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Transcription Factor TEAD2 Is Involved in Neural Tube Closure

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Abstract

Summary—TEAD2, one of the first transcription factors expressed at the beginning of mammalian development, appears to be required during neural development. For example, *Tead2* expression is greatest in the dorsal neural crest where it appears to regulate expression of *Pax3*, a gene essential for brain development. Consistent with this hypothesis, we found that inactivation of the *Tead2* gene in mice significantly increased the risk of exencephaly (a defect in neural tube closure). However, none of the embryos exhibited spina bifida, the major phenotype of *Pax3* nullizygous embryos, and expression of *Pax3* in E11.5 *Tead2* nullizygous embryos was normal. Thus, *Tead2* plays a role in neural tube closure that is independent of its putative role in *Pax3* regulation. In addition, the risk of exencephaly was greatest with *Tead2* nullizygous females, and could be suppressed either by folic acid or pifithrin- α . These results reveal a maternal genetic contribution to neural tube closure, and suggest that *Tead2*-deficient mice provide a model for anencephaly, a common human birth defect that can be prevented by folic acid. *genesis* 45:577–587, 2007.

Keywords

exencephaly; anencephaly; TEAD; *Pax3*; fetal brain

INTRODUCTION

Mammals express four closely related and evolutionarily conserved transcription factors, the prototype for which was isolated from human cells as TEF1 (Davidson *et al.*, 1988) and later renamed TEAD1 by the human genome project. A mouse homologue was subsequently identified (TEAD1/TEF1; Blatt and DePamphilis, 1993; Shimizu *et al.*, 1993), as well as three additional members of the TEAD family (*TEAD2*/TEF4; Jacquemin *et al.*, 1996; Kaneko *et al.*, 1997; Yasunami *et al.*, 1995, *TEAD3*/TEF5; Kaneko *et al.*, 1997; Yasunami *et al.*, 1996, and *TEAD4*/TEF3; Jacquemin *et al.*, 1996; Yasunami *et al.*, 1996; Yockey *et al.*, 1996). These transcription factors are defined by the presence of the 72 amino acid TEA DNA binding domain in their N-terminal half that is virtually identical among TEAD proteins and by the conspicuous homology among their C-terminal halves (Kaneko and DePamphilis, 1998). This conservation of structure is reflected in the ability of these proteins to bind the same transcriptional coactivator proteins (Mahoney *et al.*, 2005; Vassilev *et al.*, 2001) and to substitute for TEAD proteins in other organisms (Deshpande *et al.*, 1997). Nevertheless, despite their structural similarities, the four mammalian TEAD proteins are not functionally redundant, because mice lacking TEAD1 fail to develop a proper heart and die between

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embryonic day 11 (E11) and E12 (Chen *et al.*, 1994), whereas mice lacking TEAD4 arrest development before E3.5 (Yagi *et al.*, 2007). Here we address the role of *TEAD2*.

The mouse *Tead2* gene is expressed first as a maternal RNA that is recruited to polysomes and presumably translated (Wang and Latham, 2000) and then subsequently transcribed at the onset of zygotic gene expression during the 2-cell stage of development (Kaneko *et al.*, 1997). *Tead2* is the most abundantly expressed TEAD protein in embryos during their first 7 days of development (Kaneko *et al.*, 1997), and its postimplantation embryonic expression pattern suggests that it plays a critical role in neural development (Milewski *et al.*, 2004; Sawada *et al.*, 2005).

Mouse *Tead2* is expressed at basal levels in most adult tissues (Jacquemin *et al.*, 1996; Kaneko *et al.*, 1997; Yasunami *et al.*, 1995), but it appears to play a critical role during development. Originally isolated as a gene that is expressed preferentially in fetal neural precursor cells (Yasunami *et al.*, 1995), *Tead2* was shown later to be expressed in proliferating fetal neuroepithelial cells, but not in postmitotic neurons (Jacquemin *et al.*, 1996; Yasunami *et al.*, 1995). In fact, *Tead2* expression decreases in perinatal brain (postnatal day 0) relative to fetal brain (E12), revealing that *Tead2* is downregulated during neural differentiation (Matsuki *et al.*, 2005). This finding is consistent with the role proposed for *Tead2* as a regulator of *Pax3* expression, a gene that is essential for cell migration and differentiation in the neural crest (Milewski *et al.*, 2004). *Tead2* may be involved in the production or maintenance of the stem cells responsible for embryonic development, because it is expressed in embryonic, neural, and hematopoietic stem cells (Ramalho-Santos *et al.*, 2002). Moreover, since these analyses compared transcripts expressed preferentially in proliferating stem cells relative to terminally differentiated cells, a common thread ties together all of the *Tead2* expression studies: *Tead2* appears to be expressed specifically in proliferating cells, although the absence of *Tead2* expression in proliferating lymphoma and myeloma cell lines (Davidson *et al.*, 1988; Kaneko *et al.*, 2004) suggests that *TEAD2* protein is not required for cells to proliferate per se but facilitates the process.

In an effort to identify a specific requirement for *TEAD2* protein during mammalian development, the *Tead2* gene was inactivated in the mouse. Consistent with its strong expression during neural development in postimplantation embryos, embryos lacking a functional *TEAD2* protein were discovered to be at risk for exencephaly, a rare and fatal defect in which the brain protrudes into the amniotic cavity as a consequence of the failure of the neural tube to close during embryonic development. Moreover, the risk of *TEAD2*-dependent exencephaly was linked to *TEAD2* nullizygous mothers, but not to *Pax3* gene expression. *Tead2* nullizygous embryos that escaped exencephaly produced viable, fertile adults with no visible abnormalities. These results reveal that *TEAD2* plays an important role during closure of the embryonic neural tube, although not necessarily through regulation of *Pax3* expression. Furthermore, *TEAD2* is not required to sustain adult animals, although it may facilitate tissue regeneration (Ramalho-Santos *et al.*, 2002; Zhao *et al.*, 2006).

RESULTS

Construction of a *Tead2* Conditional Knock-Out Allele

To create *Tead2* heterozygous mutant mice, the *Tead2* allele (Fig. 1A) was mutated by homologous recombination with a genetically altered targeting vector in mouse embryonic stem cells. The targeting vector consisted of upstream loxP site in intron 1 and downstream loxP site and a neomycin resistance gene in intron 3 (Fig. 1B; Materials and Methods). The targeting vector was linearized and then electroporated into 129/SvJ embryonic stem cells (contain genes for agouti coat color) and screened for clones that had undergone homologous recombination. They were then injected into C57BL/6J blastocysts (black coat color),

transferred to foster mothers, and chimeric pups were obtained and genotyped (data not shown). The resulting chimeras containing the mutant allele were mated with C57BL/6J females to obtain agouti-colored pups, indicative of germline transmission. Genomic DNA prepared from tails of agouti mice was screened for the presence of the mutant allele by PCR (data not shown), and the results confirmed by digesting genomic DNA with either XhoI or NheI restriction endonuclease (Fig. 1B) and then identifying the appropriate DNA fragment by Southern blotting-hybridization (Fig. 2A) using either a 5'- or 3'-specific DNA probe (Fig. 1B). Both assays confirmed that homologously recombined mutant allele was transmitted through the germline. These mice were then mated with Ella-Cre⁺ mice (Lakso *et al.*, 1996) in order to delete exons 2 and 3 from the mutant allele by Cre-dependent homologous recombination between the two loxP sites (Fig. 1B,C). Ella promoter activity in mice is restricted to oocytes and preimplantation embryos (Dooley *et al.*, 1989). Genomic DNA was prepared from tails of the progeny, and subjected to Southern blotting hybridization by digesting it with EcoRV restriction endonuclease and identifying the appropriate DNA fragment using the “KO probe” (Fig. 1C).

The net result was that exons 2 and 3, encoding the N-terminal 99 amino acids of the 445 amino acid TEAD2 protein, were deleted in the *Tead2* knock-out (KO) allele (Fig. 1C). This deletion removed the translation start codon, 82% of the TEA DNA binding domain, and the putative *Tead2* enhancer element that resides within 3'-half of intron 1 (Zhao *et al.*, 2006; data not shown). The *Tead2* transcription start site lies within exon 1, ~1.4 kb upstream of exon 2 (Kaneko *et al.*, 2004). Therefore, this deletion should reduce the overall level of *Tead2* mRNA, prevent translation of full-length *Tead2* protein, and in the event that some *Tead2* protein is produced, insure that it is not functional.

Inactivation of the *Tead2* Gene Impedes, but does not Prevent, Development

Mice that were heterozygous for the *Tead2* KO allele (Fig. 2B, “parents”) were mated. Their offspring were genotyped both by PCR analysis (Fig. 2C) and by Southern blotting hybridization analysis (Fig. 2B, “litter”). Both assays confirmed Cre-mediated deletion. The genotypic ratio of *Tead2*^{+/+}, *Tead2*^{+/-}, and *Tead2*^{-/-} mice from these heterozygous matings revealed a skewed ratio in which the number of *Tead2*-nullizygous mice was less than expected from simple Mendelian segregation (Table 1), suggesting that embryonic development was hindered in the absence of TEAD2 but not completely blocked. The size, morphology, behavior, and reproduction of *Tead2* nullizygous mice were indistinguishable from wild-type (WT) mice with the same mixed genetic background, suggesting that maintenance of adult functions does not require *Tead2*.

Tead2 Nullizygous Mice do not Contain *Tead2* WT mRNA

Previous studies have reported that *Tead2* mRNA is present in embryonic stem cells (Kaneko *et al.*, 2004; Ramalho-Santos *et al.*, 2002), in fetal neural tissue from postimplantation embryos (Yasunami *et al.*, 1995), and in adult lung tissue (Kaneko *et al.*, 1997), but it is virtually undetectable in adult brain (Yasunami *et al.*, 1995). Northern blotting hybridization analysis confirmed that *Tead2* mRNA was absent from adult brain tissue (Fig. 3A, lane 1), although abundant in embryos undergoing brain development (Fig. 3A, lane 2), in embryonic stem cells (Fig. 3A, lane 4), and in adult lung (Fig. 3A, lane 5). In contrast, *Tead2* mRNA was greatly reduced in these tissues from mice lacking one or both *Tead2* genes (Fig. 3A, lanes 3, 6, 7). These results were consistent with the fact that the *Tead2* KO allele retains the native *Tead2* promoter but lacks the *Tead2* enhancer (Fig. 1A).

To determine whether or not *Tead2* exons 2 and 3 had been deleted from *Tead2* mRNA produced in *Tead2* nullizygous mice (Fig. 1C), total RNA prepared from lung or ovarian tissue was assayed for *Tead2* exons 2 through 6 (Fig. 1A) by reverse-transcription/polymerase chain

reaction (RT-PCR) (Kaneko *et al.*, 1997). As expected, the region from exons 2 through 6 was detected from either *Tead2* WT or heterozygous mice, but not from RNA in *Tead2* nullizygous mice (Fig. 3B,C). Hence, none of the mRNA produced from the *Tead2* mutant allele could express a functional TEAD2 protein.

TEAD2 is not Required for Expression of *Fgfr4*, *Tead4*, or *Pax3* Genes

TEAD2 has been hypothesized to regulate expression of the *Fgfr4* gene in the lung (Weinstein *et al.*, 1998; Zhao *et al.*, 2006), and perhaps also in granulosa cells of developing follicles where the same cells express both *Fgfr4* (Puschek *et al.*, 1997; Weinstein *et al.*, 1998) and *Tead2* genes (Kaneko *et al.*, 1997). However, no change in *Fgfr4* expression was detected either in lung (Fig. 3B) or in ovaries (Fig. 3C) from *Tead2* WT, heterozygous or nullizygous females.

Loss of TEAD2 may result in compensatory expression of other TEAD proteins. For example, *Tead4* and *Tead2* are both expressed in preimplantation embryos (Yagi *et al.*, 2007) and in adult lung (Yockey *et al.*, 1996). However, expression of *Tead4* (Fig. 3B,C) was not dependent on *Tead2* expression. Similar results were obtained with *Tead1* (data not shown). Therefore, TEAD2 does not appear to be involved in regulating expression of other *Tead* genes.

TEAD2 also has been hypothesized to regulate expression of *Pax3* (Milewski *et al.*, 2004). Hence, loss of TEAD2 would be expected to downregulate expression of the *Pax3* gene. However, when RNA was prepared from individual E11.5 embryos from a single heterozygous intercross and examined for *Pax3* expression, *Pax3* mRNA levels were the same, regardless of the presence or absence of TEAD2 (Fig. 4A).

TEAD2 is not Required for Follicular Development

Tead2 expression in ovary is restricted primarily to mural granulosa cells, suggesting that it may play a role in ovarian function (Kaneko *et al.*, 1997). To test this hypothesis, immature females that were either heterozygous or nullizygous for *Tead2* were injected with pregnant mare's serum (PMS) to mimic the action of follicle stimulating hormone. PMS induces expression of several genes in immature females, including aromatase (*Cyp19*) and leutenizing hormone/choriogonadotropin receptor (*Lhcgr*) from ovarian somatic cells (Couse *et al.*, 2005). However, contrary to this hypothesis, these enzymatic changes were not dependent on a functional *Tead2* gene either in unstimulated or PMS stimulated ovaries (data not shown).

TEAD2 is Involved in Neural Tube Closure

The fraction of *Tead2* nullizygous pups born from heterozygous intercrosses was less than expected (Table 1). This deviation from Mendelian segregation was not due simply to genetic background effects, because heterozygous intercrosses of a conditional KO allele in *Dkk11* (located 3 kb upstream of *Tead2* gene; Kaneko and DePamphilis, 2000) that was constructed in the same manner as the conditional KO allele in *Tead2* and in the same mouse strain yielded normal Mendelian segregation ratios (Kaneko *et al.*, manuscript in preparation). Deviation from Mendelian genetics could result from an increased risk for exencephaly, a developmental abnormality wherein parts of the fetal brain lie outside the neural tube (Harris and Juriloff, 2007). The "natural" incidence of exencephaly in many inbred strains is less than 0.5% (Juriloff *et al.*, 2001, and references therein). Two of 32 embryos from *Tead2* heterozygous intercrosses that were examined between E11.5 and E13.5 exhibited exencephaly (Fig. 4, data not shown). For example, as early as E11.5, a nullizygous embryo exhibited clear abnormalities in the cranial region when compared to a normal heterozygous littermate (Fig. 4A,C).

This diagnosis was confirmed by mating *Tead2* nullizygous females with heterozygous males so that ~50% of the embryos would be nullizygous for *Tead2*. Pregnant females were euthanized, and each litter was examined for abnormally developing embryos. Of 54 embryos

examined, 19% were exencephalic (Table 2A). Moreover, 30% of the *Tead2* nullizygous embryos were exencephalic, and surprisingly 7% of the *Tead2* heterozygous embryos were exencephalic. For example, several exencephalic embryos were found among litters at E13.5 (Fig. 5). Another litter at E16.5 contained an exencephalic embryo that was clearly alive (as evidenced by its beating heart; Fig. 6A), a dead exencephalic embryo that was in the process of being resorbed (Fig. 6B; as evidenced by lack of beating heart, absence of blood vessels, and skin disintegration), as well as normal embryos (Fig. 6C).

Folic acid has been reported to prevent 70% of human neural tube defects (NTDs; Fleming and Copp, 1998). To determine whether or not folic acid can suppress *Tead2*-dependent exencephaly, nullizygous pregnant mothers were injected with folic acid on E8.5 and E9.5, and the embryos were examined on E13.5. Folic acid reduced the risk of *Tead2*-dependent exencephaly about threefold (Table 3A) when compared to control treatment (Table 3C).

Pifithrin- α , an inhibitor of p53-dependent apoptosis (Murphy *et al.*, 2004), has been reported to rescue NTDs in *Pax3*-deficient embryos (Pani *et al.*, 2002). Similarly, pifithrin- α also prevented *Tead2*-dependent exencephaly to the same extent as did folic acid (Table 3B), suggesting that TEAD2 may regulate neural tube closure by inhibiting p53-dependent apoptosis, rather than by inducing neural tube-specific gene expression.

If the risk for exencephaly rested solely on *Tead2* genotype of the conceptus, the risk for exencephaly would be the same regardless of which parent was nullizygous for *Tead2* gene. To test this hypothesis, nullizygous males were mated to heterozygous females. The result was a substantial reduction in the incidence of exencephaly. Of 54 embryos examined, only 4% were exencephalic, and both of these were *Tead2* nullizygous (Table 2B). The frequency of exencephaly was fivefold greater when the mother was nullizygous for *Tead2* rather than when the father was nullizygous. Thus, the risk for *Tead2*-dependent exencephaly is much greater when the mother is missing both copies of a functional *Tead2* gene.

DISCUSSION

Exencephaly in mice is a defect in neural tube closure that is equivalent to anencephaly in humans. NTD, which includes anencephaly, appears in one out of one thousand births making it the most common form of birth abnormality in humans. Although specific mutations in mice can cause NTDs, multiple genes are thought to be involved in human NTDs (Juriloff and Harris, 2000). Therefore, the mouse models most relevant for clinical implications are those that exhibit incomplete penetrance (Juriloff *et al.*, 1989).

Neural tube closure begins when the epithelial cells lining the neural folds “elevate,” and fusion begins at four separate sites from the cranial to the caudal regions (Juriloff and Harris, 2000). Exencephaly results from failure of the cranial neuroepithelial cells to elevate at or near Closure 2. The extent of penetrance (risk) for exencephaly in *Tead2* nullizygous embryos was consistent with that observed for other murine genetic defects that give rise to exencephaly (Harris and Juriloff, 2007). For example, only 30% of mice that completely lack Closure 2 develop exencephaly even though one would expect 100% penetrance (MacDonald *et al.*, 1989). However, genetic background can also greatly affect penetrance in exencephaly (Juriloff *et al.*, 1991). For example, only 30% of *Pax2* mutants develop exencephaly in C57BL/6J mice, but 100% do so in 129/Sv mice (Schwarz *et al.*, 1997). Therefore, it is possible that penetrance of *Tead2*-dependent exencephaly can be increased by backcrossing to another strain.

Studies both in humans and in mice have shown that administration of a variety of metabolites to pregnant females can suppress NTDs (Blom *et al.*, 2006; Harris and Juriloff, 2007). However, little is known regarding the mechanism by which these supplements work, since either the mother and/or the conceptus could be the primary target. Moreover, whether or not

neural tube closure requires a unique maternal genetic contribution is not yet clear. In this regard, it is noteworthy that *Tead2*-dependent exencephaly was dependent on the maternal genotype (Table 2). The incidence of exencephaly was 5-times greater when the mother was *Tead2*^{-/-} compared to when the father was *Tead2*^{-/-}. Moreover, when the mother was *Tead2* nullizygous, a fraction of exencephalic embryos were *Tead2* heterozygous, revealing that, under these conditions, even a single *Tead2* mutant allele could increase the incidence of exencephaly. In contrast, when the mother was *Tead2* heterozygous, all of the exencephalic embryos were *Tead2* nullizygous, regardless of whether the *Tead2*^{+/-} females had been mated to *Tead2*^{-/-} (Table 2B) or *Tead2*^{+/-} (Fig. 4, data not shown) males. The fact that embryos produced by *Tead2* nullizygous females have a greater incidence of exencephaly than those from heterozygous females reveals the existence of a maternal contribution for neural tube closure. To our knowledge, only one other study has examined the maternal genetic contribution to exencephaly, and they concluded that exencephaly is controlled largely by genes active in the pregnant female (Beck, 1999). Finally, the risk of exencephaly was reduced about threefold by intraperitoneal administration of folic acid to pregnant females (Table 3), suggesting that the maternal effect in *Tead2*-dependent exencephaly is linked to folic acid metabolism, as previously reported for anencephaly in humans (Fleming and Copp, 1998). Hence, the maternal genetic effect observed in *Tead2* nullizygous females might provide an important model for human NTDs.

Previous studies strongly implicated TEAD2 as a regulator of *Pax3* expression in the neural crest (Milewski *et al.*, 2004). This is consistent with the fact that *Tead2* is expressed exclusively in mitotic neuroepithelial cells during fetal brain development (Jacquemin *et al.*, 1996; Yasunami *et al.*, 1995). Moreover, as previously reported for *Pax3* mutants (Pani *et al.*, 2002), injection of pregnant females with pifithrin- α , an inhibitor of p53 signaling (Murphy *et al.*, 2004), suppressed exencephaly in *Tead2* nullizygous females (Table 3B), suggesting that inhibition of apoptosis can rescue this NTD. However, the NTD seen in *Tead2* nullizygous mice contrasts with that observed in *Pax3* nullizygous mice. *Pax3* mutants not only exhibit complete penetrance of a NTD, but they exhibit both exencephaly and spina bifida aperta (Harris and Juriloff, 2007; Juriloff and Harris, 2000). The absence of a more severe phenotype in *Tead2* nullizygous embryos reflects the fact that expression of *Pax3* at E11.5 was not suppressed in embryos lacking *Tead2* (Fig. 4A,B), revealing that alternative mechanisms are available for maintaining *Pax3* expression. Nevertheless, the fact that only some *Pax3* mutants show exencephaly is consistent with low penetrance by *Tead2* inactivation. Perhaps the exencephalic defect in *Tead2* nullizygous embryos is mediated through reduction in *Pax3* expression specifically at areas around neural crest cells at or near Closure 2.

The results presented here confirm that TEAD2 plays a role in brain development through its role in neural tube closure, but reveal that TEAD2 is indispensable neither for this process nor for the production and maintenance of viable, fertile adult animals. Two explanations are possible. First, TEAD2 is simply one of several factors that determine the level of an effector gene, and therefore the absence of TEAD2 reduces, but does not eliminate, production of the effector gene product. Second, other transcription factors, possibly other members of the TEAD family, can substitute for TEAD2 function. Furthermore, although *Tead2* mRNA is detectable in adult tissues (Jacquemin *et al.*, 1996; Kaneko *et al.*, 1997), the levels are much lower in comparison to *Tead2* mRNA in embryonic cells (Fig. 3). Thus, once embryonic development is completed, the need for TEAD2 in adult tissues may be minimal. Nevertheless, given the high level of *Tead2* expression in adult neural stem cells (Ramalho-Santos *et al.*, 2002; data not shown) and in regenerating muscle (Zhao *et al.*, 2006), defects in *Tead2* nullizygous mice may only manifest themselves with age.

Finally, it is notable that of the four *Tead* genes in mammals, the biological roles for three of them (*Tead1*, *Tead2*, and *Tead4*) have now been identified through genetic mutations.

Remarkably, despite the fact that all four genes have identical DNA binding domains and bind the same transcriptional coactivators, each of them plays a unique role in mammalian development. TEAD1 is involved in cardiovascular development (Chen *et al.*, 1994). TEAD4 is involved in preimplantation development (Yagi *et al.*, 2007), and TEAD2 is involved in brain development (this report).

METHODS

Conditional *Tead2* Knockout Allele

The targeting vector (Fig. 1B) was constructed as described using recombineering methods (Liu *et al.*, 2003; described in detail at <http://recombineering.ncifcrf.gov/>). Briefly, BAC clone 75.6i (Genome Systems; Kaneko and DePamphilis, 2000) was introduced into bacterial strain EL350 by electroporation. Region upstream of *Tead2* promoter (nucleotides 1055501–1056417; Genbank NW_000319.1) was amplified using primers D and E (Table 4), Pfu Taq polymerase (Strata-gene) and BAC clone 75.6i as template. This amplicon was digested with *SpeI* and *HindIII*. Region within *Tead2* intron 7 (nucleotides 1065309–1065555; Genbank NW_000319.1) was amplified using primers F and G, and the amplicon was digested with *HindIII* and *XbaI*. These two amplicons were combined and then ligated into pL253 that had been digested with *XbaI* and *SpeI*. The resulting plasmid was digested with *HindIII*, purified with a Qiagen miniprep spin column, and electroporated into EL350 containing BAC clone 75.6i, previously treated at 42°C for 15 min to obtain “gap repaired plasmid.”

Oligonucleotides encoding the FLAG-HA polypeptide (primers H and I; Table 4) were annealed, phosphorylated, and ligated to gap repaired plasmid that had been digested with *BamHI*. This placed the FLAG-HA sequence just downstream of the first ATG (methionine) codon within exon 2 and in the same reading frame as the *Tead2* gene.

A loxP site was introduced into intron 1 (Fig. 1B) using a mini-targeting vector that contained phosphoglycerol kinase promoter driving a neomycin resistance gene (PGK-Neo) flanked by loxP sites. The “right-arm” was constructed by combinatorial PCR. PCR was done by either using primers J and K (Table 4), and BAC 75i6 as template, or using primers H and L, and FLAG-HA containing gap repaired plasmid as template. There was an 18-nucleotide overlap between primers H and K. After purification through Qiagen miniElute PCR purification kit, the two amplicons were combined, and PCR was performed using primers J and L. The PCR product was digested with *SaII* and *EcoRI*.

The “left-arm” was constructed by PCR using primers M and N, and BAC 75i6 as template. This amplicon was then digested with *EcoRI* and *NotI*, mixed with digested “right-arm” amplicon, and ligated to pBluescript (Strata-gene) that had been digested previously with *NotI* and *SaII*. LoxP flanked PGK-Neo cassette was released from pL452 by digesting the plasmid with *EcoRI* and *BamHI*, purified by agarose gel electrophoresis, and then ligated to a mini-targeting vector that had been digested with *BlgII* and *EcoRI*. This mini-targeting vector was then digested with *NotI* and *SaII*, and the targeting cassette was purified by agarose gel electrophoresis. This targeting cassette and FLAG-HA containing gap repaired plasmid were electroporated into EL350 bacteria that had been treated previously at 42°C for 15 min. The resulting plasmid contained a loxP-flanked PGK-Neo cassette within intron 1 together with FLAG-HA epitope tag immediately downstream of the ATG translation start site within exon 2. This plasmid was then electroporated into EL350 bacteria, previously induce to produce Cre recombinase in the presence of 10% L-(+)-arabinose (Liu *et al.*, 2003), in order to excise the PGK-Neo cassette, thereby leaving a single loxP site within intron 1.

A second mini-targeting vector was constructed to introduce a PGK-Neo/loxP cassette flanked by FRT sites into intron 3 (Fig. 1B). “Left-arm” was constructed by PCR using primers O and

P, and BAC 75i6 as template. This fragment was digested with *NotI* and *EcoRI*. The “right-arm” was constructed by PCR using primers Q and R, and BAC 75i6 as template. This fragment was digested with *EcoRI* and *Sall*. The two fragments were combined and ligated into pBluescript that had been digested previously with *NotI* and *Sall*. The FRT flanked loxP/PGK-Neo cassette was purified from pL451 after digesting the plasmid with *EcoRI* and *BamHI*. Purified fragment was then ligated to the second mini-targeting vector that had been digested previously with *BamHI* and *EcoRI*. This mini-targeting vector was digested with *NotI* and *Sall*, and the targeting cassette was purified by agarose gel electrophoresis. This targeting cassette and loxP-FLAG-HA containing gap repaired plasmid were electroporated into heated-treated EL350 to produce the final targeting vector (Fig. 1B). Altered sequences and additions in the targeting vector were confirmed by sequencing.

The targeting vector was linearized with *KpnI*, electroporated into GSI-1 ES line (Genome Systems; derived from 129/SvJ strain mice), and selected with G418 and gancyclovir (Ohtsubo *et al.*, 2004). Genomic DNA prepared from surviving colonies was screened by restriction digestion and Southern blotting hybridization using probes outside of the targeting vector (data not shown; Fig. 1B, see below). Positive clones were injected into C57BL/6J blastocysts and surgically transferred to foster mother. Chimeric mice containing the mutant allele were then mated with C57BL/6J females to obtain germline-transmitted agouti-coat color pups.

Mice

For experiments involving embryo isolation, genotyped males and females (6–10 weeks old) were mated and the females were checked for the presence of a vaginal plug (designated E0.5). Pregnant females were euthanized at the indicated day, uteri were dissected and placed in PBS. Decidua were dissected and placed in separate dishes containing PBS. Embryos were dissected out of placenta/decidua tissue, and visually examined under the microscope for presence of exencephaly or other abnormalities. Part of either embryonic tail or amnion was saved for genomic DNA analysis. For total RNA preparation, the entire embryo or dissected telencephalon of E13.5 embryos was frozen in liquid nitrogen and stored at -80°C until RNA isolation (see below). All animal care and procedures were done according to guidelines provided by the IACUC.

Genomic DNA Analysis

Genomic DNA was prepared from mouse tail clippings or embryonic tail/chorion using Puregene DNA purification kit (Gentra), and the precipitated DNA was hydrolyzed in 30 μl 10 mM Tris—EDTA, pH 8.0 and analyzed as follows.

Multiplex genomic DNA PCR analysis—1/30 of each sample was assayed using 0.2 μM primer A + C (Table 4), 0.4 μM primer B, and Platinum Taq polymerase (Invitrogen) according to the manufacturer’s instructions. PCR reactions of 25 μl were incubated for 5 min at 95°C followed by 40 cycles of 30 s at 95°C , 30 s at 60°C , and 30 s at 72°C with a final extension of 7 min at 72°C . Aliquots of 10 μl were subjected to electrophoresis in 5% polyacrylamide gels containing Tris—borate—EDTA buffer (BioRad Ready Gel). DNA was visualized with ethidium bromide.

Southern blotting hybridization analysis—To confirm germline transmission of the recombined allele (Figs. 1B and 2A), 1/3 of the genomic DNA sample was digested with 120 U of either *XhoI* or *NheI* (Roche Biochemicals). To confirm the Cre-mediated deletion allele (Figs. 1C and 2B), 1/3 of the genomic DNA sample was digested with 120 U *EcoRV* (Roche Biochemicals). Digested DNA was fractionated by electrophoresis in 0.8% agarose gel containing Tris—Borate—EDTA buffer. *XhoI* digested DNA was hybridized with radiolabeled 5'-probe (nucleotides 1052380–1053246; Genbank NW_000319.1), *NheI*

digested DNA with 3'-probe (nucleotides 1065550–1066861, Fig. 1B), and EcoRV digested DNA with “KO” probe (nucleotides 1059178–1059702, Fig. 1C). DNA probes were prepared by standard PCR technology using either BAC 75i6 or subclone fragment isolated from BAC 75i6, and radiolabeled using Ready-To-Go DNA labeled Beads and [³²P]-dCTP (Amersham) (Kaneko and DePamphilis, 2000).

Total RNA Analyses

Embryos, as well as adult and fetal tissues, were frozen in liquid nitrogen upon isolation. Total RNA was isolated as previously described (Kaneko *et al.*, 1997). After addition of RNAgents denaturation solution (Promega), samples were homogenized before proceeding with the manufacturer's protocol. Total RNA (15 µg) was analyzed by Northern blotting hybridization as previously described (Kaneko *et al.*, 1997). Alternatively, a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out, as previously described (Kaneko *et al.*, 2004). After the initial cycle and at every two cycles thereafter as indicated, a 5 µl aliquot was saved before PCR was continued. Aliquots were then fractionated simultaneously by electrophoresis in 5% polyacrylamide gels containing Tris—borate—EDTA buffer. DNA primers for amplification of *Tead2* exons 2–6 and *Gapdh* have been described (Kaneko *et al.*, 2004). Primers for other genes were: *Tead4* (S and T), *Fgfr4* (U and V), *Pax3* (W and X). *Tead4* primers amplify either a 396 bp WT gene segment, or a 267 bp splice variant gene segment (Yockey *et al.*, 1996). Primers for *Fgfr4* and *Pax3* amplify 316 bp and 434 bp DNA fragments, respectively.

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FIG. 1.

Construction of a *Tead2* conditional KO mouse. The mouse *Tead2* gene is located at 23 cM on chromosome 7 in ~30 kb region that includes the *Dkk11* and *CD37* genes. (A) Schematic depiction of the features of the *Tead2* gene that are relevant for construction of the knockout allele. Locations of cleavage sites for EcoRV (RV), Nhe I (N), and Xho I (X) restriction endonucleases are indicated. Locations of the 5' and 3'-sequence specific DNA probes used in Southern blotting-hybridization analysis are indicated. Locations of primers A, B, and C used for multiplex genomic DNA PCR assays are indicated. Primer sequences are in Table 4. A putative *Tead2* gene enhancer ("E") is located within intron 1. (B) The targeting vector spanned the region from just upstream of the *Tead2* promoter through part of intron 7. LoxP sites (closed triangles) were inserted within introns 1 and 3. A neomycin resistance gene (Neo) driven by the phosphoglycerol kinase (PGK) gene promoter, flanked by Flip recombinase target (FRT) sites (closed ovals), was inserted into intron 3 just upstream of 3'-loxP site. Newly created restriction sites are indicated with an asterisk. (C) Exposure of the targeted allele to Cre recombinase results in excision of the region between the two loxP sites. The deleted allele was screened by multiplex genomic DNA PCR (primer A/C) as well as by Southern blotting-hybridization analysis after digesting with EcoRV enzyme and probing with KO probe.

FIG. 2.

Genomic DNA analysis confirmed the presence of the *Tead2* mutant allele. **(A)** Genomic DNA was extracted from the tails of pups born from matings between chimeric males and C57BL/6J females. DNA was digested with the indicated restriction endonuclease, subjected to Southern blotting-hybridization, and then detected with the indicated probe (Fig. 1A, B). Lanes 2–4 contain DNA from agouti pups. Lane 1 contains DNA from a black littermate. XhoI-5' probe gives rise to a 19.9 kb DNA fragment from the wild type (Wt) allele, and a 4.6 kb DNA fragment from the mutant allele (Mt). NheI-3' probe gives rise to 8.4 kb fragment from the Wt allele, and a 9.5 kb fragment from the mutant allele. Genomic DNA from WT ES cells was included as a positive control. **(B)** Mice containing the mutant allele were mated to EllaCre⁺ mice, and

resulting pups were assayed for the deleted/KO allele. Genomic DNA was digested with EcoRV restriction endonuclease, subjected to Southern blotting-hybridization, and the DNA detected with “KO probe” (Fig. 1C). The Wt allele generates a 7.2 kb DNA fragment (Wt). The knockout allele generates a 8.5 kb DNA fragment (KO). Result shown is an example from such matings (“parents”). These *Tead2* heterozygous parents (male and female) were then mated to each other and their litters of five pups were similarly assayed for the presence of KO allele. (C) The DNA samples (“litter”) in panel B were also subjected to multiplex genomic DNA PCR analysis. Primers A and B (Fig. 1B) amplified a 165 bp fragment from the Wt allele. Primers A and C (Fig. 1C) amplified a 210 bp fragment from the KO allele.

FIG. 3.

Full-length *Tead2* mRNA was absent in *Tead2* nullizygous mice. *Tead2* heterozygous mice were mated, and DNA extracted from the tails of littermates was genotyped. **(A)** Total RNA (15 μ g) was prepared from adult forebrain (lane 1) and fetal brain (E13.5; lane 2) from *Tead2* WT mouse, and fetal brain (E13.5) from a *Tead2* nullizygous mouse (lane 3), fractionated by agarose gel electrophoresis, transferred to nylon membrane, and hybridized with radiolabeled *Tead2* cDNA (Kaneko *et al.*, 1997). Similarly, total RNA (15 μ g) was prepared from the lungs of a *Tead2* homozygous (lane 5), a heterozygous (lane 6) and a nullizygous (lane 7) littermate. Samples were subjected to Northern blotting-hybridization analysis. Total RNA (15 μ g) isolated from WT CCE ES cells (lane 4), which abundantly express *Tead2* (Kaneko *et al.*, 2004), was used as a positive control. Ethidium bromide stained 28S rRNA was used as loading control. 0.24–9.5 kb RNA ladder from Invitrogen was used as marker. **(B, C)** *Tead2* KO mice produce no detectable level of *Tead2* mRNA, but still express the *Fgfr4* and *Tead4* genes. Total RNA from lung (B) or ovary (C) tissue was isolated from mice that were WT, heterozygous, or nullizygous at *Tead2* allele and then used as a template for cDNA synthesis. PCR was performed for the indicated number of cycles using *Tead2* primers that amplified the region from exons 2 to 6 (Kaneko *et al.*, 1997). The same aliquot of RNA was used to amplify *Fgfr4*-specific and *Tead4*-specific RNA. DNA marker used was 1 kb ladder from Invitrogen. *Gapdh* control primers confirmed that relatively equivalent RNA samples were amplified (data not shown).

FIG. 4.

Exencephalic embryos were detected as early as E11.5, although *Pax3* expression was not altered by the absence of TEAD2. **(A)** Total RNA was prepared from 11 embryos at E11.5 that were obtained from a single heterozygous female mated to a heterozygous male. This RNA was assayed for *Pax3* sequences using RT-PCR. RNA from E13.5 fetal brain served as a positive control, while RNA from ES cells served as a negative control. One embryo that exhibited exencephaly (asterisk) is shown in panel C. **(B)** Multiplex PCR analysis was used to determine the genotype of each embryo. **(C)** One normal (*Tea2*^{+/-}) and one exencephalic (*Tea2*^{-/-}) embryo from the same litter at E11.5 are shown using bright-field illumination. Arrows indicate sites where neural tube failed to close.

FIG. 5.

Exencephalic embryos were clearly evident by E13.5. *Tead2* nullizygous females were mated to a heterozygous male, and embryos were harvested at day E13.5. Two exencephalic and one normal embryo from the same litter are shown using dark-field illumination.

FIG. 6.

By E16.5, some exencephalic embryos are alive while others are being resorbed. *Tead2* nullizygous females were mated to a heterozygous male, and embryos were harvested at day E16.5. (A) *Tead2*^{-/-} exencephalic embryo. (B) *Tead2*^{-/-} exencephalic embryo undergoing resorption. (C) *Tead2*^{+/-} littermate.

Table 1*Progeny from Tead2+/- X Tead2+/- Matings*

	Male	Female	Total		
Total Mice	111	118	229		
Genotype	Male	Female	Sum	% Total	Theoretical
Wild-type	24	38	62	27.1	25
Heterozygous	66	56	122	53.3	50
Nullizygous	21	24	45	19.7	25

Summary of genotyped offspring obtained from heterozygous intercross. Tail samples from pups born from heterozygous inter-cross were used to analyze for their genotype. The numbers indicate compilation of 32 litters.

Table 2
Tead2-Dependent Exencephaly

	Tead2+/-	Tead2-/-	Total
(A) ♂ <i>Tead2</i>+/- X ♀ <i>Tead2</i>-/-			
Total mice	27	27	54
Exencephalic mice	2	8	10
% Exencephalic	7	30	19
(B) ♂ <i>Tead2</i>-/- X ♀ <i>Tead2</i>+/-			
Total mice	30	22	52
Exencephalic mice	0	2	2
% Exencephalic	0	9	4

(A) *Tead2* nullizygous females were mated with heterozygous males, and embryos at E11.5 to E17.5 were examined for neural tube defects and genotyped. Results from seven litters were combined. (B) *Tead2* nullizygous males were mated with heterozygous females, and postimplantation stage embryos were examined for NTDs and genotyped. Results from six litters were combined. The difference between “% Exencephalic (Total)” in panels A and B was statistically significant ($P < 0.02$). The data were analyzed by testing the difference in proportions using normal approximation to the binomial.

Table 3
Suppression of Exencephaly by Either Folic Acid or Pifithrin- α

	Tead2+/-	Tead2-/-	Total
(A) ♂ <i>Tead2</i>+/- X ♀ <i>Tead2</i>-/- (+ folic acid)			
Total mice	8	8	16
Exencephalic mice	0	1	1
% Exencephalic	0	13	6
(B) ♂ <i>Tead2</i>+/- X ♀ <i>Tead2</i>-/- (+ pifithrin-α)			
Total mice	9	11	20
Exencephalic mice	0	1	1
% Exencephalic	0	9	5
(C) ♂ <i>Tead2</i>+/- X ♀ <i>Tead2</i>-/- (control)			
Total mice	11	13	24
Exencephalic mice	0	4	4
% Exencephalic	0	31	17

Tead2 nullizygous females were mated with heterozygous males, injected with supplements, and embryos at E13.5 were examined for neural tube defects and genotyped. (A) At E8.5 and E9.5, pregnant females were injected intraperitoneally with 10 mg folic acid per kg weight (Fleming and Copp, 1998), and the embryos were examined at E13.5 for NTDs and genotyped. Results from three litters were combined. (B) At E8.5 and E9.5, pregnant females were injected intraperitoneally with pifithrin- α (Pani et al., 2002), and the embryos were examined at E13.5 for NTDs and genotyped. Results from three litters were combined. (C) Mice were mated in parallel with (A) and (B) but treated with saline instead of either folic acid or pifithrin- α .

Table 4

PCR Primers and Inserted Sequences

Name	Sequence
A	tgtctcttctgctggccacatg
B	cccaaagtctctgagtcacac
C	ccccacaacttagagaaaagcc
D	ggactagtctctgctctgtaggatatac
E	gtcaagcttaactcacaacccaagcagcatcccgattc
F	gtcaagcttagggctcacatcttgatctg
G	aagcccatctctagacctg
H	gatcccgactacaaggacgacgatgacaagtaccctacgac
I	gtgcccgactacgcc
J	gatcggcgtagtctgggcacgtcgtagggttactgtcatcgt
K	cgctctgtagtctgg
L	ggaaattcagatcttgttcacctaacctctctc
M	gtcctttagtctgggatcccatctgggcctg
N	acgcgtcgactcaccatacatcttgccctc
O	ataagcggccgcaaccctaccagtccttagatgg
P	gtcgaattcatctcaagcccagcccagtgctg
Q	ataagcggccgcttggaaacttgggtctgggg
R	gtcgaattcacacaacaaggccagaaagacg
S	ggaaattcggatccggcttctctaagttgtgggg
T	acgcgtcgactcttccacaggtcacacagc
U	ctggcaaagctccttgccaaaa
V	aatgatgcagaggggtatggagc
W	gagaactctctgggtagcattc
X	tatactctctgcatctcagc
	atagtggagatggccaccatg
	tcatctgactgaggtgcagagg