Published in final edited form as:

Nat Genet. 2005 April ; 37(4): 373–381. doi:10.1038/ng1525.

Altered Twist1 and Hand2 dimerization is associated with Saethre-Chotzen syndrome and limb abnormalities

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Abstract

Autosomal dominant mutations in the bHLH transcription factor *Twist1* are associated with limb and craniofacial defects in humans with Saethre-Chotzen syndrome (SCS). The molecular mechanism underlying these phenotypes is poorly understood. We show that the ectopic expression of the related bHLH factor *Hand2* phenocopies *Twist1* loss-of-function phenotypes in the limb, and that they display a gene dosage-dependent antagonistic interaction. *Twist1* and *Hand2* dimerization partner choice can be modulated by PKA and protein phosphatase 2A-regulated phosphorylation of conserved helix I residues. Interestingly, multiple *TWIST1* mutations associated with SCS alter PKA-mediated *Twist1* phosphorylation, suggesting that misregulation of *Twist1* dimerization via either stoichiometric or posttranslational mechanisms underlies SCS phenotypes.

Studies of developing vertebrate limbs have yielded many insights into the process of embryonic pattern formation. Prominent among these are the identification of a growing catalog of transcription factors that orchestrate limb patterning. While the genetic and biochemical interactions of these transcription factors are clearly important for integrating patterning information, these interactions are poorly understood. *Twist1* and *Hand2* are basic helix-loop-helix (bHLH) transcription factors within the Twist family, and are attractive candidates for investigating such interactions. Each is required for distinct yet subtly related aspects of limb development, and biochemical studies have revealed a complex regulation of their protein-protein interactions^{1–3}.

Early limb bud expression of *Twist1* is observed primarily in the peripheral mesenchyme, and *Twist1* is required for maintenance of the overlying apical ectodermal ridge (AER)^{4–7}. *Twist1* haploinsufficiency in mice and humans is associated with a range of limb abnormalities. *Twist1* heterozygous null mice display a partially penetrant preaxial polydactyly^{8,9}. Human

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TWIST1 nonsense, missense and null mutant alleles^{8,10–12} are associated with distal limb abnormalities (including polydactyly, brachydactyly and syndactyly) as seen in patients with the autosomal dominant craniosynostosis disorder Saethre-Chotzen Syndrome (SCS)¹². While some *TWIST1* missense alleles display deficient nuclear localization or impaired DNA binding *in vitro*^{13,14}, a mechanistic understanding underlying many of the *TWIST1* SCS mutations is lacking.

Following the onset of limb bud outgrowth, *Hand2* is expressed primarily in the posterior limb mesenchyme, where *Hand2* expression is both dependent on, and necessary for, normal *sonic hedgehog* (*Shh*) expression^{15–17}. Furthermore, ectopically expressed *Hand2* within the anterior limb mesenchyme induces preaxial polydactyly and activates the *Shh* pathway, although it does not always induce ectopic expression of *Shh*^{15–17}. While *Hand2* appears to act through a maintenance loop with *Shh*, additional functions for *Hand2* are possible, as the *Hand2* expression domain is broader than that of *Shh*^{15–17}. Interestingly, both *Twist1* and *Hand2* are associated with polydactylous limb phenotypes, raising the issue of whether they may interact genetically and/or biochemically.

Although direct interactions between Twist1 and Hand2 have not been described, numerous genetic and functional studies of Twist-related bHLH factors have been reported^{1,3,18}. A number of Twist family proteins, including Twist1 and Hand2, not only dimerize with ubiquitously expressed E proteins but also exhibit promiscuous dimerization with other class B bHLH proteins, and can form stable homodimers^{19–21}. Furthermore, the dimerization partner choice of a related Twist family member, Hand1, can be modulated via the phosphorylation state of specific conserved residues in helix I (ref. 20). Thus, Twist1/Hand2 interactions might depend on partner availability as well as regulatory posttranslational modifications that influence protein-protein interactions¹⁸.

Here we investigate the biochemical and genetic interactions between Twist1 and Hand2 both *in vitro* and during limb development. We show that PKA and B56δ-containing PP2A can regulate Twist1 and Hand2 phosphorylation at the conserved helix I residues, that hypophosphorylation and phosphorylation mimics of these residues alter bHLH dimerization affinities, and that a population of *TWIST1* mutations that cause SCS in humans exhibit dysregulation of this phosphoregulatory circuit. We also show that ectopic *Hand2* expression phenocopies multiple SCS-like limb phenotypes, that the appropriate genetic dosage of *Hand2* and *Twist1* is critical for proper limb development, and that these interactions require the phosphoregulated helix I residues. These findings support a mechanism where the Twist family dimerization partner choices are modulated by both the relative levels of gene expression and the phosphorylation state of key helix I residues, thereby dictating changes in the regulation of their downstream target genes. We propose that the disruption of this regulatory mechanism underlies the phenotypes observed in a subset of *TWIST1* mutants that cause SCS.

RESULTS

PKA and PP2A: B56δ regulate Twist1 and Hand2 phosphorylation

Sequence comparisons indicate that the phosphorylation-regulated threonine and serine residues in helix I of Hand1 are highly conserved among the bHLH domains of Twist family proteins, and are conserved among *Twist1* homologs as distant as *Drosophila Twist* (Fig. 1a). By contrast, threonine and serine residues at this position within helix I are not generally found in bHLH proteins outside of the Twist family. This striking conservation raised the possibility that modulating the phosphorylation state of these residues might be a general mechanism that controls the activity not only of Hand1 (ref. 20), but also of Twist1, Hand2 and other Twist-related factors.

To test whether the conserved helix 1 residues of Twist1 and Hand2 could be phosphorylated by PKA, we coexpressed wild type Hand2 and Twist1 or Hand2 T112;S114A and Twist1 T125;S127A double alanine substitution mutants with constitutively active PKA in metabolically-labeled HEK293 cells (Fig. 1b). Immunoprecipitation of radiolabeled proteins shows that PKA can phosphorylate Hand2 and Twist1; moreover, specific mutation of the conserved helix 1 residues reduces the level of ^{32}P incorporated into either Hand2 or Twist1 (Fig. 1b).

We next asked if Twist1 and the PP2A regulatory subunit B56 δ can form a complex by coexpressing Twist1 with either B56 δ or B56 α in HEK293 cells (Fig. 1c). Immunoprecipitation (IP) followed by immunoblot analysis shows that Flag-tagged Twist1 co-immunoprecipitates significant amounts of B56 δ , while interaction between Twist1 and the related PP2A subunit B56 α , is significantly weaker (Fig. 1c). Interestingly, Twist1 appears to form a stronger interaction with B56 δ than Hand1 (Fig. 1b). Thus, similar to observations reported for Hand proteins²⁰, Twist1 can be phosphorylated by PKA and interacts specifically with the PP2A B56 δ regulatory subunit.

We next sought to determine if the conserved helix I threonine and serine residues are PKA phosphorylation targets, and if B56 δ facilitates specific dephosphorylation of Twist1 and Hand2. We performed phosphopeptide-mapping analyses of both wild type and hypo-phosphorylation mutants of these proteins coexpressed with active PKA and/or B56 δ or B56 α in metabolically labeled HEK293 cells (Fig. 2). PKA coexpression results in *de novo* detection of two Hand2 phosphopeptides and increased phosphorylation of an additional phosphopeptide (Fig. 2a,b). Addition of B56 α reduces ^{32}P incorporation in a single peptide compared to Hand2 with PKA (Fig. 2c,l). This suggests that B56 α can moderately affect Hand2 phosphorylation despite the lack of evidence for direct protein-protein interaction. Coexpression of Hand2, PKA and B56 δ results in a more robust reduction (38% of Hand2 plus PKA) in the phosphorylation of this peptide (Fig. 2d,l). Moreover, mutation of the conserved T112 and S114 residues to alanines reduces ^{32}P incorporation into this peptide to undetectable levels (Fig. 2e,l).

In order to further define the phosphorylated residues of Hand2, we raised polyclonal antisera against a Hand protein helix I peptide that contains a phosphoserine at the S114 position, and used it for immunoblotting extracts of HEK293 cells transfected with various Hand2 mutants in combination with PKA (Fig. 2m). This antiserum detects a wild type Hand2 band only when Hand2 is coexpressed with PKA (Fig. 2m). The T112;S114A mutation reduces this phosphospecific band to undetectable levels, while the basic domain T103A mutation has no effect. These data provide additional evidence that the S114 residue of Hand2 is phosphorylated by coexpression of Hand2 with constitutive PKA.

Coexpression of Twist1 with active PKA also results in increased protein phosphorylation (Figs. 1b;2f,g). The addition of B56 α has no significant effect on Twist1 phosphorylation (94% of wild type Twist1 phosphorylation levels; Fig. 2h,l). In contrast, coexpression of Twist1 with PKA and B56 δ reduces the phosphorylation of a single Twist1 phosphopeptide by 75% (Fig. 2i,l), while mutation of T125 and S127 to alanines reduces this phosphorylation to undetectable levels (Fig. 2j). Thus, Hand2 and Twist1 can be post-translationally modified by PKA and B56 δ -containing PP2A, consistent with a possible regulatory circuit that utilizes the helix I phosphorylation state to control the activity of these proteins.

Twist1 SCS mutations alter T125 and S127 phosphorylation

Missense mutations of several basic domain residues in human *TWIST1* are associated with SCS^{8,10–12,22,23}. As the mutated residues are adjacent to the phosphorylated helix I residues, this raised the possibility that these mutations might disrupt the Twist1 PKA-PP2A regulatory

circuit. We therefore assessed the phosphorylation of Twist1 mutated at each basic domain residue (R118, R120, Q121, R122) individually (Fig. 3a–h). We found that each mutation greatly reduced the phosphorylation of T125;S127 by PKA in metabolically labeled HEK293 cells (Fig. 3).

Three of these mutations replace positively charged residues, suggesting that the degree of PKA-mediated phosphorylation is proportional to the basic character of the basic domain. By contrast, a dominant negative *C. elegans* Twist E29K mutation²⁴ increases the alkalinity of the basic domain, and thus might enhance PKA-mediated phosphorylation. Phosphopeptide mapping analysis of the analogous Twist1 E117K mutant indicates that this is indeed the case (Fig. 3i,j). Taken together, these data demonstrate that Twist1 helix I phosphorylation can be modified by mutation of nearby residues, including residues with mutations found in SCS patients.

Twist1:Hand2 dimerization is regulated at T125 and S127

We next tested how differential phosphorylation might impact the activities of Hand2 and Twist1. Previous reports demonstrate that Hand1 and Hand2 can form homo- or heterodimers, and that Hand1 dimerization choices can be phosphoregulated^{19,20}. These observations raise the possibility that interactions between Twist1 and other Twist family members might be similarly regulated. We therefore asked whether Twist1 can heterodimerize with Hand1 or Hand2, and if mimicking phosphorylation at the conserved threonine and serine residues can regulate Twist1 dimerization affinities.

To assess the ability of Twist1 to complex with Hand proteins, we performed IP-immunoblot analysis on extracts of HEK293 cells cotransfected with epitope-tagged forms of Twist1, Hand1 and Hand2 (Fig. 4a). Expression of Flag-tagged Twist1 with myc-tagged Hand1 or Hand2 results in specific co-immunoprecipitation of Twist1 with either Hand protein. As expected, Hand protein heterodimeric and homodimeric complexes are also readily detectable when epitope-tagged Hand1 or Hand2 are coexpressed.

Fluorescence resonance energy transfer (FRET) occurs between donor and acceptor fluorescent molecules when they are in close proximity, and can be used to demonstrate direct protein-protein interactions. To compare interactions between various Twist1 dimerization partners, we used acceptor photobleaching FRET to measure the fraction of donor fluorescence that is absorbed by the acceptor (FRET efficiency)²⁵. We generated both CFP and YFP carboxy-terminal fusion proteins of E47, Hand2, wild-type and T125;S127 mutant forms of Twist1, coexpressed them in HEK293 cells and measured FRET efficiencies (Fig. 4b). Our results show that wild type Twist1 forms dimers with itself, E47, and most strongly with Hand2. The Twist1 phosphorylation mimic T125;S127D displays a general reduction in dimerization, altering E47/Twist1 heterodimers and Twist1 homodimers to a greater extent than the Hand2/Twist1 heterodimer. Altered FRET efficiency is also observed in the hypophosphorylation mutant T125;S127A. While this mutant also shows a general reduction in heterodimerization, the Hand2/Twist1 heterodimers are more severely affected than E47/Twist1. By contrast the Twist1 T125;S127A homodimer is stabilized compared to wildtype Twist1. This results in distinctly different relative FRET efficiencies than those observed with wild type and Twist1 T125;S127D. Taken together, these findings suggest that the alteration of the phosphorylation state of Twist1 at T125 and S127 affects the dimerization affinity of Twist1 for its various potential partners, including Hand2.

Twist1 and Hand2 limb bud expression domains overlap

Having established that Twist1 and Hand2 can interact *in vitro*, we next sought to determine if they might interact *in vivo* during embryonic development. We chose to examine the

developing vertebrate limb bud, as previous studies indicated that both genes are expressed in the limb^{6,7,15–17}. There is overlap in the RNA expression of *Twist1* and *Hand2* in both chick and mouse limb mesenchyme throughout limb morphogenesis (Fig. 5). *Twist1* is expressed broadly in the peripheral mesenchyme from Hamburger-Hamilton stage 19 through stage 30 (ref. 26) in chick and from E9.5 through E12.5 in mouse, with elevated levels observed at the anterior and posterior margins. The major *Hand2* expression domain is initially restricted to posterior mesenchyme, and expands progressively towards the distal anterior. A minor anterior proximal *Hand2* expression domain is also visible. While *Twist1* and *Hand2* are initially coexpressed within the posterior limb mesenchyme, the region of overlap expands by stage 24 or E11.5 to include much of the distal mesenchyme underlying the AER (Fig. 5a–h,q–t). At the time of digital condensation, extensive interdigital expression of both genes is observed (Fig. 5i–l). Subsequently, overlapping expression is seen in the peridigital mesenchyme at stage 30 of the chick and E12.5 in the mouse (Fig. 5m–p). These results demonstrate that *Twist1* and *Hand2* are coexpressed within distinct regions of the developing vertebrate limb, consistent with the idea that these two factors may functionally interact.

Hand2 misexpression phenocopies Twist1 loss of function phenotypes

If *Hand2* and *Twist1* act together in the limb, then *Hand2* misexpression might cause phenotypes related to those seen in *Twist1* mutants. To test this, we used a replication-competent retrovirus to misexpress wild type *Hand2* in developing chick limbs by general or focal infection at stages 10–20, and examined cartilage, soft tissue and molecular phenotypes through E10 (Fig. 6). We observed preaxial polydactyly in wings stained for cartilage patterning. Affected anterior digit identities varied widely, ranging from cartilage blebs and posterior transformations through induction of ectopic digit 3s (Fig. 6a–c). While these observations are consistent with previous reports, the extent of the digit duplications is more severe than previously described following *Hand2* misexpression in chick limbs^{15,16}. We also observed several novel limb phenotypes that are distinct from those previously associated with *Hand2* misexpression. These include frequent cases of syndactyly (Fig. 6d,e), shortened cartilage elements in digits and long bones (Fig. 6f,g), and occasional bent digits (Fig. 6h,i). We also detected AER disruptions, as indicated by gaps in the expression of the AER marker *Fgf8* (Fig. 6j,k), and regions of reduced distal mesenchyme outgrowth (Fig. 6l,m), that are consistent with disrupted AER maintenance. This combination of limb phenotypes caused by ectopic *Hand2* expression resembles those seen in *Twist1*^{+/-} mice or humans with reduced or altered *TWIST1* activity (syndactyly, brachydactyly, hallux valgus, AER disruption and polydactyly)^{4,12}. The similarities between the phenotypes resulting from *Twist1* loss of function mutations and *Hand2* gain of function experiments are consistent with the idea that these genes have antagonistic functions in limb development.

Genetic interactions between Twist1 and Hand2

We next examined genetic interactions between *Twist1* and *Hand2* to test for functional antagonism. If they do interact antagonistically, then either reducing *Hand2* levels to compensate for reduced expression of *Twist1*, or increasing *Twist1* levels when misexpressing *Hand2*, might change their respective effects on limb development. We first asked whether lowering *Hand2* levels would suppress the effects of reduced *Twist1*. While *Hand2* heterozygous null mice are normal, *Twist1* heterozygous null mice display a partially penetrant preaxial polydactyly of the hindlimbs (Fig. 7a; refs. 8, 9). We intercrossed *Twist1*^{+/-} and *Hand2*^{+/-} mice to determine if lowering *Hand2* gene dosage would rescue the *Twist1*^{+/-} mediated polydactyly (Fig. 7b). 18 of 43 (42%) F1 *Twist1*^{+/-} animals had hindlimb polydactyly, while their F1 *Hand2*^{+/-} siblings were all normal (53 of 53). By contrast, polydactyly was never observed (0 of 44) in compound F1 *Hand2*^{+/-} *Twist1*^{+/-} offspring ($p < 0.01$, χ^2). These results reveal a novel dose-dependent requirement for *Hand2*, and show that the relative levels of *Hand2* and *Twist1* critically affect limb development.

We then tested whether increased *Twist1* expression could compensate for increased *Hand2* levels by misexpressing *Hand2* either in combination with *Twist1*, or with *GFP* as a control. We coinfecting the anterior of stage 20 chick wing buds with two viruses that allow simultaneous transduction of two genes. We noted the identities of ectopic digits at E10, scored each limb based on the digit with the most posterior identity, and compared the frequency distribution for each virus combination (Fig. 7c). As the degree of digit posteriorization is proportional to the strength of polarizing signal or *Shh* pathway activation^{27,28}, this allowed us to quantify the activity of the virally expressed proteins. *Hand2* plus *GFP* virus infection induced a range of duplications, with an ectopic digit 3 noted in 64% of limbs, digit 2 in 21% and no ectopic digit in 15%. The extent of duplications induced by *Hand2* plus *Twist1* was significantly weaker, with an ectopic digit 3 in only 38% of limbs, a digit 2 in 28% and no ectopic digit in 35% ($p < 0.01$, Mann-Whitney). Focal anterior *Twist1* virus infection alone had no effect on limb morphology, although *Twist1* did cause gross cartilage abnormalities when misexpressed throughout the limb bud, providing independent confirmation of *Twist1* virus activity (Fig. 7c, data not shown). Thus increasing *Twist1* levels can suppress the effects of increased *Hand2*.

We also tested whether the conserved helix I threonine and serine residues are required for the ability of *Twist1* to suppress the *Hand2* misexpression phenotype. We generated a retrovirus carrying the *Twist1* helix I double alanine substitution, and tested it in the anterior limb coinfection assay. As observed with wildtype *Twist1*, anterior misexpression of the mutant *Twist1* alone did not cause patterning defects. Unlike the WT gene, however, the mutant *Twist1* had no effect on the ability of *Hand2* to cause digit duplications (*Hand2* plus *GFP*, 69% ectopic digit 3; *Hand2* plus *Twist1* mutant, 69% ectopic digit 3; Fig. 7d). Thus the same helix I mutation that prevents *Twist1* phosphorylation and that alters *Twist1* dimerization partner preference also disrupts *Twist1* biological activity *in vivo*.

DISCUSSION

In this study, we describe two mechanisms by which the activities of *Twist1* and *Hand2* can be regulated, either by changes in their relative levels of expression or through phosphorylation. Specific threonine and serine residues in helix I shared by *Twist1* and *Hand2* are conserved among a majority of *Twist* family members. We find that these residues can be post-translationally modified via the actions of PKA and B56 δ containing PP2A, and provide evidence that dimerization affinity can be altered by such modifications. We also find that ectopic *Hand2* expression phenocopies limb phenotypes resulting from mutations in *Twist1*, including those presented in patients with SCS. We furthermore demonstrate a gene dose-dependent interaction between *Twist1* and *Hand2* in both combination gain-of-function and combination loss-of-function experiments and show that a mutation that alters *Twist1* dimerization and phosphorylation *in vitro* also alters its genetic interactions with *Hand2* *in vivo*. These data strongly support models in which *Twist* family bHLH protein dimerization partner choice is crucial for normal development.

Early models of bHLH function posited that competition between transcriptionally active E proteins and transcriptionally inactive HLH proteins for dimerization with tissue specific bHLH proteins was the primary mechanism regulating the formation of bHLH complexes^{18, 29}. However, it is now clear that regulation by both phosphorylation and interactions with a wider spectrum of dimerization partners are also significant additions to this model. We previously showed that PKA and B56 δ -containing PP2A regulate *Hand1* dimerization affinities for other bHLH proteins through phosphorylation of helix I (ref. 20). The current demonstration that *Twist1* and *Hand2* are similarly modified suggests that other *Twist*-family proteins that share the phosphorylated helix I residues also share this regulatory mechanism. The embryological significance of the *Twist1* post-translational modifications is reflected in the phenotypes observed in SCS patients¹², in *C. elegans* models and in the data presented

here (Fig. 7) in which Twist1 point mutations that disrupt the PKA/PP2A circuit alter the developmental activity of Twist1 (ref. 24). These observations provide a potential mechanistic explanation for several mutations found in SCS patients. These include five basic domain mutations and mutation of the conserved phosphorylation-regulated helix I serine^{10,12,24}.

The use of phosphorylation to regulate Twist family dimerization may extend beyond interactions with the Twist family and E proteins. For example Twist1 can bind the myogenic factor MyoD, and negatively regulate its activity^{30,31}. Interestingly, a Twist1 R120A;R122A;R124A triple mutation was reported to disrupt interactions between MyoD and Twist1 (ref. 30). Our results show that mutating these residues individually also disrupts PKA-mediated phosphorylation of Twist1 helix I (Fig. 3). These data are consistent with the idea that the MyoD and Twist1 interaction may be regulated in part by phosphorylation of Twist1.

Although the Twist1 basic domain mutations can affect the ability of PKA to phosphorylate the helix I residues, it is possible that the developmental effects may result solely from changes in DNA binding specificity. We feel this is unlikely for several reasons. First, Twist carrying several of the SCS mutations tested here remains capable of DNA binding as *C. elegans* Twist-Daughterless heterodimers²⁴. Nevertheless, DNA binding is not required for all Twist1 activity, as a Twist1 mutant with a non-DNA binding basic domain can still inhibit transcriptional activation by MyoD and Mef2 (ref. 31). Similarly, Hand2 that lacks the basic domain remains competent to induce polydactyly when misexpressed in mouse limbs³². Thus multiple lines of evidence point toward Twist1 having DNA binding-independent activities that may be influenced by properties of its basic domain.

While proper *Twist1* gene dosage has long been appreciated as critical for normal development, the molecular basis for this is less clear^{8,9,11,12}. Our results indicate that at least in the limb, reduced *Twist1* or increased *Hand2* dosage disrupts a critical antagonistic balance between *Twist1* and *Hand2*. *Hand2* and *Twist1* are expressed in the limb in overlapping domains, and these proteins can form heterodimers with each other, form homodimers, or form heterodimers with other partners. Changing the *Twist1:Hand2* dosage probably alters the relative amounts of the various possible protein combinations, leading to changes in target gene expression and consequent developmental defects. While we currently do not know which complexes are most critical, there is precedent for specific Twist dimer complexes differentially regulating transcription of target genes²¹ and thus each unique dimer likely exhibits unique biological activities. Studies in *Drosophila* using forced dimers show that Twist homodimers specify mesoderm and the somatic myogenic lineage while Twist-daughterless heterodimers repress the transcription of genes required for somatic myogenesis²¹. A similar experimental strategy may help define the functions of the various Twist1 and Hand2 dimer combinations in the limb.

Some of the ectopic *Hand2* limb phenotypes reported here may reflect a normal developmental mechanism where high levels of Hand2 are required to antagonize Twist1 activity. The AER disruption associated with Hand2 misexpression is one example. AER disruption can be observed adjacently to ectopic outgrowths that correspond to sites of Hand2 virus infection, and thus presumably is due to high levels of Hand2 expression (Fig. 6, data not shown). As Twist1 is normally required in the mesenchyme for the function of an FGF signaling loop between mesenchyme and ectoderm that maintains the AER^{4,5,33}, the *Hand2* virus phenotype may be caused by interference with this Twist1 activity. Interestingly, *Hand2* expression is normally highest in the posterior limb, adjacent to ectoderm just proximal of the AER, and from which the AER has regressed in concert with distal limb outgrowth. Thus, one normal function of high level *Hand2* expression may be to limit the posterior-proximal extent of the AER.

Bialek et al.³⁴ recently showed that a novel carboxy-terminal ‘Twist domain’ conserved in Twist1 and Twist2 binds Runx proteins and inhibits Runx transcriptional activity. As Runx2 promotes ossification of cartilaginous and membranous bone, Twist proteins thereby act to delay the onset of ossification. It is proposed that in SCS patients, either mutation of the Runx binding domain or reducing Twist1 levels generates excess free Runx2 protein that promotes premature ossification, resulting in the craniofacial and skeletal defects of SCS. How Hand2 activity fits into this paradigm is unclear. Hand2 levels in the limb may modulate the availability of Twist1 for interaction with Runx proteins. Or, as Runx mutants with polydactyly have not been described^{35–38}, interactions between Hand2 and Twist1 in the early limb may operate independently of Runx pathways. Whether changes in *Hand2* gene dosage can alter osteogenic SCS phenotypes is an interesting question. Similarly, the Twist1 double alanine mutation tested here does not suppress the Hand2-induced polydactyly phenotype and also does not cause gross cartilage defects. Whether the loss of this latter activity is due to defective Hand2 interactions or reflects a requirement for these residues for broader aspects of Twist1 functionality remains to be determined. These and future studies will expand our understanding of how combinatorial interactions among broadly expressed transcription factors drive highly context-specific developmental programs.

METHODS

Constructs

Twist1 and *Hand2* retroviruses were constructed by subcloning *cTwist1*, *cTwist1T109;S111A* or *mHand2* cDNAs into the RCAS BP(A) and RCAS BP(B) vectors³⁹. The *cTwist1* constructs contain an amino-terminal FLAG epitope tag (Sigma). RCAS BP(A) *GFP* was described previously³⁹. pIRES FLAG-*Hand1* and *Hand2* encode amino terminal FLAG epitope tag fusions cloned into pIRES NEO (Clontech). B56 α and B56 δ cDNAs were cloned into the expression vector pCEP4-l. Full length *E12* and *E47* were a gift from Y. Kee and M. Bronner-Fraser. Myc- and FLAG-tagged mouse *Twist1* were a gift from E. Olson. E-protein and Hand YFP and CFP fusion proteins were made as described²⁰. Twist1 YFP and CFP fusion proteins were cloned by inserting the *mTwist1* cDNA beginning from codon 3 in frame into pEYFP-N1 and pECFP-N1 (Clontech). *Hand2* and *Twist1* point mutants were generated using the Quick-Change Mutagenesis kit (Stratagene) following the manufacturer’s protocols.

Cell culture, metabolic labeling and immunoprecipitations

EK293 cells were grown and transfected as described²⁰ with the indicated constructs. In some experiments a single CaPO₄ precipitate was split onto duplicate plates, with one used for metabolic labeling and one for immunoblot analysis. For metabolic labeling 48 hours post transfection cells were incubated with 1mCi of ³²P orthophosphoric acid (NEN)/ml of phosphate-free DMEM supplemented with dialyzed FBS for 4 hours. Cells were washed in 20mM Na HEPES pH 7.4 and 150mM NaCl and lysed in 20mMNaPO₄, 150mMNaCl, 2mMMgCl₂, 0.1%NP40, 10% glycerol, 3 μ g/ml leupeptin, 3 μ g/ml pepstatin, 1mM PMSF, 50 μ M NaVO₄, 5mM NaF, 100nM Okadaic acid, and 5mM Beta glycerol phosphate and equal amounts of protein were immunoprecipitated with agarose-conjugated FLAG M2- or myc beads (Sigma) for 2 hours. Samples were resolved through a 12% SDS PAGE gel, and exposed to a phosphorimager screen.

Immunoblotting

Cell lysates were collected, and immunoprecipitations performed as described²⁰. Cell lysates or immunoprecipitated proteins were run through 12% SDS PAGE gels, electroblotted and incubated with the indicated antibody as described²⁰. Blots were visualized using the Super

Signal Luminescent detection protocol (Pierce). Rabbit anti-phosphoHand antisera was commercially prepared by Sigma using the peptide shown in Fig. 2 as antigen.

2-dimensional phosphopeptide mapping

HEK293 cells were grown, transfected, labeled with ^{32}P , and immunoprecipitated exactly as described²⁰. TLC and gel exposures were visualized by phosphoimager. Quantification of peptide intensities was performed using Image Quant phosphoimager software. Equal-sized volume boxes were placed over variable experimental and invariant control phosphopeptides, and integrated volumes and volume ratios of affected to control peptides were calculated.

FRET

HEK293 cells were transfected on glass cover slips with the indicated constructs, grown for 48 hrs, fixed and mounted. Measurement of acceptor photo-bleaching of FRET efficiency was performed exactly as described²⁵. The fluorescence emission from the donor and the acceptor were examined in each cell to ensure that assayed cells expressed equivalent levels of CFP and YFP. The fluorescence emission from the donor and the acceptor were collected sequentially. Average fluorescence intensities of the donor were measured before and after bleaching. A minimum of 30 data points for each dimer pair were collected. The FRET efficiency was calculated by $E_T = (1 - (I_{DA}/I_D)) * 100$, where I_{DA} and I_D represent the steady state donor fluorescence in the presence and the absence of the acceptor.

In situ hybridization

Non-radioactive whole mount and section *in situ* hybridization were performed as previously described⁴⁰. Probes for the following genes were used: *mHand2* (ref. 41), *cHand2* (ref. 42), *cTwist1* (ref. 6), *mTwist1* (ref. 4).

Virology

SPF White Leghorn chick embryos (Charles River) were used for all experiments. Concentrated virus stocks of the indicated viruses were generated as described, and all viruses had titers of at least 5×10^8 infectious particles/ml³⁹. Flag-cTwist1 and Flag-cTwist1T109;S111A protein expression and nuclear localization were confirmed by anti-Flag M2 immunostaining. For single virus experiments, stage 10 through stage 20 embryos were either generally or focally infected in the fore and hind limb fields using standard procedures³⁹. For double virus experiments, (A) and (B) coat viruses were combined to equal titers, and stage 20–21 wing buds were focally infected under the anterior AER. Embryos were harvested through E10 for subsequent analyses. E10 chick limb skeletal and soft tissue morphologies were revealed by alcian green staining followed by clearing in BABB (1:2 v/v benzyl alcohol:benzyl benzoate). Digit identities were scored as described⁴³. Statistical comparisons were performed using the Mann-Whitney test for nonparametric distributions.

Mice

Outbred Swiss Webster mice (Taconic) were used for wild type embryo collection. Noon on the day of a mating plug was considered E0.5. *Hand2*^{+/-} mice⁴¹ were obtained on a mixed 129.B6 background and backcrossed to B6 through N5. *Twist1*^{tm1Bhr/+} mice⁴ (Jackson Laboratory) were maintained on a B6 background. We observe 44% penetrance (N=124 *Twist1*^{tm1Bhr/+} animals) of hindlimb polydactyly on this background. *Hand2*^{+/-} B6(N2) to B6 (N5) and *Twist1*^{tm1Bhr/+} animals were intercrossed, and hindlimbs of F1 progeny scored for the presence of ectopic digits. As the *Hand2*^{+/-} B6 backcross generation had no effect on the frequency of polydactyly in *Twist1*^{tm1Bhr/+}; *Hand2*^{+/-} animals, data from all generations were pooled. Animals were pcr-genotyped for *Twist1* (ref. 9) and *Hand2* alleles⁴¹ as described. Alizarin red staining of skeletal preparations was performed as described⁴⁴. Experimental

protocols involving vertebrate animal use were approved by the Columbia University College of Physicians and Surgeons Institutional Animal Care and Use Committee.

Accession numbers

The following sequences were used for protein comparisons: mouse Twist1, M63649; mouse Twist2, U36384; mouse Hand1 S79216; mouse Hand2, U40039, mouse Paraxis, U18658, mouse Scleraxis, S78079; mouse Ferd3l AF517121; chick Twist1, AF093816; chick Twist2, AJ13110; chick Hand1, U40041; chick Hand2, U40040, chick Paraxis, U72641; human TWIST1, U80998; human TWIST2, G01204; rat Twist1, AF266260; *Xenopus* Twist1, NM_204084; *D. melanogaster* Twist, X14569; *C. elegans* Twist, NP_509367; zebrafish Twist1, AF205258; zebrafish Twist2, AF205259; zebrafish Hand, AF228334; mouse HRT1 (hey1) AF172286; mouse Myogenin, M84918.1.

Acknowledgements

We thank K. Dionne and I. Messina for excellent technical assistance, N. Vargesson for help with the *Hand2* experiments and S. Weiner for help with *in situ* analyses. We also thank L. Field, C. Tabin, T. Jessell, A. Kania, D. Vasilaiuskas, L. Zeltser and members of the Laufer lab for comments on the manuscript. This work was supported by the National Institutes of Health (to A.B.F., D.V., D.K.), March of Dimes Birth Defects Foundation (A.B.F.), American Cancer Society (P.C.), American Heart Association (P.C.), and Howard Hughes Medical Institute Research Resources Program for Medical Schools (E.L.).

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Figure 1. Conservation of helix I threonine and serine residues within the Twist family of bHLH proteins

(a) Amino acid alignment of bHLH domains. All are Twist family proteins except Ferd31, Hey1 and Myogenin. Conserved residues are black background. Basic and helix I domains are overlined, PKA consensus site is underlined, TWIST1 SCS mutated residues are boldface, and residues mutated in this study are gray background. m, mouse; c, chick; h, human; r, rat; x, *Xenopus*; dm, *D. melanogaster*; ce, *C. elegans*; z, zebrafish. (b) Immunoprecipitation of wild type (WT) or mutant FlagHand2 and FlagTwist1 from metabolically-labeled HEK293 cells cotransfected +/- PKA. Immunoblots below show protein expression in HEK293 cells transfected with the same CaPO₄ precipitates as the radioactive experiment. (c) Twist1

associates with PP2A B56 δ subunit *in vivo*. IP-immunoblot of FlagTwist1 or FlagHand1 coexpressed with HA-tagged B56 α or B56 δ in HEK293 cells. Upper blot shows HA immunoblot of Flag immunoprecipitated protein. (•) Indicates coimmunoprecipitated protein. Lower panels show immunoblot of total extract.

Figure 2. Hand2 and Twist1 are phosphorylated/dephosphorylated by PKA and B56δ-containing PP2A on conserved helix I residues

Phosphopeptide analysis of (a–e) Hand2 phosphorylation or (f–j) Twist1 phosphorylation. (a) Hand2 alone. (b) Adding PKA increases phosphorylation of three peptides. (c) Adding B56α slightly reduces while (d) adding B56δ significantly reduces phosphorylation of one peptide (dashed circle). (e) Mutating Hand2 to Hand2T112;S114A eliminates this phosphopeptide. (f) Twist1 alone. (g) Adding PKA increases phosphorylation. (h) Adding B56α does not alter Twist1+PKA phosphorylation, while (i) B56δ reduces phosphorylation of one peptide (dashed circles). (j) Mutating Twist1 to Twist1T125;S127A eliminates this phosphopeptide. (*) Affected phosphopeptide in (b,e,g,j). (k) Residues phosphoregulated in Hand1, Hand2 and Twist1 (•). (l) Quantification of ³²P incorporation into experimental (dashed circles) and control (dashed boxes) phosphopeptides. Values are normalized to WT Hand2 +PKA or Twist1+PKA signals. H2: Hand2; Tw: Twist1. (m) Anti-Hand phosphoS114-specific antiserum detects phosphoHand2 on immunoblot of non-radioactive cell lysates (Fig. 1b) only when Hand2 is coexpressed with PKA. Phosphorylation is lost with Hand2T112;S114A mutation, but unaffected by Hand2T103A mutation. PhosphoHand2 is detected in the presence of B56α and B56δ, although subtle changes in levels are unlikely to be measurable by this method. Anti-Flag and anti-tubulin immunoblots show loading of Hand2 and total protein. The phosphoHand peptide sequence is shown below the immunoblot.

Figure 3. *Twist1* point mutations within the basic domain exhibit altered phosphorylation by PKA (a–f) *Twist1* mutations found in SCS patients¹² engineered into *mTwist1* coexpressed with or without constitutively active PKA. Sequence of *mTwist1* aa 120–128 is shown. Significantly reduced phosphorylation of T125;S127 is observed in R120W (a–b), R122H (c–d) and Q123P (e–f) mutants. (g–h) In *Twist1* R124A the third R of the basic domain is substituted with an uncharged residue. R124H corresponds to the position of a human SCS patient mutation¹². Coexpression of PKA (h) shows no significant increase in phosphorylation compared to pcDNA1 (g). (i–j) *Twist1* E121K corresponds to a *C. elegans* *Twist* E29K mutation²⁴ that increases the alkalinity of the basic domain. This substitution results in increased PKA-mediated ³²P incorporation. (k–l) Phosphorylation of WT *Twist1* is provided as reference. Panels (k) and (l) are duplicated from Fig. 2. Dashed circles denote the PKA-phosphorylated

phosphopeptide. (**) hyperphosphorylation compared to wild type Twist1 (*). (**m**) anti-Flag immunoblot shows equivalent expression of the different Twist1 proteins, and anti-tubulin immunoblot serves as a loading control.

Figure 4. Regulated heterodimerization between Twist1 and Hand proteins

(a) Twist1 can form heterodimers with Hand1 and Hand2. IP-immunoblot using extracts of HEK293 cells coexpressing Flag-tagged Hand1 or Twist1 and myc-tagged Hand1 or Hand2. Flag antibody-immunoprecipitated protein was immunoblotted with anti-myc or anti-Hand1 antibodies. Hand1 and Hand2 form homo- and heterodimers, as previously reported. Additionally Twist1 forms heterodimers with both Hand1 and Hand2. (b) Acceptor photobleaching FRET analysis of Twist1 and Twist1 point mutants demonstrates that dimerization affinities are affected by post-translational modifications at T125;S127. Wildtype and mutant Twist1 YFP fusion proteins were coexpressed with Hand2, E47 and wild type and mutant Twist1 CFP fusion proteins in HEK293 cells. Protein-protein interaction was then determined by the acceptor photobleaching method of FRET detection. Acceptor photobleaching FRET efficiencies of 30%–40% are indicative of strong interactions, while baseline values are typically below 2%. The phosphomimic Twist1 mutant has similar relative dimer preference as WT, while dephosphomimic preferences are reversed. Error bars denote standard error.

Figure 5. Coexpression of Twist1 and Hand2 in the developing limb

Whole mount and section in situ hybridization analysis of chick and mouse *Twist1* and *Hand2* RNA expression. Whole mount (**a, b, e, f, i, j, m, n**) and serial sections (**q–t**) of chick limbs hybridized with *Twist1* and *Hand2* probes at developmental stages as indicated. Whole mount mouse (**c, d, g, h, k, l, o, p**) limbs hybridized with *Twist1* or *Hand2* probes at embryonic ages as indicated. Note regions of overlap in distal, posterior and interdigital mesenchyme. Anterior to top; distal to right.

Figure 6. Chick limb phenotypes caused by *Hand2* misexpression

(a, d, f, h, j, l) Contralateral controls of wild type *Hand2* virus-infected limbs; images are flipped for clarity. (b, c, e, g, i, k, m) Wild type *Hand2* virus-infected limbs. (a–c) Preaxial mirror-image digit duplications in infected wings. (a) WT control (pattern 234). (b) Ectopic digit 2 (2*234). (c) Ectopic digit 3 (3*234). Note that first phalange of ectopic 3* is consistently shortened. (e) Hindlimb syndactyly. Note excess interdigital tissue in absence of gross patterning defects (^). (g) Hindlimb with shortened long bones. Red bars (f, g) indicate relative lengths of 3rd tarsometatarsals. (i) Hindlimb with bent digit 1 (^). (k) Disrupted AER denoted by gap in *Fgf8* expression (^). (m) Reduced distal mesenchyme growth (^) despite anteroposterior duplication. *Hoxd13* probe.