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Twist1 homodimers enhance FGF responsiveness of the cranial sutures and promote suture closure

Jeannette Connerney [a,b], Viktoria Andreeva [a], Yael Leshem [a,1], Miguel A. Mercado [a], Karen Dowell [a], Xuehei Yang [a], Volkhard Lindner [a,b], Robert E. Friesel [a,b], Douglas B. Spicer [a,b,*]

[a] Center for Molecular Medicine, Maine Medical Center Research Institute, 81 Research Drive, Scarborough, ME, 04074, USA
[b] Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, 5735 Hitchner Hall, Orono, ME 04469, USA

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ABSTRACT

Haploinsufficiency of the transcription factor *TWIST1* is associated with Saethre–Chotzen Syndrome and is manifested by craniosynostosis, which is the premature closure of the calvaria sutures. Previously, we found that Twist1 forms functional homodimers and heterodimers that have opposing activities. Our data supported a model that within the calvaria sutures Twist1 homodimers (T/T) reside in the osteogenic fronts while Twist1/E protein heterodimers (T/E) are in the mid-sutures. *Twist1* haploinsufficiency alters the balance between these dimers, favoring an increase in homodimer formation throughout the sutures. The data we present here further supports this model and extends it to integrate the Twist1 dimers with the pathways that are known to regulate cranial suture patency. This data provides the first evidence of a functional link between Twist1 and the FGF pathway, and indicates that differential regulation of FGF signaling by T/T and T/E dimers plays a central role in governing cranial suture patency. Furthermore, we show that inhibition of FGF signaling prevents craniosynostosis in *Twist1*$^{+/-}$ mice, demonstrating that inhibition of a signaling pathway that is not part of the initiating mutation can prevent suture fusion in a relevant genetic model of craniosynostosis.

Introduction

The flat bones of the skull remain separated by openings termed sutures that function as the growth centers of the bones, allowing growth of the skull during fetal and postnatal development. At the edges of these bones are the osteogenic fronts that contain highly proliferative cells expressing osteogenic markers such as *Runx2* and *alkaline phosphatase* (Cohen, 2000; Opperman, 2000). The mid-suture region is composed of mesenchymal precursor cells. In the human, the sutures gradually close from infancy to young adult. The premature closure of sutures, termed craniosynostosis, is a fairly common disorder, occurring in 1 in 2500 births. Non-syndromic craniosynostosis is most common, however approximately 20% of all cases are associated with mutations in the gene *TWIST1* or one of the fibroblast growth factor receptor (FGFR) genes (Cunningham et al., 2007; Lenton et al., 2005; Rice, 2005). There is a complex relationship between Twist1 and FGF signaling which is not completely understood. Increases in *Twist1* expression and FGF signaling have both been associated with an inhibition of osteoblast differentiation in vitro, yet craniosynostosis occurs due to activating mutations of the FGFR genes and haploinsufficiency of *TWIST1* (Cunningham et al., 2007; Lenton et al., 2005; Rice, 2005). Eighty percent of patients with Saethre–Chotzen Syndrome (SCS), which is one of the most common autosomal dominant disorders of craniosynostosis, have mutations in *TWIST1*, however mutations in *FGFR2* and *FGFR3* have also been reported in some patients that have phenotypes consistent with SCS, exemplifying this relationship and further indicating that haploinsufficiency of *TWIST1* gives a similar phenotype as activation of FGFR signaling (Chun et al., 2002; el Ghouzzi et al., 1997; Howard et al., 1997; Paznekas et al., 1998). Receptor tyrosine kinase signaling by FGF, IGF1, and HGF induces the expression of *Twist1* (Dupont et al., 2001; Fang et al., 2001; Isaac et al., 2000; Leshem et al., 2000; Rice et al., 2000), while Twist can affect the expression of FGF receptor genes. In *Drosophila* and *Caenorhabditis elegans*, Twist induces the expression of the FGFR homolog *DFR1* and *egl-15*, respectively (Castanon and Baylies, 2002); however, the relationship between Twist1 and FGFR in vertebrates has been less clear. In the cranial sutures, *FGFR2* expression is normally only detected in the osteogenic fronts but it extends into the mid-suture of *Twist1*$^{+/-}$ mice, suggesting that Twist1 may normally inhibit *FGFR2* expression (Connerney et al., 2006; Rice

* Corresponding author. Center for Molecular Medicine, Maine Medical Center Research Institute, 81 Research Drive, Scarborough, ME, 04074, USA. Fax: +1 207 885 8179.
E-mail address: spiced@mmc.org (D.B. Spicer).
[1] Current address: Savyon Diagnostics, 3 Habosem Street, Ashdod, 77610, Israel.

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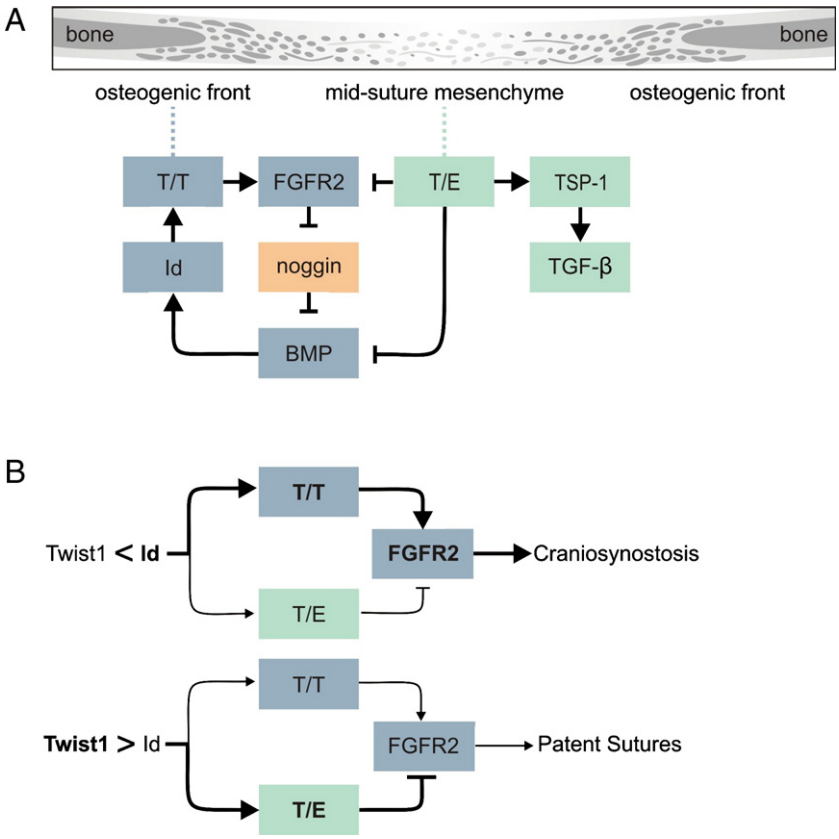

Fig. 1. Model. (A) Drawing showing parts of a suture going from a more differentiated bone to a less differentiated mid-suture mesenchyme. The model shows T/T dimers up-regulating FGFR2 expression resulting in increased FGF signaling toward the middle of the suture. FGF signaling inhibits the expression of the BMP antagonist *noggin*, resulting in increased BMP signaling which up-regulates Id1 expression, further enhancing T/T homodimer formation. On the other hand, T/E dimers inhibit FGFR2 expression, as well as BMP signaling through binding to Smad proteins. T/E dimers also up-regulate TSP1 expression, which activates latent TGFβ. This could have differing outcomes dependent on which TGFβ isoform is present. (B) The ratio of T/T to T/E determines the functional output of Twist1 expression, and this is determined by the relative expression of Twist1 and Id1 proteins. When Id1 levels are greater that Twist1 this balance is shifted toward T/T formation resulting in up-regulation of FGFR2 leading to craniosynostosis. When Twist1 levels are higher than Id1 the balance is toward an increase in T/E formation, inhibiting FGFR2 expression and resulting in patent sutures.

et al., 2000). However, calvaria cells isolated from a SCS patient with a mutation in *TWIST1* had decreased *FGFR2* levels, which were increased when Twist1 was ectopically expressed (Guenou et al., 2005). Therefore, the relationship between Twist1 and FGFR2 may be context dependent. Consistent with this, we recently found that Twist1 forms different dimer complexes that have opposing effects on *FGFR2* expression (Connerney et al., 2006).

Twist1 is a basic-Helix–Loop–Helix (bHLH) transcription factor that plays both positive and negative roles in the regulation of early morphogenesis and differentiation of mesenchymal tissues (O'Rourke and Tam, 2002). We found that, unlike most other bHLH proteins, Twist1 can form functional homodimers (T/T) as well as heterodimers with ubiquitously expressed bHLH E proteins (T/E). These dimers have distinct activities and regulate the expression of different sets of genes. The ratio between these dimers is determined by the relative levels of Twist1 and the HLH Id proteins (Connerney et al., 2006). Id proteins preferentially dimerize with E proteins and disrupt functional Class I/II bHLH heterodimers from forming (Massari and Murre, 2000). We found that Twist1 formed T/E dimers in the absence of Id, and formed T/T dimers when Id levels were increased (Connerney et al., 2006). Consistent with this, we found that in the sutures, genes that are regulated by T/T dimers, such as *FGFR2* and *periostin*, are expressed in the osteogenic fronts where Twist1 and Id1 are co-expressed, while T/E-regulated genes, such as *thrombospondin 1* (*TSP-1*), are expressed in the mid-sutures where only Twist1 is expressed. In the sutures of *Twist1*$^{+/-}$ mice, the ratio between these dimers is altered, favoring an increase in homodimers and an expansion into the mid-suture of the expression of T/T-regulated genes, and a complete absence of T/E-regulated genes. Furthermore, we were able to inhibit craniosynostosis in *Twist1*$^{+/-}$ mice by modulating the balance between these dimers toward T/E formation, by either increasing the expression of E2A E12 or by decreasing Id1 expression (Connerney et al., 2006).

Here we propose a model that integrates Twist1 dimer formation with the signaling pathways known to regulate cranial suture patency (Fig. 1A). Our model predicts that the decrease in *Twist1* expression due to *Twist1* haploinsufficiency results in a higher T/T to T/E ratio extending beyond the osteogenic fronts due to Id1 levels out-competing Twist1 for dimerization with E proteins. The increase in T/T dimers expands the expression of *FGFR2*, resulting in increased FGF signaling. T/E dimers have recently been shown to inhibit BMP signaling (Hayashi et al., 2007), which is predominantly active in the osteogenic fronts (Warren et al., 2003), and therefore the decrease in T/E dimers in *Twist1*$^{+/-}$ sutures, would allow for an expansion of BMP signaling as well. BMP signaling induces Id expression (Rice et al., 2000), which would further promote T/T formation. This positive feedback loop then causes the sutures to close prematurely, resulting in craniosynostosis. Our data support this hypothesis and illustrate that the sutures of *Twist1*$^{+/-}$ mice have increased FGF and BMP signaling, and that inhibition of FGF signaling prevents craniosynostosis in *Twist1*$^{+/-}$ mice. Furthermore, enhanced T/T expression on a wild type background leads to a similar suture closure phenotype as *Twist1*$^{+/-}$ mice, indicating that T/T dimers play an active role in promoting craniosynostosis, and that the balance between the Twist1 dimers dictates suture patency.