Twist1 Dimer Selection Regulates Cranial Suture Patterning and Fusion

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Saethre-Chotzen syndrome is associated with haploinsufficiency of the basic-helix-loop-helix (bHLH) transcription factor TWIST1 and is characterized by premature closure of the cranial sutures, termed craniosynostosis; however, the mechanisms underlying this defect are unclear. Twist1 has been shown to play both positive and negative roles in mesenchymal specification and differentiation, and here we show that the activity of Twist1 is dependent on its dimer partner. Twist1 forms both homodimers (T/T) and heterodimers with E2A E proteins (T/E) and the relative level of Twist1 to the HLH inhibitor Id proteins determines which dimer forms. On the basis of the expression patterns of Twist1 and Id1 within the cranial sutures, we hypothesized that Twist1 forms homodimers in the osteogenic fronts and T/E heterodimers in the mid-sutures. In support of this hypothesis, we have found that genes regulated by T/T homodimers, such as FGFR2 and periostin, are expressed in the osteogenic fronts, whereas genes regulated by T/E heterodimers, such as thrombospondin-1, are expressed in the mid-sutures. The ratio between these dimers is altered in the sutures of Twist1/H11545/H11546 mice, favoring an increase in homodimers and an expansion of the osteogenic fronts. Of interest, the T/T to T/E ratio is greater in the coronal versus the sagittal suture, and this finding may contribute to making the coronal suture more susceptible to fusion due to TWIST haploinsufficiency. Importantly, we were able to inhibit suture fusion in Twist1/H11545/H11546 mice by modulating the balance between these dimers toward T/E formation, by either increasing the expression of E2A E12 or by decreasing Id expression. Therefore, we have identified dimer partner selection as an important mediator of Twist1 function and provide a mechanistic understanding of craniosynostosis due to TWIST haploinsufficiency. Developmental Dynamics 235:1345–1357, 2006. © 2006 Wiley-Liss, Inc.

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INTRODUCTION

The growth of the cranium occurs through a complex interplay between the intervening suture mesenchyme, the osteogenic fronts, and the underlying dura mater and brain and involves several different signaling pathways and transcriptional regulators (Opperman, 2000; Jiang et al., 2002). Although there has been quite a bit of progress made toward the identification of many of the genes that are important in this process, much less is known about how these pathways interact with each other to mediate these events (Ishii et al., 2003; Rice et al., 2005). Here, we focus on the role of the transcription factor Twist1 in mediating cranial suture patency.

Craniosynostosis is a fairly common disorder occurring in approximately 1 in 2,500 individuals. Nonsyndromic
craniosynostosis is most common; however, 20% of all cases are associated with mutations in the gene TWIST1 or one of the fibroblast growth factor receptor (FGFR) genes. TWIST1 haploinsufficiency is associated with Saethre-Chotzen syndrome, which is the most common autosomal dominant disorder of craniosynostosis (el Ghouzzi et al., 1997; Howard et al., 1997). More than 50 different mutations have been identified in the TWIST1 gene and are predicted to cause loss of function (Gripp et al., 2000), which is also indicated by Twist1+/− mice presenting a similar phenotype (el Ghouzzi et al., 1997; Bourgeois et al., 1998; Carver et al., 2002). Conversely, a family has been identified with trisomy at the TWIST1 locus resulting in cranium bifidum, which is characterized by a persistent calvarial foramen and open sutures (Stankiewicz et al., 2001). These two opposing clinical phenotypes resulting from either half or one and a half times the normal amount of Twist1, respectively, illustrate the critical requirement for the tight regulation of Twist1 expression.

We and others have shown that Twist1 has both positive and negative functions regulating mesenchymal cell specification and differentiation. Twist was originally identified in Drosophila as a gene required for gastrulation and mesoderm formation but also has a seemingly opposing but evolutionarily conserved function of inhibiting the differentiation of mesenchymal tissues, including muscle and bone (Castanon and Baylies, 2002; O’Rourke and Tam, 2002). The mechanisms underlying these disparate functions are unclear but may be dependent on the dimer partner of Twist.

Basic-helix–loop–helix (bHLH) transcription factors are classified into different categories based on their tissue distribution, partner choice, and DNA-binding and structural properties. Twist falls into Class II, which contains tissue-specific bHLH proteins such as MyoD. These proteins form heterodimers with Class I bHLH proteins, termed E proteins, which are widely expressed in many tissues. Id proteins represent a third class of HLH proteins that lack the basic domain and, therefore, cannot bind DNA. Id proteins preferentially dimerize with E proteins and disrupt functional Class I/II bHLH heterodimers from forming (Massari and Murre, 2000). The majority of Class II bHLH proteins do not form stable homodimers (Vinals et al., 2004; Vinals and Ventura, 2004); however, studies in Drosophila suggest that homodimers of Twist mediate mesoderm formation (Castanon et al., 2001). Therefore, Twist may uniquely form functional heterodimers (T/E) and homodimers (T/T) that may have different activities and may account for the opposing actions ascribed to Twist1.

The cranial sutures are the growth centers separating the bones of the skull and are composed of two opposing osteogenic fronts and an intervening mesenchyme (Fig. 1). Twist1 is expressed throughout the suture and in differentiating osteoblasts (Funato et
We hypothesized that Id competes with Twist1 for dimerization with E proteins in the osteogenic fronts, forcing Twist1 to form homodimers, while in the intervening suture mesenchyme, where Id is absent, Twist1 forms T/E heterodimers. These dimers then differentially regulate gene expression and cell behavior in these areas. Our data support this hypothesis and indicate that there is dynamic regulation of Twist1 dimer formation in the cranial sutures that is altered in Twist1+/− mice. Twist1 haploinsufficiency increases the ratio of T/T to T/E, and conditions that promote T/E formation in the sutures prevent fusion in Twist1+/− mice.

RESULTS
Id Levels Can Modulate Twist1 Dimer Composition

Twist1 is expressed in both the osteogenic fronts and in the mid-suture (Johnson et al., 2000; Rice et al., 2000; Oshima et al., 2002), whereas Id1 is only in the osteogenic fronts (Rice et al., 2000), and, therefore, it was suggested that Id inhibits Twist1 activity in the osteogenic fronts. Given that Id preferentially dimerizes with E proteins rather than class II HLH proteins like Twist1 (Benezra et al., 1990), we have tested an alternative hypothesis, that Id expression promotes Twist1 homodimer formation in the osteogenic fronts. To determine whether increasing Id levels would drive Twist1 to form T/T over T/E dimers, we developed a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis where we could detect the Twist1 dimers that formed. The 293T HEK cells were transfected with constructs expressing myc-tagged Twist1, myc-tagged Id1, and E2A E12 in different combinations. Protein extracts were subjected to nonreducing SDS-PAGE gels followed by Western blot analysis without heating the samples before loading the gels (Fig. 2a, middle and right gels). When either Twist1 or E2A E12 was transfected alone (lanes 2 and 3, respectively), two bands were detected in the nonreducing gels (middle and right gels) corresponding to the size of the monomers (T and E) and the respective homodimers (T/T...
Cotransfection of Twist1 and E2A E12 led to the formation of a new band corresponding to the size of T/E heterodimers that was detected with both anti-myc and anti-E2A E12 antibodies, along with the disappearance of the homodimer bands (lane 4, middle and right gels). Increasing amounts of Id1 protein caused the T/E band to decrease and a band corresponding to T/T homodimers to appear (lane 4 – 8, middle gel). No dimers were detected when extracts were heated before loading and run under reducing conditions (Fig. 2a, left gel). These data support previous work indicating that Id preferentially interacts with E proteins (Benezra et al., 1990) and does not efficiently interact with Twist1, which we have also confirmed using GST fusion and in vitro translated proteins (data not shown). Therefore, Id levels determine the amount of free E protein that is available to dimerize with Twist1 and, hence, can determine which Twist1 dimer is formed.

Forced Dimers of Twist1 Are Resistant to Competition by other HLH Proteins

To determine whether T/T homo- and T/E heterodimers have different activities, we constructed “forced” homodimers of Twist1 (TT) and heterodimers of Twist1 and E2A E12 (TE) where the two monomers are linked by a flexible glycine–serine polylinker (Fig. 2b). This strategy was originally used with MyoD and the E protein E2A E47, where it was shown that the tethered dimer was more resistant to inhibition by Id than the separate monomers (Neuhold and Wold, 1993). The strategy has now been used successfully with several different transcription factors (Sigvardsson et al., 1997; Castanon et al., 2001; Bakiri et al., 2002). Because we have found no difference in the activity of Twist1 heterodimers composed of E2A E12, E2A E47, or HEB E proteins (data not shown), we used E2A E12 for our studies here. To confirm that the tethered dimers could bind to a target DNA-binding sequence (E box) and were resistant to inhibition by other HLH proteins, we performed an electrophoretic mobility shift assay (EMSA) comparing the “forced” dimers to the separated monomers in their ability to bind oligos containing an E box. Using in vitro translated proteins, we found that both tethered dimers (TT and TE) formed specific complexes with the E box probe (Fig. 2c,d). These complexes were slightly smaller than the complexes formed by the separated monomers (T/T and T/E), and this size difference is consistent with each of the separated monomers having myc epitope tags whereas only the amino-terminal partner of the tethered dimers has myc tags. As expected, the addition of increasing amounts of Id led to dissociation of the T/E monomer complex, whereas DNA binding by the TE tethered dimer was not affected by Id (Fig. 2c). Similarly, addition of the E2A E12 monomer quickly decreased the T/T homodimer complex with a commensurate increase in a T/E complex bound to DNA. The TT tethered dimer complex, however, was more resistant to dissociation by increasing levels of E2A E12 (Fig. 2d).

Figure 3. Twist1 dimers differentially regulate gene expression (a–e) 10T1/2 cells were infected with pWZL retroviral constructs to stably express Twist1 (T), the T/T homodimer (TT), or the T/E heterodimer (TE). Control 10T1/2 cells were infected with insertless pWZL virus (WZL). a: TT enhanced periostin expression, whereas TE cells inhibited it. The 10T1/2 cell lines were grown in 10% fetal bovine serum (FBS) without or with 100 ng/ml bone morphogenetic protein-7 (BMP7) for 4 days and then assayed for periostin and β-actin expression by Western blot analysis. b: Id1 is up-regulated by BMP signaling. 10T1/2 cells were grown in 10% FBS without or with 100 ng/ml BMP7 for 4 days and then assayed for Id1, Id3, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression by reverse transcriptase-polymerase chain reaction (RT-PCR). c: TE induced thrombospondin 1 (TSP-1) expression. 10T1/2 cell lines were grown in 10% FBS for 48 hr and then assayed for TSP-1 expression by immunofluorescence. d: 10T1/2 cell lines were grown in 0.2% FBS for 48 hr, and then the conditioned medium was assayed for TSP-1 expression by Western blot analysis after concentration on a heparin–sepharose column and elution with 1.5 M NaCl. e: TT and TE dimers differentially regulate fibroblast growth factor receptor-2 (FGFR2) expression. 10T1/2 cell lines were grown without or with 100 ng/ml BMP7 for 4 days and were analyzed for FGFR1, FGFR2, and GAPDH expression by RT-PCR. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
can also effectively bind these DNA probes and their complexes with DNA are more stable than the separate monomers when challenged by other HLH proteins.

**Twist1 Dimers Differentially Regulate Gene Expression**

While Twist1 has been implicated in the regulation of cell specification and differentiation little is known about what genes Twist1 regulates to mediate these effects. We, therefore, wanted to determine whether T/T and T/E dimers regulated different sets of genes. We also reasoned that the expression patterns of genes that are differentially regulated by the Twist1 dimers could be used to identify regions in the sutures where each dimer resides. We previously had performed microarray analysis comparing control C3H10T1/2 embryonal fibroblasts (10T1/2 cells) with ones overexpressing Twist1. We had used 10T1/2 cells because they are a multipotential mesenchymal cell line able to undergo chondrogenesis and osteogenesis, but unlike primary calvarial cells, the endogenous levels of Twist1 are very low, allowing us to better determine the specific effects of the expression of different forms of Twist1. We found that periostin, a member of the fascin clin I protein family, was significantly repressed by Twist1 and these data were confirmed by RT-PCR (data not shown) and Western analysis (Fig. 3a). Of interest, periostin was identified recently as a gene significantly induced by Twist1 in SAOS2 osteosarcoma cells (Oshima et al., 2002). We reasoned that our differing results might be due to the different culture conditions favoring the formation of either Twist1 homodimers or heterodimers with endogenous E proteins, and these dimers may differentially affect periostin expression. Indeed we found that 10T1/2 cells expressing TT enhanced periostin expression, whereas expression of TE inhibited it (Fig. 3a). Cells expressing the Twist1 monomer more resembled TE-expressing cells and expressed less periostin. We next asked whether increasing Id levels in the Twist1-expressing cells would promote more of a TT phenotype. Bone morphogenetic proteins (BMPs) induce Id1 expression in the calvarial sutures (Rice et al., 2000) as well as in many cell lines including 10T1/2 cells (Ogata et al., 1993). We confirmed that BMP7 induced Id1 expression in 10T1/2 cells and found that Id3 expression was also slightly induced in these cells (Fig. 3b). Id2 and Id4 were not detected (data not shown). Consistent with our hypothesis, in the presence of BMP7 where Id levels were high, Twist1-induced periostin expression (Fig. 3a, compare lanes 2 and 6).

Another gene that we found to be differentially regulated by the Twist1 dimers was thrombospondin 1 (TSP-1). Recently, TSP-1 was shown to be up-regulated in mesenchymal tissues of Id1–/– mice (Volpert et al., 2002). Because Id does not directly regulate gene expression, we hypothesized that there are more free E proteins available in the Id1–/– mice, which may lead to increasing amounts of T/E dimers formed and these dimers may mediate the induction of TSP-1 expression. Consistent with this interpretation, we found that TSP-1 expression was significantly induced in the 10T1/2 cells expressing TE (Fig. 3e). There was also significantly more TSP-1 in the conditioned media of TE-expressing cells than in control or TT cells and the Twist1-expressing cells had an intermediate level of TSP-1 (Fig. 3d). Therefore, TT dimers induced periostin expression, whereas TE dimers inhibited periostin and induced TSP-1 expression. Furthermore, Twist1 cells behaved similar to TE cells under conditions where Id levels were low but acted like TT cells when Id levels were increased.

**Twist1 Dimers Differentially Regulate FGFR2 Expression**

FGFR2 is normally expressed in the osteogenic fronts, and its expression expands into the mid-suture of Twist1+/- mice (Rice et al., 2000), possibly suggesting that Twist1 normally represses FGFR2 expression. However, Twist1 has been shown recently to bind to the FGFR2 promoter and induce its expression (Guenou et al., 2005). Furthermore, Twist induces FGFR expression during Drosophila gastrulation (Shishido et al., 1993), and this finding may be mediated by Twist homodimers (Castanon et al., 2001). We, therefore, examined our 10T1/2 cell lines to see if either of the Twist1 dimers affected the expression of FGFR2. As can be seen in Figure 3e, TT-expressing 10T1/2 cells induced FGFR2 expression while there was no expression in TE and control cells. Unlike the case with periostin and TSP-1 where the Twist1-expressing cells behaved more similarly to the TE cells, FGFR2 was induced in the TE cells similar to the TT cells. Because there is most likely a mix of T/T and T/E dimers in the TT cells, this finding suggests that genes are differentially sensitive to the ratio or amount of the two Twist1 dimers. FGFR1, whose expression does not overlap with Twist1 in the sutures (Johnson et al., 2000; Rice et al., 2000), was expressed equivalently in all of the cell lines. The addition of BMP7 induced FGFR2 expression in control 10T1/2 cells; however, TE expression inhibited this induction. Therefore, T/T and T/E dimers have opposing effects on FGFR2 expression.

**T/E and T/T Regulated Genes Are Expressed in the Predicted Domains Within the Cranial Sutures**

We next asked whether the expression pattern of these genes that are differentially regulated by the Twist1 dimers could be predicted in the cranial sutures based upon the expression patterns of Twist1 and Id1. As outlined above, because Twist1 is expressed in both the osteogenic fronts and in the mid-suture while Id1 is only in the osteogenic fronts (Johnson et al., 2000; Rice et al., 2000; Oshima et al., 2002)(Fig. 4n), we predicted that T/T dimers would be formed in the osteogenic fronts whereas T/E dimers would be in the mid-sutures. Consistent with our hypothesis, we observed periostin expression in the osteogenic fronts (Fig. 4a), while TSP-1 was expressed in the mid-suture area (Fig. 4e). Of interest, TSP-1 was only significantly expressed in the sagittal and frontal sutures and not in the coronal suture (Fig. 4e,f,m), while periostin was expressed in all the sutures (Fig. 4a,b,m). Based upon our model (see Fig. 1), this finding suggested that the coronal suture may have a higher ratio of T/T to T/E. We, therefore, analyzed the expression patterns of Twist1 and Id
proteins in the sagittal and coronal sutures to see if they would suggest a difference in dimer formation. Twist1 was similarly expressed in the osteogenic fronts and middle of both sutures (Fig. 4n); however, Id proteins were more extensively expressed in the coronal versus the sagittal suture (Fig. 4n). In the sagittal suture, Id proteins were highly expressed in the osteogenic fronts, with little or low expression in the mid-suture while in the coronal suture, only a few cells in the mid-suture did not express Id. Consistent with an increase in T/T formation in this area, there was only a small area in the mid-coronal suture that did not express periostin (Fig. 4b). We propose that this difference may be part of the reason that it is primarily the coronal suture that fuses due to TWIST1 haploinsufficiency (see below).

**T/T and T/E Regulated Genes Are Altered in Twist1+/− Mice**

The mechanism that promotes craniosynostosis due to TWIST1 haploinsufficiency is still unclear, but we suggest that the decrease in the level of Twist1 alters the balance between T/T and T/E dimers in the sutures, resulting in a change of gene expression and cell behavior. The expansion of FGFR2 expression into the mid-suture (Rice et al., 2000) suggests that there is an increase in T/T formation in the sutures of Twist1+/− mice. We propose that this is due to Id levels being relatively higher than Twist1 in a larger area resulting in less free E proteins being available for dimerization with Twist1 (see Fig. 1b). To test this hypothesis, we examined the expression of T/T and T/E-regulated genes in the sutures of wild-type and Twist1+/− mice. As was identified previously (Rice et al., 2000), we found that in Twist1+/− mice the expression domain of FGFR2 in the sagittal suture was expanded into the mid-suture (Fig. 4, compare i with k). As was identified previously (Rice et al., 2000), we found that in Twist1+/− mice the expression domain of FGFR2 in the sagittal suture was expanded into the mid-suture (Fig. 4, compare i with k). We also found that, whereas periostin expression barely extended to the tip of the calvaria bones in wild-type mice, its expression was extended both ventrally and medially toward the mid-suture of Twist1+/− mice (Fig. 4, compare a with c). There was a similar, but more subtle, effect in the coronal suture. Although there seemed to be a decrease in the level of periostin expression in the coronal suture of Twist1+/− mice, the expression domain of both periostin and FGFR2 expanded to cover the entire suture (Fig.
4, compare b with d, and j with l). More dramatically, there was a significant decrease in TSP-1 expression in the sagittal suture of Twist1+/− mice (Fig. 4, compare e with g, and Fig. 4o).

This increase in FGFR2 and peristin and decrease in TSP-1 is consistent with our hypothesis that, in the sutures of Twist1+/− mice, there is an increase in the ratio of T/T to T/E.

Because decreasing Twist1 levels resulted in an increase in the T/T to T/E ratio, we next performed the converse experiment by increasing Twist1 expression in the cranial neural crest, which gives rise to these sutural cells (Jiang et al., 2002), to see if...
this promoted T/E dimer formation. To achieve this objective, we used Wnt1-cre transgenic mice to activate an inducible transgene expressing Twist1. Consistent with our hypothesis, we found that TSP-1 expression was induced in the osteogenic fronts of these mice (Fig. 4p). TSP-1 expression was also lost from the mid-suture of these mice, suggesting a change in dimer formation there as well. The full phenotype of these mice will be described elsewhere. Therefore, decreased and increased levels of Twist1 in the cranial sutures change the expression of the genes regulated by the T/T and T/E dimers in a predicted manner that supports our hypothesis.

**Twist1 Requires Heterodimerization to Inhibit Osteoblast Differentiation**

Overexpression of Twist1 has been shown to negatively regulate osteoblast differentiation (Lee et al., 1999; Funato et al., 2001), and osteoblasts isolated from individuals that were haploinsufficient for TWIST1 differentiated faster than control cells (Yousfi et al., 2001). Furthermore, Twist1 has been implicated recently in directly inhibiting the activity of the transcription factor Runx2, which promotes osteoblast differentiation (Bialek et al., 2004). Runx2-dependent osteoblast differentiation, however, initiates in the osteogenic fronts where Twist1 is expressed. Primary calvaria cells were infected with adenovirus expressing Twist1 or E2A E12 alone or in combination to determine whether Twist1 required heterodimerization to inhibit osteoblast differentiation. Equal levels of myc-tagged Twist1 and E2A E12 were expressed after 2 weeks of differentiation (Fig. 5a). Surprisingly, we found that cells transduced with a Twist1-expressing adenovirus alone were not inhibited from differentiating as Runx2, \( \alpha(I) \) collagen, bone sialoprotein (BSP), and osteocalcin (OC) transcripts were all induced (Fig. 5b). The combination of Twist1 and E2A E12, however, inhibited the later markers of osteoblast differentiation (BSP and OC) but did not affect the expression of the early markers (Runx2 and \( \alpha(I) \) collagen). E2A E12 alone inhibited the expression of OC, and this inhibition may have been due to it dimerizing with the endogenous Twist1 proteins that were expressed at high levels at that time (Fig. 4b). These results indicate that, similar to its inhibition of myogenesis (Spicer et al., 1996), the inhibitory effect of Twist1 on osteogenic differentiation is mediated by the T/E dimer.

**Promotion of T/E Formation Prevents Suture Fusion in Twist1+/−/− Mice**

Our data suggest that there is an increase in the ratio of T/T to T/E in the sutures of Twist1+/−/− mice that drives craniosynostosis. We, therefore, asked whether we could prevent suture fusion in these mice by altering E protein or Id levels to promote the formation of T/E dimers. To increase E protein expression, we infected the sutures of Twist1+/−/− mice with an adenovirus expressing E2A E12. As in
human, \( \text{Twist1} \) haploinsufficiency in mice promotes the fusion of primarily the coronal suture; therefore, we focused our analysis on this suture. Because the coronal suture of live pups is hard to discern, we used explant culture of the skull (Ogle, 2000). The sutures of \( \text{Twist1}+/- \) mice do not fuse until 3–4 weeks after birth (Carver et al., 2002), so we altered the culture conditions to try and maintain skull morphology during 4–5 weeks in culture. This was achieved by sectioning the skull horizontally from the base of the nose to the occipital bone, leaving the dura mater and brain intact (Fig. 6). After 5 weeks in culture the skulls of wild-type mice looked remarkably normal, with both the sagittal and coronal sutures remaining patent (Fig. 6c). The coronal sutures of \( \text{Twist1}+/- \) mice are patent and relatively normal at birth (Fig. 6b); however, after 5 weeks in culture, distinct fusion of this suture was observed. In three of four uninjected \( \text{Twist1}+/- \) control explants and three of four \( \text{Twist1}+/- \) explants injected in the right coronal suture with adenovirus expressing green fluorescent protein (GFP), both the left and right coronal sutures had fused and the sagittal suture remained patent (Fig. 6d,e). However, the right coronal suture failed to fuse in four of five \( \text{Twist1}+/- \) skulls when injected with adenovirus expressing E2A E12 at the time of explant (Fig. 6f).

As another means to promote T/E formation, we lowered Id levels by crossing \( \text{Twist1}+/- \) mice with \( \text{Id1}^-/-;\text{Id3}+/- \) mice. Id3 has a similar expression pattern as Id1 in the cranial sutures (data not shown) and because \( \text{Id1}^-/-;\text{Id3}+/- \) mice are viable (Lyden et al., 1999), we decided to remove from one to three alleles of \( \text{Id1} \) and \( \text{Id3} \) genes on the \( \text{Twist1}+/- \) background. As can be seen in Table 1, there was a significant decrease in the percentage of mice showing any coronal suture fusion as the number of Id alleles was removed. Approximately 87% of \( \text{Twist1}+/- \) mice had craniosynostosis 5 weeks after birth, and this rate decreased to 0% when three Id alleles were removed. To achieve a more accurate assessment of the degree to which craniosynostosis was rescued on the different genetic backgrounds, we used a craniosynostosis index (CI) similar to one used to analyze \( \text{Twist1} \) and \( \text{Snail} \) genetic interactions (Oram and Gridley, 2005). Left and right coronal sutures were assessed individually and assigned a number between 0 (completely unfused) to 3 (completely fused), and the CI given in Table 1 indicates the average degree of craniosynostosis for a coronal suture on the indicated genetic background. As can be seen from the large standard deviation of the CI for \( \text{Twist1}+/- \) mice, the degree of suture fusion was quite variable. This finding was also true with \( \text{Twist1}+/-;\text{Id3}+/- \) mice; however, there was a noticeable decrease in the severity of suture fusion, but the difference was not significant. The removal of two Id alleles, either \( \text{Twist1}+/-;\text{Id1}^-/-;\text{Id3}+/- \) or \( \text{Twist1}+/-;\text{Id1}^-/-;\text{Id3}+/- \), however, produced almost a full rescue, decreasing the CI from 1.64 for \( \text{Twist1}+/- \) to 0.07. We have only obtained 5 \( \text{Twist1}+/-;\text{Id1}^-/-;\text{Id3}+/- \) mice so far, but all of these have completely patent and normal looking sutures. Therefore, conditions that promote T/E formation, either by increasing E proteins or decreasing Id levels, result in the inhibition of suture fusion in \( \text{Twist1}+/- \) mice.

**DISCUSSION**

We and others have shown that Twist has both positive and negative functions regulating mesenchymal cell specification and differentiation (Castanon and Baylies, 2002; O’Rourke and Tam, 2002). The mechanisms underlying these disparate functions are still unclear, but we suggest that they may be dependent on the dimer partner of Twist. We previously found that Twist1 heterodimers inhibited myogenesis (Spicer et al., 1996); however, in *Drosophila*, Twist homodimers are thought to mediate mesoderm formation (Castanon et al., 2001), indicating that Twist may be unique among Class II bHLH proteins in forming functional heterodimers and homodimers. Therefore, we thought it essential to determine whether vertebrate Twist1 formed functional homodimers and if they had different activities from Twist1 heterodimers. We found that these two dimers do form and that the relative level of Twist1 to Id determines which dimer forms. We have characterized the activities of T/E and T/E dimers and have found that they differentially regulate cell behavior and gene expression. Because the two dimers regulate different sets of genes, it creates the possibility for a more dynamic control of gene expression by modulating the availability of E proteins for dimerization. This regulation can be quite complex as many different HLH proteins can compete with Twist1 for dimerization with E proteins.
chose the cranial sutures to test our hypothesis, because changes in Twist1 levels are known to affect this region and there are only a few HLH proteins that are known to be expressed there.

On the basis of the expression patterns of HLH genes within the cranial sutures, we hypothesized that Twist1 forms homodimers in the osteogenic fronts and Twist1/E heterodimers in the mid-sutures. We tested this hypothesis by determining if genes that are differentially regulated by these dimers are expressed in the predicted domains within the sutures. Our results support this hypothesis, and importantly, we have found that the Twist1 dimers differentially regulate the expression of mediators of the signaling pathways that regulate suture patency (Nah, 2000; Wilkie et al., 2001). Twist1 homodimers induce the expression of FGFR2, whereas T/E heterodimers induce the expression of TSP-1. Of interest, one of the functions of TSP-1 is to activate latent transforming growth factor-beta (TGFβ; Annes et al., 2003). TGFβ signaling plays an integral role in the regulation of suture patency (Opperman, 2000); however, the mechanism of latent TGFβ activation in the sutures has not been investigated and TSP-1 may play this role.

**Twist1 and Craniosynostosis**

It has been paradoxical that the gene families that are associated with the majority of craniosynostosis syndromes (TWIST1 and FGFRs) are both defined as inhibitors of in vitro osteoblast differentiation, yet craniosynostosis occurs due to activation of the FGFRs and haploinsufficiency of TWIST1 (Nah, 2000; Wilkie et al., 2001). The mechanisms underlying craniosynostosis are not well understood, and we propose a model that explains why TWIST1 haploinsufficiency leads to craniosynostosis and links Twist1 expression with FGFR signaling (Fig. 1a). Although Twist1 has been shown to regulate FGFR expression in Drosophila (Shishido et al., 1993), the relationship between Twist1 and FGFR in vertebrates has been less clear. We have found the relationship to be quite complex, with T/T dimers inducing FGFR2 expression and T/E dimers inhibiting it. This antagonistic regulation between the Twist1 dimers may help set up a more distinct boundary of gene expression. Due to increased competition by Id in Twist1+/− mice (Fig. 1b), this boundary is altered favoring T/T formation and FGFR2 expression is extended into the mid-sutural mesenchyme (Rice et al., 2000). Conversely, we found a decrease in the T/E-regulated gene TSP-1 in the mid-suture of Twist1+/− mice (Fig. 4). Importantly we were able to inhibit suture fusion in Twist1+/− mice by promoting T/E formation by either increasing the expression of E2A E12 or decreasing Id expression (Fig. 6; Table 1).

Recently, Twist1 has been shown to interact with and inhibit the bHLH protein Hand2 (Firulli et al., 2005). In addition to craniosynostosis, Twist1 haploinsufficiency is also associated with limb abnormalities. Of interest, crossing Twist1+/− mice with Hand2+/− mice rescues these limb defects but not craniosynostosis (Firulli et al., 2005), indicating that these two phenotypes may be mediated by different mechanisms. Consistent with this, we have not found that there is a significant rescue of these limb defects in Twist1+/− mice with differing numbers of functional Id alleles (data not shown).

**Twist1 and Osteoblast Differentiation**

Twist1 has been shown recently to directly inhibit the activity but not the expression of the osteogenic transcription factor Runx2 (Bialek et al., 2004). Our data agree with this as we found that Twist1 inhibited the expression of the later osteoblast differentiation markers BSP and OC without affecting the expression of Runx2. The inhibition of Runx2 by Twist1, however, was shown to only require the C-terminus and not the bHLH domain of Twist1 (Bialek et al., 2004), indicating that Twist1 does not need to dimerize or bind DNA to affect Runx2 function. Our data here do not support this conclusion, as Twist1 required the presence of E2A E12 to inhibit the differentiation of calvaria cells (Fig. 5). Whereas many TWIST1 mutations found in Saethre-Chotzen syndrome (SCS) patients result in the deletion of the carboxy terminus, most of these mutations also delete part or all of the bHLH domain. Furthermore, other SCS mutations are point mutations within the bHLH region that disrupt DNA binding and/or dimerization and leave the carboxy terminus intact (Gripp et al., 2000), suggesting that the transcriptional activity of Twist1 is required for normal regulation of suture patency. We suggest that the regulation of osteoblast differentiation by Twist1 may be at least partially indirect through the regulation of other genes, such as FGFR2, periostin, and TSP-1. Of interest, TSP-1 has been shown to inhibit osteoblast differentiation (Canfield et al., 1996), whereas periostin enhances certain aspects of osteoblast recruitment and differentiation (Horiuchi et al., 1999; Litvin et al., 2004). Therefore, the transition from T/E to T/T in the osteogenic fronts may result in a switch from inhibition of differentiation to induction of at least the early phases of osteoblast differentiation. It is still unclear whether all of these genes are directly transactivated by Twist1 or what is the mechanism that differentiates transactivation by T/T vs. T/E dimmers; however, initial promoter analysis with periostin (Oshima et al., 2002) and TSP-1 (data not shown) suggests that at least these two genes may be directly regulated by Twist1.

Here, we have identified dimer partner selection as an important mediator of Twist1 function. Small changes in the level of Twist1 expression alter the ratio of T/T to T/E, which can have dramatic phenotypic consequences such as craniosynostosis. Twist1 plays important roles in other areas during embryogenesis (Castanon and Baylies, 2002; O’Rourke and Tam, 2002) and has been implicated recently in promoting tumor metastasis (Yang et al., 2004); we anticipate that the regulation of Twist1 dimer formation plays critical roles in these processes as well.

**EXPERIMENTAL PROCEDURES**

**Plasmds and Viral Constructs**

CS2-mTwist1 and CSA-E2A E12 were described previously (Spicer et
Electrophoretic Mobility Shift Assays

EMSA analysis was performed using in vitro translated proteins and double-stranded oligonucleotides containing the E box-binding site from the muscle creatine kinase (MCK) enhancer as a probe as previously described (Lassar et al., 1991).

Cell Culture and Gene Transfer

C3H10T1/2 cells (10T1/2) and 293T HEK cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin–streptomycin. The 293T HEK cells were cultured in DMEM supplemented with 10% fetal calf serum, bovine serum and penicillin–streptomycin. The 293T HEK cells (ATCC) were cultured in DMEM supplemented with 10% fetal calf serum, bovine serum and penicillin–streptomycin.

Immunostaining

10T1/2 cells were analyzed by immunofluorescence as previously described (Leshem et al., 2000) using monoclonal anti-MBP (1:1,000; Sigma); rabbit polyclonal anti-Periostin (1:1,000; a gift from Roger Markwald); and monoclonal anti-TSP1 (Ab-1; 1:250, NeoMarker); rabbit polyclonal anti-E2a (sc-349, Santa Cruz Biotechnology). The following primary antibodies were used: monoclonal anti-β actin (1:1,000 Sigma); monoclonal anti-TSP1 (Ab-1; 1:750, NeoMarker); rabbit polyclonal anti-Periostin (1:1,000; a gift from Roger Markwald); and monoclonal anti-TSP1 (Ab-1; 1:250, NeoMarker).

Mice

Twist1+/− and Wnt1-Cre mice were obtained from the Jackson Laboratory. A cre-responsive transgene CAGCAT-Twist1 was constructed by replacing the lacZ region of CAG-CAT-Z (Araki et al., 1995) with the murine Twist1 cDNA. This construct was used for microinjection to establish a transgenic line. Neural crest-specific transgene expression was achieved by crossing the CAGCAT-Twist1 mice with Wnt1-Cre mice. Id1−/−-Id3+/− mice (gift from R. Beneze; Lyden et al., 1999) were crossed with the Twist1+/− mice to obtain Twist1+/− mice with varying numbers of functional Id alleles.
In Situ Hybridization

Skulls from newborn mice (P1) were fixed in 4% paraformaldehyde, cryosectioned, and analyzed by in situ hybridization as in (Yoshida et al., 2005). In situ probes were against peristin (gift from C. Vary), TSP-1 (gift from P. Bornstein), and FGFR2 (gift from C. Basilio) as indicated. For each probe, sections from wild-type and Twist1+/− skulls were processed in the same container for the same period of time to be able to directly compare gene expression. Whole-mount in situ analysis was performed on P1 skulls after removal of the skin and brain. The in situ protocol was as described by Isaac et al. (2000) with the modification that the skulls were digested with proteinase K digestion for 45 min.

Calvarial Explants

Heads of P1 pups were de-skinned and sectioned horizontally below the nose. Calvaria were placed on Falcon cell culture inserts (PET pore size 0.4 μm) in six-well dishes containing DMEM:Ham's F-12K 50:50, 10 mg/ml streptomycin. Explants were incubated in a CO2 incubator at 37°C with medium changed daily.

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