heterozygote intercrosses were collected at different times of gestation and genotyped (Table 1). No homozygous mutant embryos were recovered at, or after, E8.5 (Table 1), and the percentage of resorptions at these times was unusually high (25%; Table 1). At E8.0 and E7.5, *TIF1β*$^{-/-}$ mutants were identified by PCR (Table 1 and Fig. 2A,B). These mutants were either severely growth-retarded or undergoing resorption (Fig. 2C and data not shown).

When preimplantation embryos (morulae and blastocysts) collected by uterine flushing at E3.5 were examined and genotyped, we found 23% (12/52) homozygous null embryos that were indistinguishable from heterozygous and wild-type embryos (Table 1). These E3.5 embryos were examined for the presence of TIF1β protein using whole-mount immunofluorescence. Almost all nuclei of wild-type blastocysts, in both the inner cell mass and the trophectoderm, were found to express TIF1β (Fig. 2D). In contrast, no TIF1β staining was detected above background level in null blastocysts (Fig. 2D), suggesting that the survival of these embryos is not due to the persistence of a maternal pool of TIF1β protein. Taken together, results of this retrograde genotypic analysis indicate that TIF1β plays a critical role in early postimplantation development and is most probably non-essential for preimplantation cell viability.

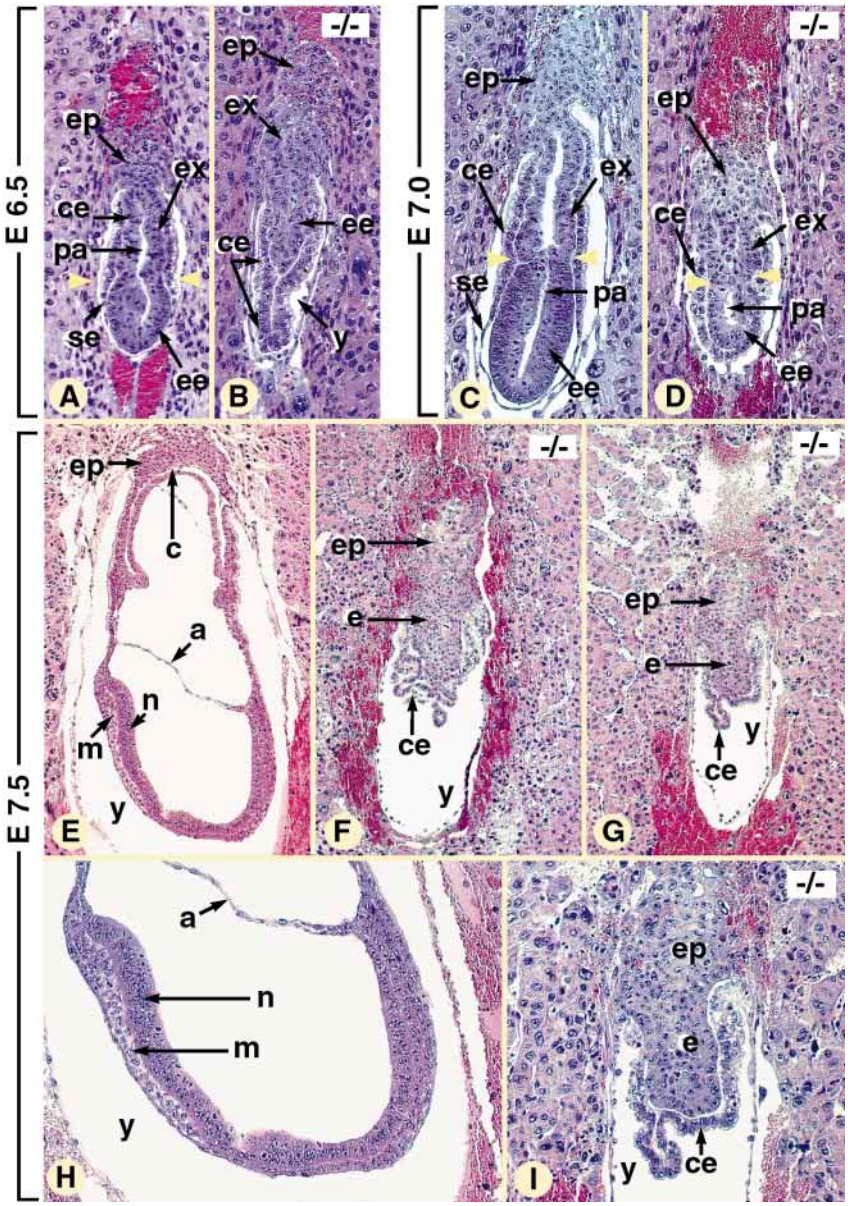

Fig. 3. Histological sections of normal (A,C,E,H) and presumptive *TIF1β*$^{-/-}$ (B,D,F,G,I) embryos at E6.5 (A,B), E7.0 (C,D) and E7.5 (E-I). a, amnion; c, ectoplacental cavity; ce, cuboidal visceral endoderm; e, ectoderm; ee, embryonic ectoderm; ep, ectoplacental cone; ex, extraembryonic ectoderm; m, embryonic mesodem; n, neurectoderm; pa, proamniotic cavity; se, squamous visceral endoderm; y, yolk sac cavity. Arrowheads in A, C and D indicate the position of the circular furrow. ×50 (A-D,H,I); ×25 (E-G).

TIF1β$^{-/-}$ mutant embryos do not undergo gastrulation

Histological sections of embryos from heterozygote intercrosses were analyzed at several developmental stages. At E5.5 no overt abnormalities were found in a total of 39 embryos. The earliest defects were observed at E6.5 and E7.0 in 23% (5 out of 21) of the embryos. Importantly, these defects were not found in embryos from wild-type crosses or *TIF1β*$^{+/-}$/wild-type intercrosses.

In normal E6.5-E7.0 embryos, the proamniotic cavity (pa, Fig. 3A,C) has formed, and the primitive embryonic ectoderm (ee) and primitive extraembryonic ectoderm (ex) are

separated by a circular furrow (yellow arrowheads in Fig. 3A,C). The visceral endodermal cells, which are in close contact with the ectodermal cells, can be subdivided into distinct subpopulations: squamous cells surrounding the embryonic ectoderm (se, Fig. 3A,C) and cuboidal cells, with characteristic apical vacuoles, surrounding the extraembryonic ectoderm (ce, Fig. 3A,C). Abnormal presumptive *TIF1β*$^{-/-}$ embryos at E6.5 (Fig. 3B) and E7.0 (Fig. 3D) were small in size; their proamniotic cavity was barely distinguishable (pa, Fig. 3D) or was absent (Fig. 3B). The frontier between the embryonic and extraembryonic ectoderms was poorly defined (arrowheads in Fig. 3D). The ectoderm, notably its embryonic portion, contained fewer cells than in control embryos (compare Fig. 3C,D). The visceral endoderm consisted exclusively of cuboidal, vacuolated cells, as it is normally the case in E5.5 embryos. Moreover, the presumptive mutant endoderm formed large folds, protruding into the yolk sac cavity (y, Fig. 3B), as if the growth of this tissue continued in the absence of expansion of the overlying primitive ectoderm.

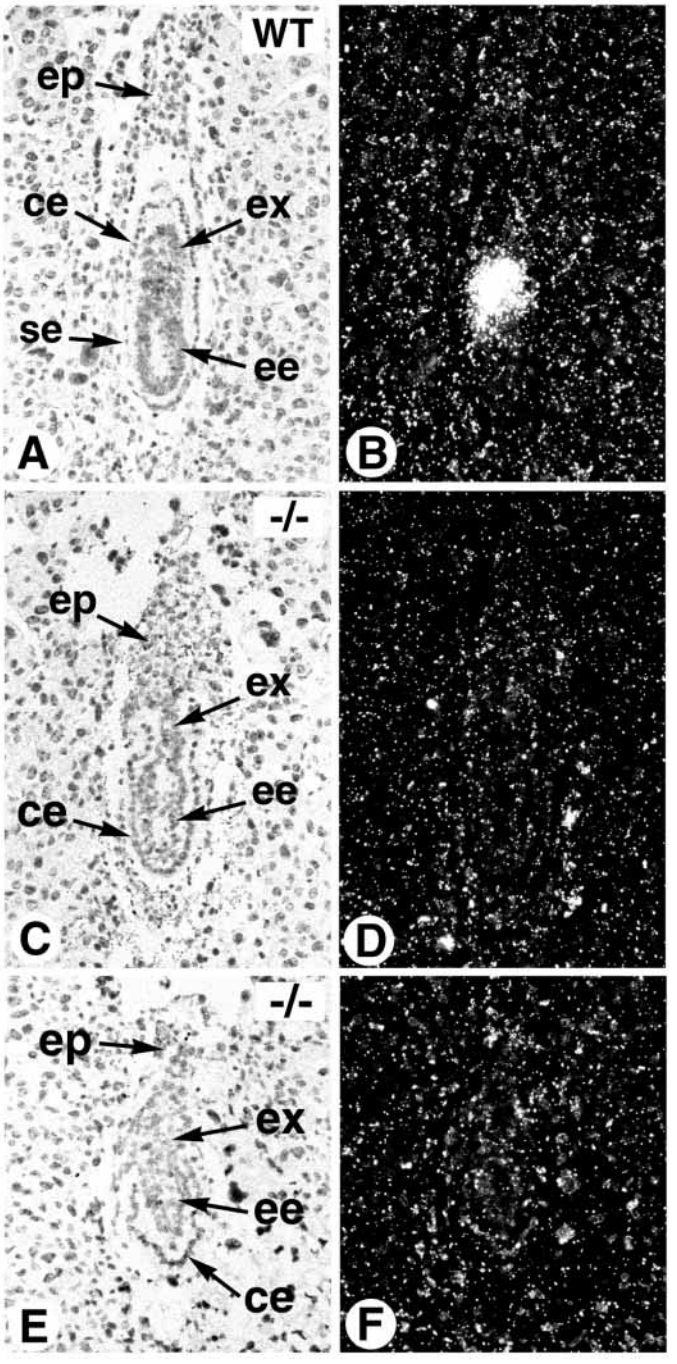

Fig. 4. In situ hybridization analysis of *Brachyury* expression in wild type (WT) (A,B) and *TIF1β*$^{-/-}$ (C-F) littermate embryos collected at E 7.0. (B,D,F) are dark-field views of (A,C,E), respectively, and show the hybridization signal grains as white dots. Mutant embryos were identified by the cuboidal appearance of the entire visceral endoderm layer, and their genotype was confirmed by PCR analysis. The control embryo (A,B) shows a strong signal at the embryonic/extraembryonic junction (i.e the early primitive streak), in contrast to the null embryos which only exhibit background grain. ce, cuboidal visceral endoderm; ee, embryonic ectoderm; ep, ectoplacental cone; ex, extraembryonic ectoderm; se, squamous visceral endoderm. ×100.

At E7.5, control embryos have formed mesoderm (m, Fig. 3E,H) and neurectoderm (n, Fig. 3E,H), as a consequence of gastrulation. Moreover, their extraembryonic ectoderm has organized into an ectoplacental cavity (c, Fig. 3) and amnion (a, Fig. 3E,H). The three presumptive E7.5 *TIF1β*$^{-/-}$ embryos (collected in a litter of 12 embryos) were still alive as assessed by the presence of mitotic figures and absence of large numbers of picnotic nuclei. However, they had not progressed in their development. In particular, they never contained mesodermal cells, their extraembryonic ectoderm was not organized and the visceral endoderm did not differentiate into the squamous cell type (Fig. 3F,G,I).

To further investigate whether mesoderm induction takes place in *TIF1β*$^{-/-}$ embryos, we examined the expression of the gene *Brachyury*, an early marker of the mesoderm lineage, by in situ hybridization (ISH) on sections of embryos collected at E7.0 and genotyped by PCR (Zeitlin et al., 1995). All heterozygous and wild-type embryos analyzed (n=10) exhibited the expected expression of *Brachyury* in the posterior part of the egg cylinder, defining the early primitive streak (Fig. 4A,B, and data not shown). However, no *Brachyury* expression was detected in three *TIF1β*$^{-/-}$ embryos (Fig. 4C-F and data not shown), including one mutant embryo whose size was comparable to that of its control littermates (Fig. 4C,D).

Altogether, these results show that TIF1β is required for egg-cylinder organization and mesoderm induction, and that in its absence, development cannot progress further than the E5.5 stage.

TIF1β expression during early mouse development

The early postimplantation defects of the *TIF1β*$^{-/-}$ embryos prompted us to examine the pattern of *TIF1β* expression before and during gastrulation by ISH. In agreement with the detection of the TIF1β protein in preimplantation E3.5 blastocysts (Fig. 2D), high expression levels of *TIF1β* transcripts were detected in E4.5 implanting embryos (Fig. 5A,B). A weaker ISH signal was seen in uterine stromal cells, while the uterine epithelium was not labelled (Fig. 5A,B). A similar expression pattern was observed in early postimplantation embryos at E5.0 and E5.5 (data not shown). At E6.5, *TIF1β* transcripts continued to be highly expressed in the embryonic ectoderm (ee, Fig. 5C-F). Lower expression was seen in the extraembryonic ectoderm (ex, Fig. 5E,F) and in the ectoplacental cone (ep, Fig. 5E,F). Furthermore, the visceral

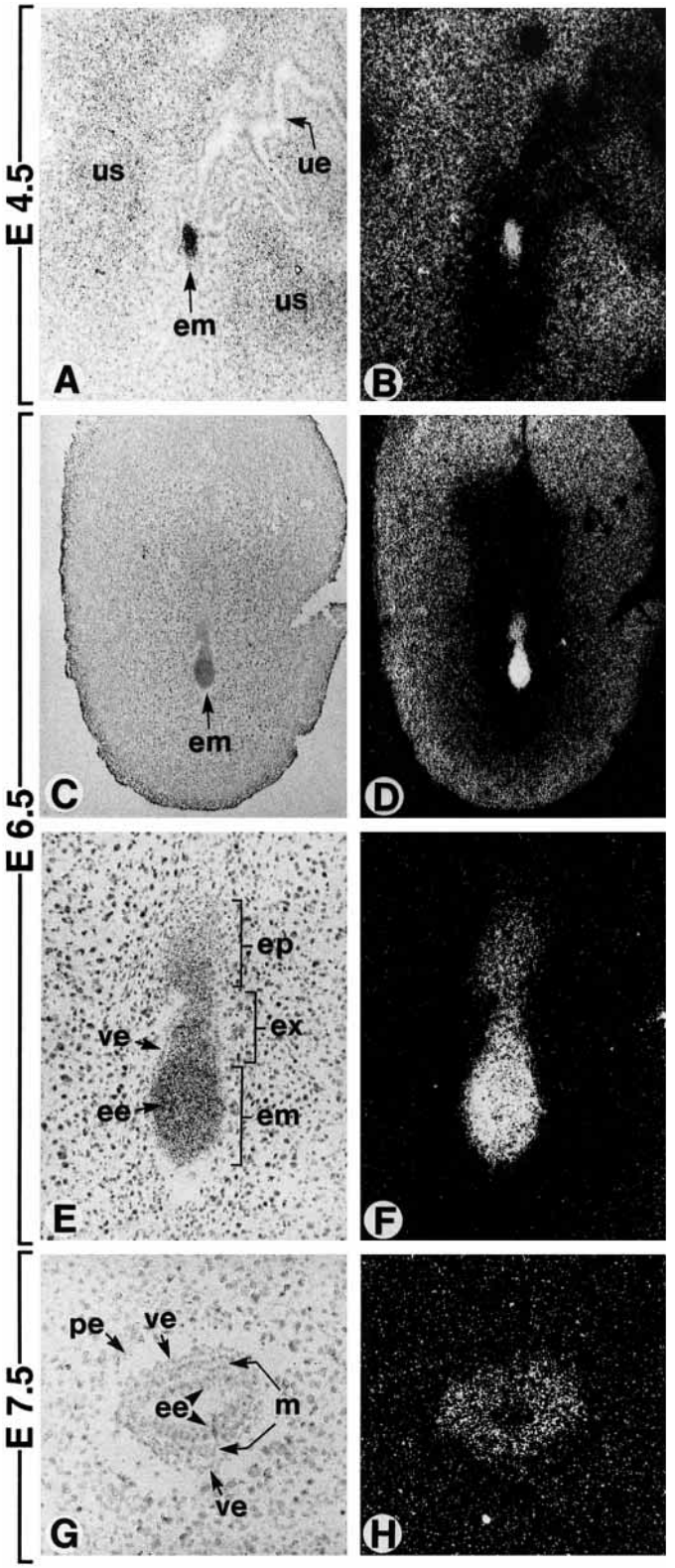

Fig. 5. In situ hybridization analysis of *TIF1β* expression during early mouse development. Selected sections are displayed under bright-field (left) and dark-field (right) illumination, revealing the signal grain in white. (A,B) E4.5 embryo within the uterine lumen. (C,D) Overview of an implantation site at E6.5. (E,F) High-power magnification of the same conceptus. (G,H) Transverse section of an E7.5 embryo. ee, embryonic ectoderm; em, embryo; ep, ectoplacental cone; ex, extraembryonic region; m, mesoderm; pe, parietal endoderm; ue, uterine epithelium; us, uterine stroma; ve, visceral endoderm. ×100 (A,B,E-H); ×40 (C,D).

endoderm layer exhibited little, if any, labelling (ve, Fig. 5E,F). At E7.5, *TIF1β* expression was detected throughout the ectodermal and mesodermal (m) layers, but not the parietal (pe) or visceral endoderm, of the embryos (Fig. 5G,H, and data not shown). Thus, *TIF1β* appears to be highly expressed in E4.5-E6.5 postimplantation embryos prior to gastrulation.

DISCUSSION

We have examined the developmental consequences of a null mutation in the *TIF1β* gene and found that *TIF1β*-deficient embryos implant in the uterine wall, but arrest in their development just prior to gastrulation and are completely resorbed by E8.5, indicating that TIF1β exerts cellular function(s) essential for early postimplantation development.

The earliest stage at which morphologically abnormal embryos were identified in heterozygote intercrosses is E6.5, which corresponds to the onset of gastrulation. Abnormal *TIF1β*$^{-/-}$ embryos showed a reduced cell number in the embryonic ectoderm, no evidence of mesoderm formation, and an altered morphology in their visceral endoderm that fails to acquire the flattened morphology normally appearing at the early gastrulation stage. Interestingly, a lack of the squamous cell population of visceral endoderm is also associated with embryonic defects in null mutants for the *TGFβ*-related gene *nodal* (Iannaccone et al., 1992), the type I activin receptor *ActRIB* gene (Gu et al., 1998), the EGF-CFC family gene *Cripto* (Ding et al., 1998), the tumor suppressor gene *SMAD2* (Weinstein et al., 1998) and the X-ray cross-complementing *Xrcc1* gene (Tebbs et al., 1999). The molecular basis of this abnormal endodermal phenotype is unknown. The finding that this abnormality was retained in a number of *Xrcc1/Trp53* double-mutant embryos, which initiated gastrulation, indicates that it does not result from a defect in initiation of gastrulation (Tebbs et al., 1999). However, it may be the consequence of a non-cell-autonomous effect in the case of *nodal* (Conlon et al., 1994), *ActRIB* (Gu et al., 1998) and *Cripto* (Ding et al., 1998) mutants, as these genes are not expressed in visceral endoderm at the early gastrulation stage. Similarly, the abnormal endodermal phenotype of *TIF1β* null mutants may reflect a non-cell-autonomous defect, as *TIF1β* transcripts do not appear to be expressed in visceral endoderm, as judged from ISH experiments. Studies with chimeric embryos are required to determine whether the primary defect of the *TIF1β* mutation is of embryonic or extraembryonic origin.

In agreement with our *TIF1β* expression data in ES cells, the TIF1β protein could be revealed in preimplantation embryos at the blastocyst stage (E3.5). As it could not be detected in *TIF1β*$^{-/-}$ blastocysts, its synthesis must correspond to an early event in mouse embryogenesis. Note that even though they do not contain any detectable TIF1β protein, the mutant blastocysts are morphologically indistinguishable from their wild-type littermates. Moreover, they are able to hatch from the zone pellucida, adhere and develop trophoblastic giant cells after 4 days of outgrowth (our unpublished results). Thus, *TIF1β* is not required for cell viability at preimplantation stages.

TIF1β belongs to the family of *TIF1* genes whose physiological functions are unknown (Le Douarin et al., 1996; see Introduction). Our present data provides the first demonstration that a member of this family plays an essential function in vivo. Our study also provides genetic evidence that, at least during early embryogenesis, the members of this family, though structurally related, exert distinct, non-redundant functions. Based on biochemical and transient cotransfection data, TIF1β, but neither TIF1α nor TIF1γ, was reported to interact with and act as a corepressor for several members of the KRAB-ZFP family (Friedman et al., 1996; Kim et al., 1996; Moosmann et al., 1996; Venturini et al., 1999; Nielsen et al., 1999; see Introduction). Whether the embryonic lethality of *TIF1β*$^{-/-}$ embryos could be related to a failure of KRAB-ZFP-mediated silencing is unknown, but strongly suggested by several lines of evidence that point to an important role of the *KRAB-ZFP* gene family in regulating cell differentiation and development. A number of KRAB-ZFPs have been shown to exhibit temporally and spatially regulated expression patterns, at specific stages of development (Lange et al., 1995; Shannon and Stubbs, 1999), in germ cells (Bellefroid et al., 1998; Ogawa et al., 1998), in hematopoietic lineages (Mark et al., 1999; Liu et al., 1999), as well as in a limited number of organs (Witzgall et al., 1993; Mazarakis et al., 1996; Tekki-Kessaris et al., 1999). Moreover, several *KRAB-ZFP* genes have been involved in human diseases on the basis of their chromosomal location (Mannens et al., 1996; Dreyer et al., 1998; Rippe et al., 1999), or associated with embryonic lethal mutations (Shannon and Stubbs, 1999). In view of their possible important biological functions and their abundance in the genome, a dysfunction of KRAB-ZFPs may underlie the severe mutant phenotype of *TIF1β*-deficient embryos.

In summary, we have shown that *TIF1β* is essential for the development of the mouse embryo at the egg-cylinder stage and for subsequent gastrulation. That *TIF1β* may have additional role(s) during and after gastrulation is suggested by its persistent expression during late embryogenesis and adult life (our unpublished results; Kim et al., 1996). Conditional knockouts of *TIF1β* at specific developmental stages and in specific tissues are in progress to investigate this possibility.

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