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Mutations within Helix I of Twist1 Result in Distinct Limb Defects and Variation of DNA Binding Affinities*

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Twist1 is a basic helix-loop-helix (bHLH) factor that plays an important role in limb development. Haploinsufficiency of *Twist1* results in polydactyly via the inability of Twist1 to antagonistically regulate the related factor Hand2. The mechanism modulating Twist1-Hand2 antagonism is via phosphoregulation of conserved threonine and serine residues in helix I of the bHLH domain. Phosphoregulation alters the dimerization affinities for both proteins. Here we show that the expression of Twist1 and Twist1 phosphoregulation mutants results in distinct limb phenotypes in mice. In addition to dimer regulation, Twist1 phosphoregulation affects the DNA binding affinities of Twist1 in a partner-dependent and *cis*-element-dependent manner. In order to gain a better understanding of the specific Twist1 transcriptional complexes that function during limb morphogensis, we employ a series of Twist1-tethered dimers that include the known Twist1 partners, E12 and Hand2, as well as a tethered Twist1 homodimer. We show that these dimers behave in a manner similar to monomerically expressed bHLH factors and result in distinct limb phenotypes that correlate well with those observed from the limb expression of Twist1 and Twist1 phosphoregulation mutants. Taken together, this study shows that the Twist1 dimer affinity for a given partner can modulate the DNA binding affinity and that Twist1 dimer choice determines phenotypic outcome during limb development.

Members of the Twist family of bHLH[2] proteins are evolutionarily conserved transcription factors that play fundamental roles in the normal development of a number of tissues, including extraembryonic membranes, teeth, jaw, heart, and limbs (1–3). Although bHLH/helix-loop-helix proteins can be organized into five groups, bHLH factors can be generally classified into two groups: the ubiquitously expressed E-proteins (class A) and the tissue-specific/restricted bHLH factors (class B) (for a review, see Ref. 4). The classic model for bHLH function is that a heterodimer containing a member from class A and a member from class B interacts via the amphipathic α-helices. This interaction juxtaposes the basic domains of both proteins, such that a DNA binding domain is formed that recognizes a DNA *cis*-element termed an E-box (CANNTG) (4). The requirement for class A/class B heterodimerization for many bHLH factors is well established, and in the case of the skeletal myogenic bHLH factors (MyoD1, Myogenin, Myf5, and Mrf4), heterodimerization is critical for proper biological function (4, 5). Other class B factors, such as members of the Twist family of bHLH proteins, not only function as heterodimers with E-proteins but can also form homodimers and heterodimers with other class B bHLH proteins (1–3, 6). It is thought that the control of bHLH dimerization is a mechanism that could control transcriptional response.

Recently, we have shown that members of the Twist family of bHLH proteins contain an evolutionarily conserved threonine and serine within helix 1 that can be phosphoregulated by the actions of protein kinase A, protein kinase C, and B56δ-containing protein phosphatase 2A (2, 3). Altering the charge on these conserved residues affects the dimerization affinities that Twist family members have for their potential bHLH partners. In the case of Twist1, these partners include itself, E12/E47, and Hand2 (3, 6). Our working hypothesis is that the phosphoregulation of Twist family members alters the dimerization affinities, allowing for new bHLH complexes to form within a cell, thus driving or changing the identity program of the cells (2, 3). The mechanism for this model involves dimer choice and access and/or ability of the dimer complex to bind DNA and thus regulate transcription. Support for this hypothesis can be seen in the autosomal dominant disease Saethre-Chotzen syndrome (SCS). There are over 70 documented mutations within human *TWIST1* that are associated with the causation of SCS (7). We recently showed that a subpopulation of these TWIST1 mutations disrupts the evolutionarily conserved consensus protein kinase A site, and consequently these mutations are poorly phosphorylated. Moreover, phosphorylation mutants of Twist1 affect the dimerization characteristics of Twist1 with itself, E-proteins, and the related co-expressed Hand2 (3). Through both gain-of-function and loss-of-function analysis, we show that during limb development Twist1 and Hand2 function antagonistically; moreover, we show that a hypophosphorylation mutation in Twist1 that correlates to the phosphorylation state of Twist1 in patients with SCS cannot antagonize the actions of Hand2, supporting our idea that the bHLH dimer

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[2] The abbreviations used are: bHLH, basic helix-loop-helix; SCS, Saethre-Chotzen syndrome; EMSA, electrophoretic mobility shift assay; E17.5, embryonic day 17.5.

choice is a critical regulatory step in driving the phenotype of a cell (for a review, see Ref. 8).

The functional role of Twist1 in the developing limb is 2-fold. First, Twist1 maintains an Fgf8-Fgf10-FgfR2 autoregulatory signaling loop, which promotes the development of the apical ectodermal ridge, facilitating limb outgrowth (9). In addition, Twist1 also regulates the expression of Gli2 and Gli3, which are key positive and negative regulators of Shh (Sonic Hedgehog) activity. Shh activity defines anterior-posterior limb patterning via the zone of polarizing activity. Twist probably activates or maintains a graded pattern of Gli3 activity, which then suppresses Shh signaling within the anterior mesenchyme (9).

When the physiological ratio of *Hand2*/*Twist1* expression is perturbed by either *Twist1* haploinsufficiency or by *Hand2* overexpression, *shh* expression is expanded. This results in polydactyly via the formation of an additional/expanded zone of polarizing activity in the anterior of the forming limb (3, 9–12). Hand2-induced expression of *shh* is also associated with a direct antagonistic relationship with the *shh* repressor Gli3 (13), suggesting that a precise transcriptional balance of both Twist1 and Hand2 is essential for both normal limb outgrowth and patterning. Also involved but not currently understood is the role that E-protein expression has on modulating Twist1 and Hand2 regulation of limb formation.

In this study, we set out to investigate the consequences of Twist1 gain-of-function analysis using wild type and phosphoregulation mutants of Twist1 in the developing limb and explore the effects of Twist1 phosphoregulation on DNA binding affinities to a representative set of E-box *cis*-elements. We show that the expression of wild type Twist1, hypophosporylated Twist1 (Twist1T125;S127A), and phosphorylation mimic Twist1 (Twist1T125;S127D) indeed results in distinct phenotypes within the developing mouse limb, and, in addition to changes in dimerization affinity, altering the charge of helix I can affect DNA binding in a *cis*-element-dependent manner. Furthermore, the expression of tethered Twist1 dimers (Twist1-Twist1, Twist1-E12, and Twist1-Hand2), where the partner choice is dictated by expressing the dimer pair as a single polypeptide, show specific phenotypic effects on limb morphogenesis that correlate to those observed when expressing the monomeric wild type and phosphorylation mutant Twist1 proteins.

EXPERIMENTAL PROCEDURES

Plasmids—Expression plasmids for Twist1, Twist1T125; S127A, and Twist1T125;S127D were described previously (2, 3). Tethered dimer constructs were generated by cloning cDNAs for wild type Twist1, Hand2, and E12 into pcDNA3 (Invitrogen) modified to include a glycine-rich linker $G(SGGG)_3SGG$ that joins the two cDNAs (6). The 5′ cDNAs were PCR-amplified as HindIII-BamHI fragments with the stop codon removed to allow for translation of both cDNAs as a single protein. The 3′ cDNAs were PCR-amplified as KpnI-XbaI fragments. The glycine linker had HindIII and BamHI sites at the 5′ end and KpnI and XbaI sites at the 3′ end for cloning purposes. CATCTG E-box and CGTCTG D-box reporter constructs were previously described (14). The CATATG E-box reporter was made similarly by inserting three copies head-to-tail into the SacI and BglII sites of the luciferase vector pGL3P (Promega). Prx transgenic constructs were generated as follows. For all clones containing a Hand2 cDNA, an internal BglII site within the coding region was first mutated to destroy the site while maintaining the amino acid sequence (5′-GG AAG AAA GAG CTG AAT GAA ATC TTG AAA AGC ACA GTG AGC-3′) using the QuikChange mutagenesis kit (Stratagene) following the manufacturer's instructions. BglII sites were then added to the 5′ end of the clones and 3′ of the poly(A) signal via PCR. Sequences were verified to be free of PCR-generated mutations by sequence analysis of both strands. Verified inserts were then cloned into the BglII cloning site of the Prx promoter-containing construct. Orientation was determined by sequence analysis. Prx promoter sequence plus cDNA monomer or tethered inserts were liberated by SalI digestion and given to the IU transgenic core for gel purification and F_0 production.

Electrophoretic Mobility Shift Assays—The various bHLH and tethered bHLH proteins were *in vitro* transcribed and translated using the Promega TNT system as described in the manufacturer's protocol. Three probes were used for electrophoretic mobility shift assay (EMSA): a Hand E-box (5′-gga ttc cat tgc atc tgg att cca gag-3′), a degenerate E-box sequence shown to bind Hand1 and Hand2 termed a D-box (5′-cat tgc att ggc gtc tgg cat tgc att-3′) (14), and an E-box that was shown to bind Twist1 (5′-gat ccc tcg cat atg ttg aa-3′) (6). Single-stranded sense probes were 5′ end-labeled and then annealed to cold antisense to generate double-stranded DNA. EMSAs were performed as described using a binding buffer containing 2 mM $MgCl_2$, 2 mM dithiothreitol, 25 mM Tris, pH 7.5, 25 mM NaCl, 12% glycerol, and 0.1% Nonidet P-40 (15). Competitors used in the assay are the unlabeled oligonucleotides used for probes as specific competitors and oligonucleotides with the sequences in italic type substituted for the binding target sequences (5′-gga ttc cat tg*t gct ac*g att cca gag-3′; 5′-cat tgc att g*gg cag ag*g cat tgc att-3′; 5′-gat ccc tcg *tga ttg* ttg aa-3′) as nonspecific competitors. EMSA experiments were repeated five times using new programmed lysates, in which protein expression was verified via immunoblotting prior to EMSA analysis. Individual experiments employed a single batch of programmed lysates for each probe, and the EMSA reactions and gels were run on the same day.

Immunoblotting—In vitro programmed reticulocyte lysates were collected, and 5 μl of lysates were run through 10 or 12% SDS-polyacrylamide gels, electroblotted, and incubated with the indicated antibody as described (2). Blots were visualized using the Super Signal luminescent detection protocol (Pierce).

Cell Culture and Transfections—HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Cells were plated and transfected as described (16) with the indicated constructs using a $CaPO_4$ procedure.

Luciferase Assays—Luciferase assays were performed using the dual luciferase assay kit (Promega) following the manufacturer's protocol. Cell lysates were read using a 96-well microtiter plate luminometer (Forma). Data represent six independent experiments, and *error bars* denote S.E.

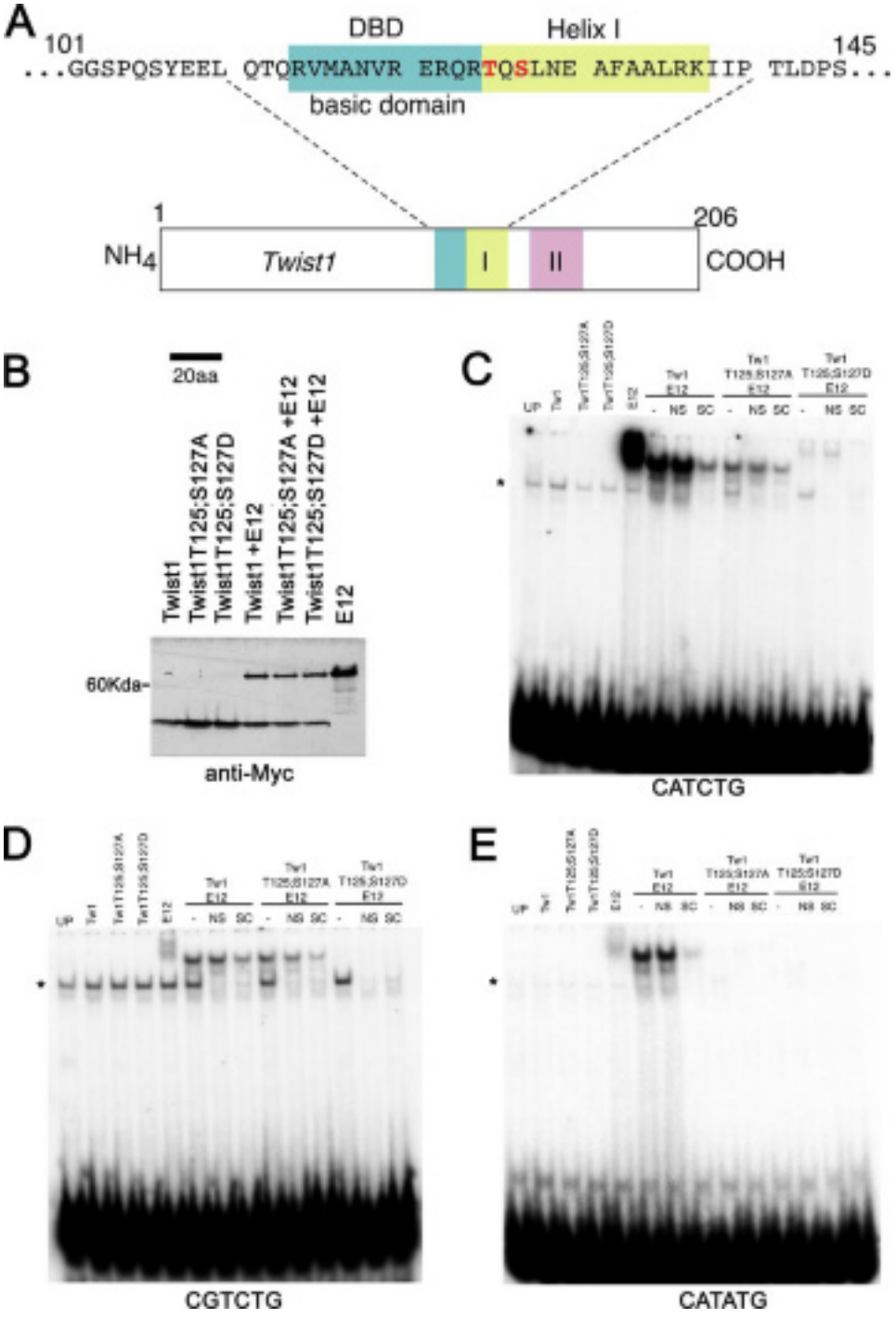

FIGURE 1. *A*, schematic of murine Twist1 showing the amino acid sequence of the basic DNA binding domain (*DBD*) and helix I. Thr[125] and Ser[127] are denoted in *red*. *B*, Western blot showing expression of Myc-tagged proteins within programmed rabbit reticulocyte lysates. Proteins expressed are denoted *above* each *lane* and represent the same amount of protein used in each EMSA assay. *C*, EMSA using the CATCTG or Hand E-box. Unprogrammed lysate (*UP*) shows the presence of nonspecific protein-DNA complexes (*). Specific DNA-protein complexes are not competed by nonspecific competitor (*NS*) and are masked by the addition of a molar excess of unlabeled probe (*SC*). *D*, EMSA analysis using a CGTCTG core D-box. *E*, EMSA analysis using a CATATG core Twist E-box.

Generation and Analysis of Transgenic Animals—Transgenic DNA was purified using resin columns (Qiagen), digested, and given to the IU transgenic facility for gel purification and microinjection into mice. Southern blotting of yolk sac DNA identified E17.5 F_0 animals carrying the transgenes of interest. Skeletal preparations were performed exactly as described (17).

RESULTS

DNA Binding Is Altered in Twist1 Helix I Phosphoregulation Mutants—As shown in Fig. 1*A*, Thr[125] and Ser[127] are positioned at the boundary between the basic DNA-binding domain and helix I of the bHLH. Our earlier findings show that the alteration of charge at Thr[125] and Ser[127] results in changes of the dimerization affinities between Twist1 and its bHLH partners, but in no case have we shown that these alterations result in the complete loss of Twist1 dimerization with another bHLH partner (3). Considering this, we sought to determine if DNA-binding was also affected in Twist1 phosphoregulation mutants. We thus performed EMSAs using *in vitro* transcribed and translated Twist1, Twist1T125;S127A, and Twist1T125;S127D alone and co-translated with E12 from cytomegalovirus-driven expression vectors containing an SP6 promoter sequence for *in vitro* transcription and translation. To control for input protein, all proteins were tagged with the Myc epitope and were *in vitro* transcribed and translated. Immunoblot analysis of the input protein shows that equivalent amounts of the protein were added to all binding reactions using each DNA probe; thus, differences in the intensity of the shifted probe reflect differences in the DNA binding affinity (Fig. 1*B*). We first looked at DNA binding to an CATCTG E-box core, which binds the related Hand factors (18, 19). No DNA binding was observed from the reticulocyte lysates programmed to express Twist1, Twist1T125;S127A, or Twist1T125;S127D, indicating that the homodimers were not binding at detectable levels (Fig. 1*C*). Twist1 homodimer formation has been demonstrated previously (3) and is discussed below. In contrast, lysates programmed to express E12 showed a strong probe shift, indicating that E12 homodimers can bind the Hand E-box. Lysates programmed to express both Twist1 and E12 also showed a smaller DNA mobility shift than that of the E12

homodimer, which was not competed away by the addition of excess nonspecific (*NS*) competitor but was competed by excess unlabeled competitor (*SC*) (Fig. 1*C*). When Twist1 was replaced with Twist1T125;S127A and co-translated with E12, a similar complex was able to form; however, the amount of shifted probe was significantly less, indicating that DNA binding affinity was diminished. Of note, we did not observe any E12 homodimer-shifted probe based on the migration pattern of the mobility shifts, indicating that the majority of the stable dimer complexes formed were indeed Twist1T125;S127A-E12 heterodimers. Conversely, lysates programmed with Twist1T125;S127D and E12 failed to shift this Hand E-box at any detectable level under our experimental conditions (Fig. 1*C*). What we did observe is a higher mobility shift that, based on its migration pattern, corresponds to an E12 homodimer and was not competed by NS but was competed by SC. Given this observation and previous FRET analysis (3), we feel that both homodimer and heterodimer complexes are able to form under these conditions and that the E12 homodimer has a stronger DNA binding affinity and/or is more stable and therefore competes out the binding of Twist1T125;S127D and E12 for the Hand E-box (Fig. 1*C*).

The next probe that we employed in our analysis was a degenerate E-box that has been termed a D-box and contains a CG in place of the CA of the CANNTG motif, which can be transactivated by Hand1 in trophoblasts (14) (Fig. 1*D*). EMSA analysis showed no homodimer DNA binding of singly translated Twist1 or helix I mutant proteins; however, we were surprised to observe that E12 homodimers shifted this degenerate sequence weakly (Fig. 1*D*). Lysates programmed with both Twist1 wild type and E12 proteins also shifted the D-box sequence, resulting in a protein-DNA complex that migrates at a slightly faster mobility than the E12 homodimer (Fig. 1*D*). The observation that Twist1 can bind this degenerate sequence known to bind Hand factors is reasonable, given the evolutionary conservation within the Twist family of factors. We were surprised to observe that lysates programmed with both Twist1T125;S127A and E12 were able to shift the D-box probe as efficiently as that of Twist1 wild type + E12. This suggests that hypophosphorylation of Twist1 does not affect DNA binding to this specific *cis*-element. Similar to what we observed using the Hand E-box probe, lysates programmed with Twist1T125;S127D and E12 failed to show any significant DNA binding to the D-box probe.

Finally, we tested a CATATG E-box sequence that was previously identified as binding *Drosophila* Twist both as a homo- and heterodimer (6). Although *Drosophila* Twist has been shown to bind this sequence as a homodimer (6), lysates programmed for mouse Twist1 and Twist1 helix I mutants failed to show any observable homodimer DNA/protein mobility shifts using this single *cis*-element as an EMSA probe (Fig. 1*E*). Similar to what we observed using the D-box, lysates programmed with E12 shifted this E-box weakly. Once again, lysates programmed with both Twist1 and E12 shifted the probe strongly (Fig. 1*E*); in contrast, neither the hypophosphorylation nor phosphorylation mimic of Twist1 shifted this probe when co-translated with E12. Of note, translated Hand2 as well as Hand2 co-translated Twist1 failed to shift any of the employed *cis*-elements; however, Hand2 co-translated with E12 shifted all three *cis*-elements (data not shown; see Fig. 2). Together, these data show that in addition to alterations in dimerization affinities, Twist1 phosphoregulation can influence DNA binding in a *cis*-element-dependent manner.

In addition to DNA binding, we employed luciferase analysis using a luciferase reporter driven by three copies of the above mentioned *cis*-elements to determine if transcriptional activity *in vitro* is affected (Fig. 2*A*). Transactivation of the CATCTG E-box showed that Twist1 increases luciferase activity ~3-fold, whereas Twist1 T125;S127A and T125;S127D showed diminished transactivation. Transactivation of the CATCTG E-box with E12 alone showed a 2.5-fold increase in luciferase activity (Fig. 2*A*). Consistent with EMSA results, co-expression of Twist1 + E12 resulted in a 6–7-fold increase in luciferase activity. Twist1T125;S127A, which can bind to this sequence when co-expressed with E12 in EMSA (albeit with less affinity than wild type Twist1), transactivated less efficiently than wild type (Fig. 2*A*). Co-expression of Twist1T125;S127D + E12 showed levels of transactivation that were nearly identical to that observed when Twist1T125;S127D was expressed alone (Figs. 1*C* and 2). Expression of Hand2 alone and when coexpressed with E12 also showed activation of the multimerized *cis*-element-driven reporter.

Transactivation of the CGTCTG D-box shows Twist1 increased luciferase activity 2.5-fold. Although the alanine point mutant showed similar transactivation, the aspartic acid mutant exhibited less transcriptional activity (Fig. 2*B*). When coexpressed with E12, there was no difference in transcriptional activity between Twist1 + E12 and Twist1T125;S127A + E12, consistent with the equivalent DNA binding affinities shown previously (Figs. 1*D* and 2*B*). Co-expression of Twist1T125;S127D + E12 did not enhance the transactivation of the D-box, reflecting the observed poor DNA binding in the EMSA analysis (Figs. 1*D* and 2*B*). Although Hand2 DNA binding was not detectable on the single D-box *cis*-element, significant activity was observed when Hand2 was expressed with the multimerized D-box reporter. This transcriptional activation was further increased when Hand2 was coexpressed with E12, correlating with its ability to bind the single D-box probe in EMSA (data not shown).

Transactivation of the *Drosophila* Twist CATATG E-box was the weakest of the tested *cis*-elements; nevertheless, the luciferase activity correlates with the observed DNA binding (Fig. 2*C*). Expression of the Twist1 and the Twist1 phosphorylation mutants resulted in 1.5-fold or less transactivation, which is not considered significant. Similar findings were observed when expressing Hand2. Although Twist1 + E12 showed a modest 2.5-fold increase in luciferase activity, neither of the Twist1 phosphorylation mutants resulted in any significant transactivation, directly correlating with the observed DNA binding with this *cis*-element in our EMSA analysis (Figs. 1*E* and 2*C*). Hand2 expressed with E12 exhibited the strongest transactivation of this reporter construct (Fig. 2). Taken together, these data show that alterations in the charge on helix I can affect both dimerization affinities (3) and DNA binding affinities for Twist1 in a *cis*-element-dependent manner. These

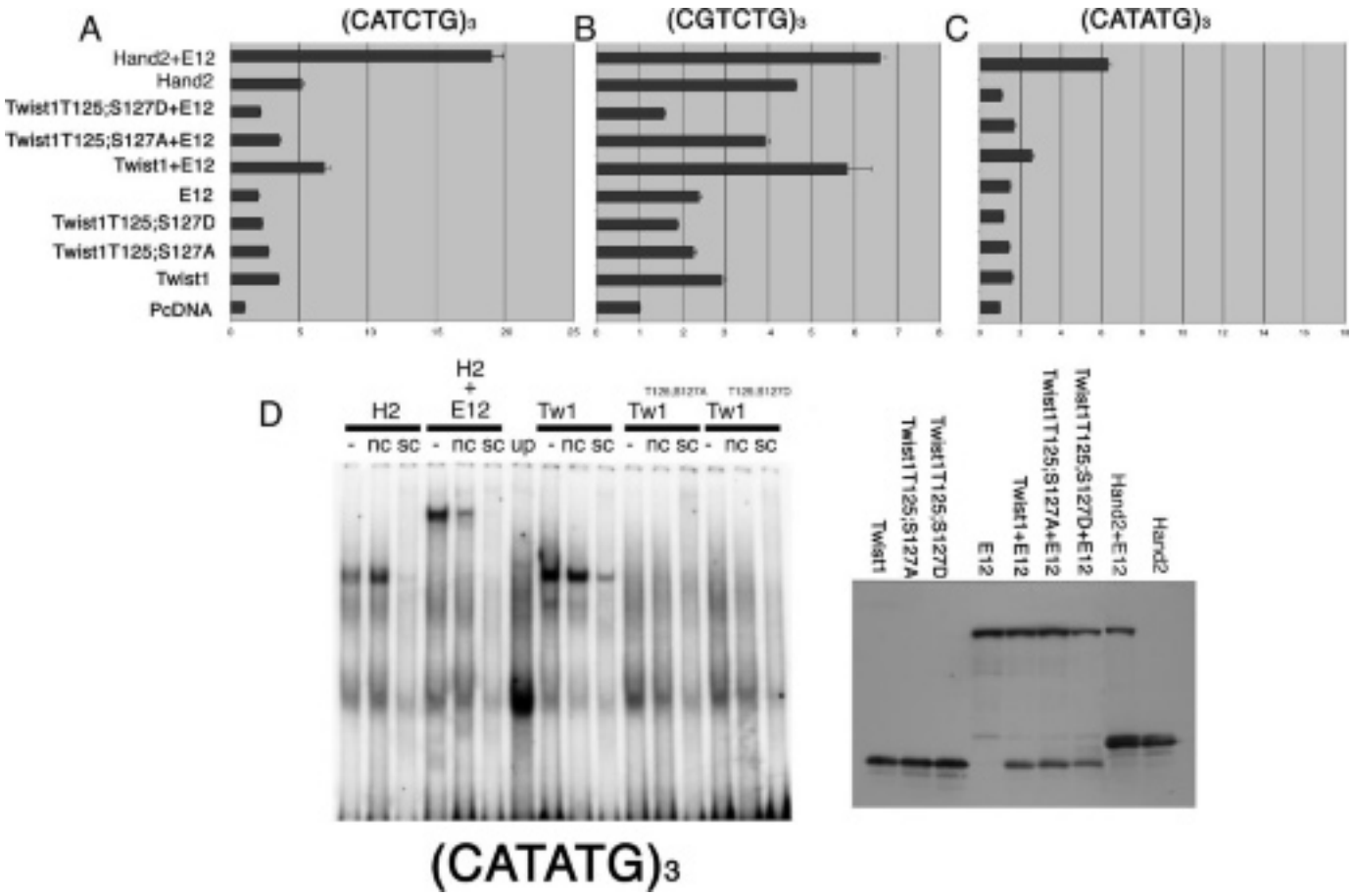

FIGURE 2. **Luciferase analysis of Twist1 monomers on the CATCTG Hand E-box.** *A*, transactivation of an SV40 minimal promoter driven by three CATCTG *cis*-elements (Hand E-box). Relative light units are shown and reflect the ratio of firefly/*Renilla* luciferase activity. *B*, transactivation of an SV40 minimal promoter driven by three CGTCTG *cis*-elements (D-box). *C*, transactivation of an SV40 minimal promoter driven by three CATATG *cis*-elements (Twist E-box). All experiments were performed at least six times, and *error bars* denote S.E. *D*, EMSA using a $(CATATG)_3$ probe showing Hand2 and Twist1 homodimer binding. Protein loading is shown by immunoblot. *ns*, nonspecific competitor; *sc*, molar excess of unlabeled probe.

differences are reflected by the transcriptional activation of these *cis*-elements in tissue culture.

Given that *Drosophila* Twist has been shown to bind as a homodimer (6) and our observed transcriptional activity shown above with Twist1 (Fig. 2), we were puzzled as to why the homodimers were not binding in our EMSA analysis. It was pointed out in review that the transcriptional assays were performed on multimerized *cis*-elements, whereas the EMSA analysis employed a single copy of the *cis*-element. Therefore, we repeated our EMSA analysis employing the same multimerized *cis*-element sequences used in the transcriptional assays. To our surprise, the EMSA analysis performed using the multimerized E-boxes increased DNA binding affinities, revealing homodimer binding (Fig. 2*D*). The use of the $(CATATG)_3$ probe showed that Hand2 and Twist1 can each specifically bind as homodimer complexes that are faster in mobility then Hand2 + E12, which was used as a control. Interestingly, neither of the Twist1 helix I point mutants showed a detectable shift of this E-box (Fig. 2*D*). Since Twist1 and Twist1 phosphorylation mutants have been shown to form homodimers (3), we conclude that decreased DNA binding affinity of T125;S127A and T125;S127D was observed. Western blot shows that equal protein levels were employed. Similar results are observed for the $(CATCTG)_3$ and $(CATCTG)_3$ probes (data not shown). These results support previous findings from *Drosophila* (6) and suggest that DNA binding is enhanced by multiple *cis*-elements within a localized region. These data also confirm that the transcriptional activation of the employed reporter clones is probably the result of the protein dimers directly binding the DNA within the cells. Therefore, to determine the effects (if any) of changing protein dimerization choice and DNA binding affinities of Twist1 *in vivo*, we made use of the *Prx* limb promoter (20) to ectopically express *Twist1* wild type and phosphoregulation mutants in F_0 transgenic mice.

In Vivo Expression of Twist1, Hypophosporylated Twist1T125; S127A, and Phosphorylation Mimic Twist1T125;S127D Helix 1 Mutants Alter Normal Limb Development in Distinct Ways—To look at the effects of modulating Twist1 phosphoregulation on limb patterning, we made use of the *Prx* limb promoter to express various forms of Twist1 in the developing limb (Fig. 1) (20). The *Prx* limb enhancer is ideal for the ectopic expression of factors in the developing limbs, since this enhancer shows robust *Prx-lacZ* limb expression throughout both fore and hind limbs at embryonic day 10.5, well prior to any observable limb patterning, and matches well with the observed *Twist1* expression (3, 20). F_0 embryos were collected at E17.5, since limb development is largely completed by this stage. Results showed that *Twist1* wild type, *T125;S127A*, and

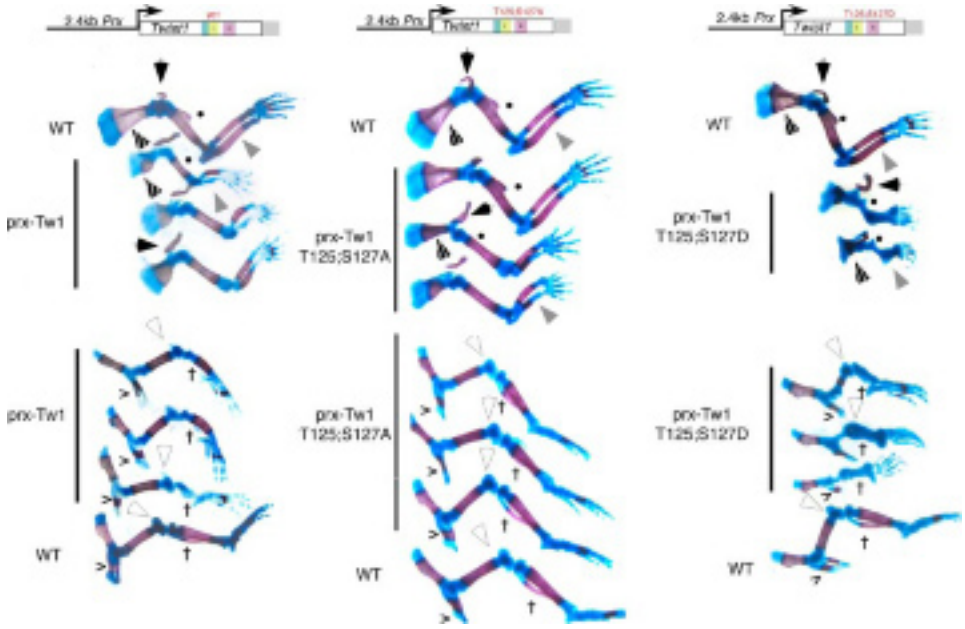

FIGURE 3. **Expression of Twist1 and phosphoregulatory mutants in mouse limbs.** Monomer-Prx constructs were used to generate E17.5 F_0 embryos. Embryos were stained with Alcian blue and alizarin S as described (17) from one wild type (*WT*) and three transgenic positive littermates. Limbs were dissected away for detailed analysis. *Filled arrowhead*, clavicle; *filled circle*, deltoid tuberosity; *gray arrowhead*, ulna; *dotted arrowhead*, scapula; *open arrowhead*, patella; *cross*, fibula; *carat*, iliac bone.

T125;S127D expression produced distinct limb phenotypes (Fig. 3). Ectopic expression of wild type *Twist1* caused an overall reduction in fore limb size but had no effect on digit number. The clavicles of these mice (*black arrowhead*) were misshapen. Both the radius and ulna were reduced in size, and the ulna in particular showed a reduction in ossification. It also appeared that the size of the ulna was disproportionate to the radius, since bending of the radius in two of three F_0 embryos suggested that growth rates were not matched. The humerus of *Prx-Twist1* wild type transgenics was smaller compared with wild type littermates and lacked the deltoid tuberosity (Fig. 3, *closed circle*). Ossification within the scapula was reduced in *Prx-Twist1* transgenics, and the superior border of the scapula was absent/reduced (Fig. 3, *dotted arrowhead*).

Hind limb defects were also observed in *Prx-Twist1* mice. Consistent with the fore limb phenotypes, hind limb digit numbers were normal; however, a loss of the fibula (Fig. 3, †) and a reduction in both the ossification and size of the tibia were evident (Fig. 3). Defects in the cartilage of the knee joint resulted in a semicircular bending of the hind limb, and the size of the patella was greatly reduced (Fig. 3, *open arrowhead*). Femurs, although shorter, appeared phenotypically normal, as did iliac (hip) bones, which displayed normal amounts of ossification (Fig. 3). Thus, ectopic *Twist1* expression did not affect digit number or distal limb development but did alter more medial regions of the limb in contrast to the phenotypes observed with *Prx-Hand2* expression (21).

We previously identified a number of Twist1 SCS mutations that compromise T125;S127 phosphoregulation and have shown that hypophosporylated Twist1 fails to antagonize Hand2 function, resulting in polydactyly (3). To look at the effect of ectopically expressing this haploinsufficient SCS allele, *Prx-Twist1T125;S127A* transgenic E17.5 F_0 embryos were generated and compared with wild type and *Prx-Twist1* mutant mice (Fig. 3). *Prx-Twist1T125;S127A* phenotypes were similar to those observed in *Prx-Twist1* fore limbs; however, they were clearly less severe. *Prx-Twist1T125;S127A* clavicles (*black arrowhead*) were misshapen in all three E17.5 embryos; however, the sizes of the radius and ulna were closer to that of wild type mice. Similar to *Prx-Twist1* fore limbs, two of three *Prx-Twist1T125;S127A* ulnas showed a reduction in ossification (Fig. 3). The humerus of the *Prx-Twist1T125;S127A* embryos was also closer in size to that of the non-transgenic embryo; however, the loss of the deltoid tuberosity (Fig. 3, *closed circle*) in two of the three fore limbs was evident. Less severe hind limb defects were also observed in *Prx-Twist1T125;S127A* embryos, as seen by a reduced limb size and loss of fibula in two of three examples (Fig. 3). These findings suggest that in addition to the inability of the Twist1 helix I mutation to antagonize Hand2 in limb development (3), Twist1 helix I hypophosphorylation F_0 embryos exhibit a reduced gain-of-function phenotype when compared with the expression of Twist1 wild type F_0 transgenic embryos, further supporting the notion that hypophospory-lated helix I functionally mimics *Twist1* SCS alleles.

Since the *Prx-Twist1* transgenic embryos had more pronounced limb defects than a *Twist1* SCS-associated mutation, we were curious about the effects of ectopically expressing a Twist1 helix I phosphorylation mimic on a limb phenotype. Consistent with our predictions, *Prx-Twist1T125;S127D* F_0 embryos exhibited the most severe limb defects (Fig. 3). Fore limbs were greatly reduced in size and showed global reductions in ossification, but in all cases, fore and hind limb digit numbers were normal. The F_0 embryos exhibited a loss of the ulna (or fusion of the ulna with the radius), and the *Prx-Twist1T125;S127D* humerus was small and lacked sites of ossification. Interestingly, the deltoid tuberosity was visible on each humerus, in contrast to the deltoid tuberosity of the *Prx-Twist1T125;S127A* and *Prx-Twist1* F_0 embryos (Fig. 3, *closed circle*). In addition, the clavicles of *Prx-Twist1T125;S127D* embryos appeared to be less severely affected than those of either the *Prx-Twist* or *Prx-TwistT125;S127A* F_0 embryos (Fig. 3).

Considering the possible variation in expression from each F_0 line generated, the resultant phenotypes were extremely consistent within each group of embryos. Moreover, the finding that hypophosphorylated Twist1 resulted in the least severe limb defects correlates well with its association with the haploinsufficient human disease SCS as a *TWIST1* null allele. Taken together, these results suggest that the differences in DNA binding and transcriptional activity observed for Twist1,

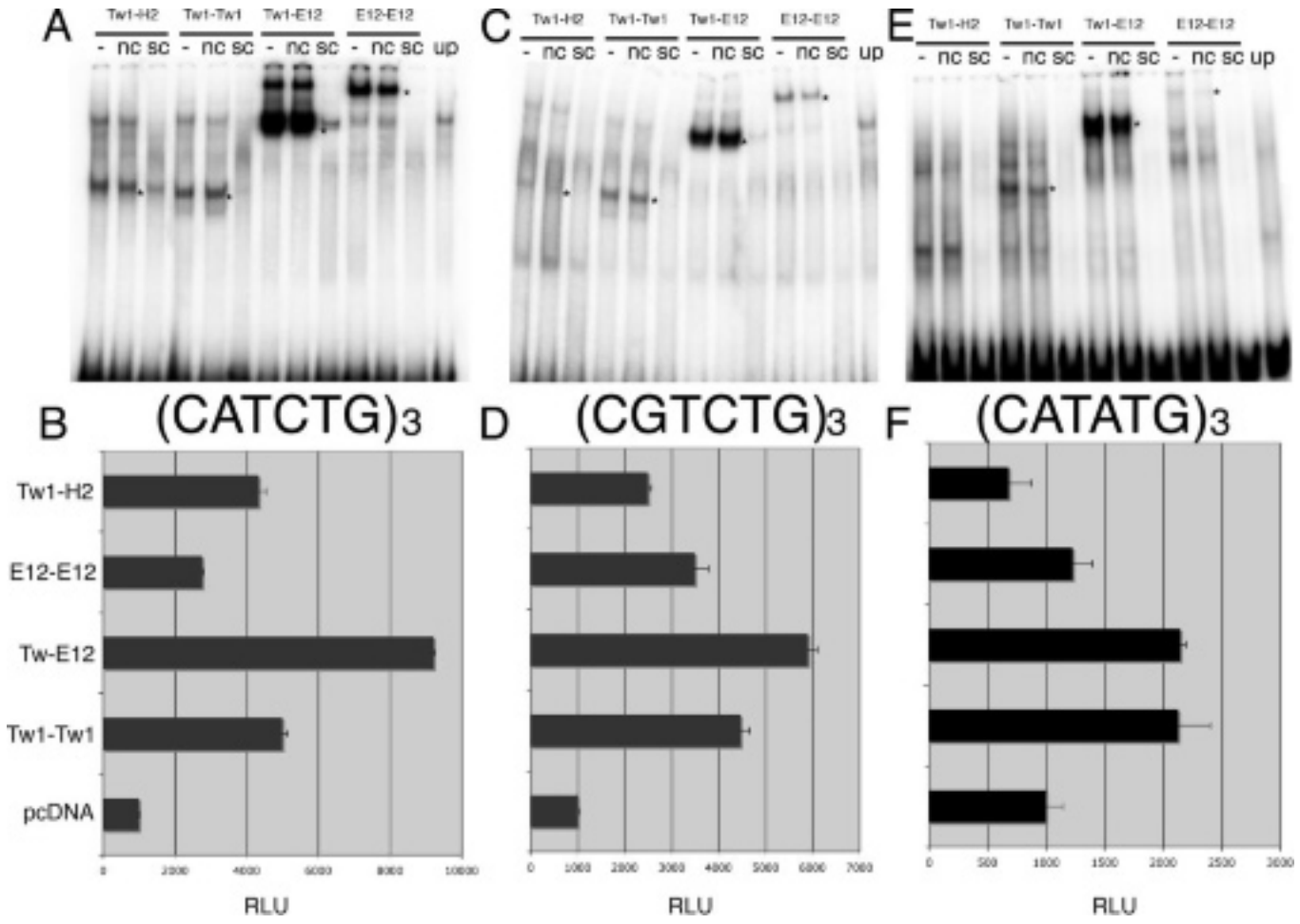

FIGURE 4. **EMSA and luciferase analysis of Twist1-tethered dimers on the $(CATCTG)_3$ E-box.** *A*, EMSA showing specific DNA-protein interactions of Twist1-Hand2 heterodimers (Tw1-H2), Twist1 homodimers (Tw1-Tw1), Twist1-E12 heterodimers (Tw1-E12), and E12-E12 homodimers. *UP*, unprogrammed lysate. Specific complexes are indicated by an *asterisk*. *ns*, nonspecific competitor; *sc*, molar excess of unlabeled probe. *B*, transactivation of an SV40 minimal promoter driven by three CATCTG *cis*-elements. Relative light units (*RLU*) are shown and reflect the ratio of firefly/*Renilla* luciferase activity. *C*, EMSA of Twist1-tethered dimers on the $(CGTCTG)_3$ D-box. *D*, transactivation of an SV40 minimal promoter driven by 3-CGTCTG *cis*-elements. *E*, EMSA of Twist1-tethered dimers on the $(CATATG)_3$ E-box. *F*, transactivation of an SV40 minimal promoter driven by three CATATG *cis*-elements. *Error bars*, S.E.

Twist1T125;S127A, and Twist1T125;S127D are in fact reflected by observable differences in limb phenotypes *in vivo*. What is still unclear is which Twist1 bHLH dimer partners are being employed to generate the different observed phenotypes. In order to gain a better insight into this question, we employed a tethered dimer approach.

Twist1-tethered Dimer Complexes Exhibit DNA Binding and Transcriptional Activity—Our first goal in testing the Twist1-tethered molecules was to determine the DNA binding affinities for the previously employed *cis*-elements. EMSA analysis was performed using tethered Twist1-Twist1 homodimers, Twist1-E12 heterodimers, E12-E12 homodimers, and Twist1-Hand2 heterodimers using the multimerized *cis*-element probes (Fig. 4, *A–F*). In addition to the DNA binding analysis, the transcriptional activity of the Twist-tethered proteins was assessed using the same E-box and D-box luciferase constructs that we employed to test the Twist1 and Twist1 phosphorylation mutant monomers (Fig. 4, *D–F*). Examining the DNA binding and transcriptional activity on the employed *cis*-elements, we found that Twist1-Hand2 tethered heterodimers, which did not bind to the monomeric *cis*-element sequence (data not shown), could bind to the $(CATCTG)_3$ and $(CGTCTG)_3$ probe but not to the $(CATATG)_3$ probe (Fig. 4, *A*, *C*, and *E*). Transcriptional activity in HEK293 cells correlated with the EMSA findings, where Twist1-Hand2 transactivated the CATCTG and CGTCTG sequences but not the CATATG *cis*-element (Fig. 4, *A–F*). Also consistent with data obtained from singly expressed proteins, both Twist1-Twist1 and Twist1-E12 tethered complexes bound to each *cis*-element in EMSA analysis (Fig. 4, *A–F*). Transactivation of the *cis*-elements showed that the Twist1-E12 tether exhibited the highest level of transactivation, directly correlating with the levels of activity observed when the proteins were individually expressed (Figs. 2 and 4, *D–F*). Together, these data show that Twist1 homodimer and Twist1-E12 and Twist1-Hand2 heterodimer complexes both bind DNA and transactivate the employed *cis*-element targets; we conclude that these artificial transcriptional complexes are functional, and we next set out to test these tethered clones *in vivo*.

Expression of Twist1-tethered Dimers in the Developing Limb Reflects Unique Roles for Different Twist Complexes—To gain a better understanding of how specific Twist1 dimers function *in*

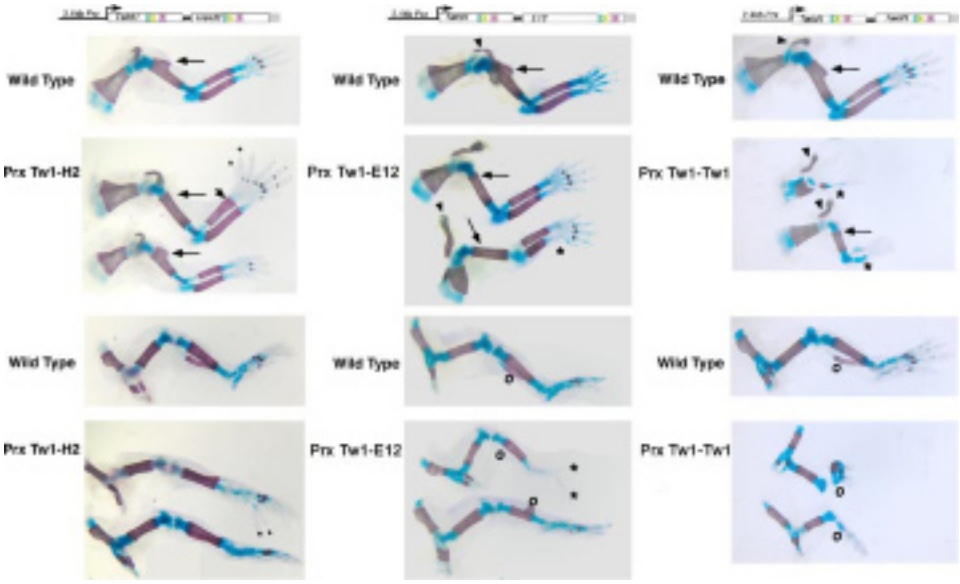

FIGURE 5. **Analysis of limb phenotypes resulting from Twist1-tethered dimer expression.** The indicated tethered dimers were used to generate E17.5 F_0 embryos. Two transgenic positive fore and hind limbs are shown for Tw1-H2 (*left*), Tw1-E12 (*center*), and Tw1-Tw1 (*right*). One wild type representative is shown followed by two transgenic embryos stained with Alcian blue and alizarin S. *Black arrowheads*, clavicle; *black arrows*, deltoid tuberosity; *small asterisk*, polydactyly; *large asterisk*, ulna; *open circle*, fibula.

vivo, we generated *Prx-Twist1-Hand2*, *Prx-Twist1-E12*, and *Prx-Twist1-Twist1* F_0 E17.5 transgenic embryos to correlate with the phenotypes observed with the *Prx*-expressing *Twist1* and *Twist1* phosphoregulation mutant F_0 transgenic embryos. When E17.5 *Prx-Twist1-Hand2* F_0 transgenic embryos were examined, we observed either no phenotype or preaxial polydactyly (Fig. 5, *left*). One of the transgenic embryos exhibited two extra digits on both fore and hind limbs. Interestingly, a thickening of the distal end of the radius was evident on the fore limbs (Fig. 5). The deltoid tuberosity was also absent from these fore limbs (Fig. 5, *arrow*). The loss of the deltoid tuberosity phenotype was not reported with *Prx-Hand2* ectopic expression (21); however, it was observed with *Prx-Twist1* expression (Fig. 3). Other than the polydactyly (a Hand2 overexpression phenotype), no other malformations were observed in the *Prx-Twist1-Hand2* hind limbs. Since polydactyly and loss of the deltoid tuberosity is observed, it suggests that the Twist1-Hand2 transcriptional complex is actively modulating transcription *in vivo*.

Analysis of the *Prx-Twist1-E12* F_0 transgenic embryos at E17.5 shows a phenotype similar to that observed with the expression of wild type Twist1 and the milder phenotype observed using Twist1T125;S127A (Fig. 5, *center*). F_0 E17.5 transgenic embryos showed the loss of the deltoid tuberosity (Fig. 5, *arrow*) as well as a misshapen clavicle (Fig. 5, *arrowhead*). In one of the two *Prx-Twist1-E12* fore limbs, we also observed a shortening of the ulna similar to that observed with wild type *Prx-Twist1* and the *Prx* expression of the hypophosporylated *Twist1* mutant shown in Fig. 2. Hind limb phenotypes included the loss of the fibula (*open circle*) and the reduced ossification of the tibia. These phenotypes were most consistent with what we observed with the *Prx-Twist1T125;S127A* F_0 embryos. Phenotypes distinct to the *Prx-Twist-E12*-expressing transgenic embryos were the presence of an ectopic outgrowth on the tibia that may be the remnant of the fibula (*open circle*) and a reduced number of digits (two marked by *asterisks*) in the hind limbs of one of the *Prx-Twist-E12* transgenic embryos. Of note, this is the first defect in autopod development that we have observed using *Prx-Twist* expression constructs that does not involve the expression of *Hand2*.

The final dimer pair tested was the *Prx-Twist1-Twist1* homodimer. Phenotypes observed with the E17.5 transgenic F_0 embryos affected the distal limb outgrowth (Fig. 5, *right*). The fore limbs displayed normal scapula and clavicle development (*arrowhead*); however, the more extreme example (*top*) exhibited a missing humorous and a single radius or ulna that extended to an autopod with a single digit, and the ossification was greatly reduced. In a less extreme example (*bottom*), the humerus was present but reduced in size and lacked a deltoid tuberosity (*arrow*). The radius and ulna lacked ossification, and the autopods were misshapen with oddly placed digits (Fig. 5, *right*). Hind limb phenotypes included a severe reduction/absence of fibula and tibia along with misshapen autopods (Fig. 5, *right*). The iliac and femur appeared relatively normal. These phenotypes correlated most closely to those observed with the *Prx-Twist1T125;S127D* expression (Figs. 3 and 5). Taken together, these data suggest that Twist1 phosphoregulation not only influences dimer choice but can also impact the DNA binding affinity to specific *cis*-elements, thereby differentially affecting developmental transcriptional programs. Furthermore, by locking Twist1 into specific dimer complexes, the phenotypic outcomes resulting from the gain-of-function analysis are as distinct as those observed with the monomeric expression of Twist1 phosphoregulation mutants, suggesting that different Twist1 dimers play specific roles in limb development.

DISCUSSION

Helix I phosphoregulation of the Twist family of bHLH proteins is a mechanism by which these factors can modulate their dimer partner choice (2, 3). Here we show that in addition to dimer control, phosphoregulation also impacts DNA-binding preferences to E-box sequences, and the impact appears to be *cis*-element-dependent (Figs. 1 and 2). Furthermore, ectopic limb expression of Twist1 and Twist1 phosphoregulation mutants produce unique phenotypes, confirming that helix I phosphoregulation can define Twist1 function *in vivo* (Fig. 3). Since the evolutionarily conserved phosphoregulated residues reside at the boundary between the basic domain and helix I, this finding suggests that protein-protein contacts within helix I are strengthened or relaxed given the phosphorylation state and bHLH partner choice, which adds or subtracts flexibility

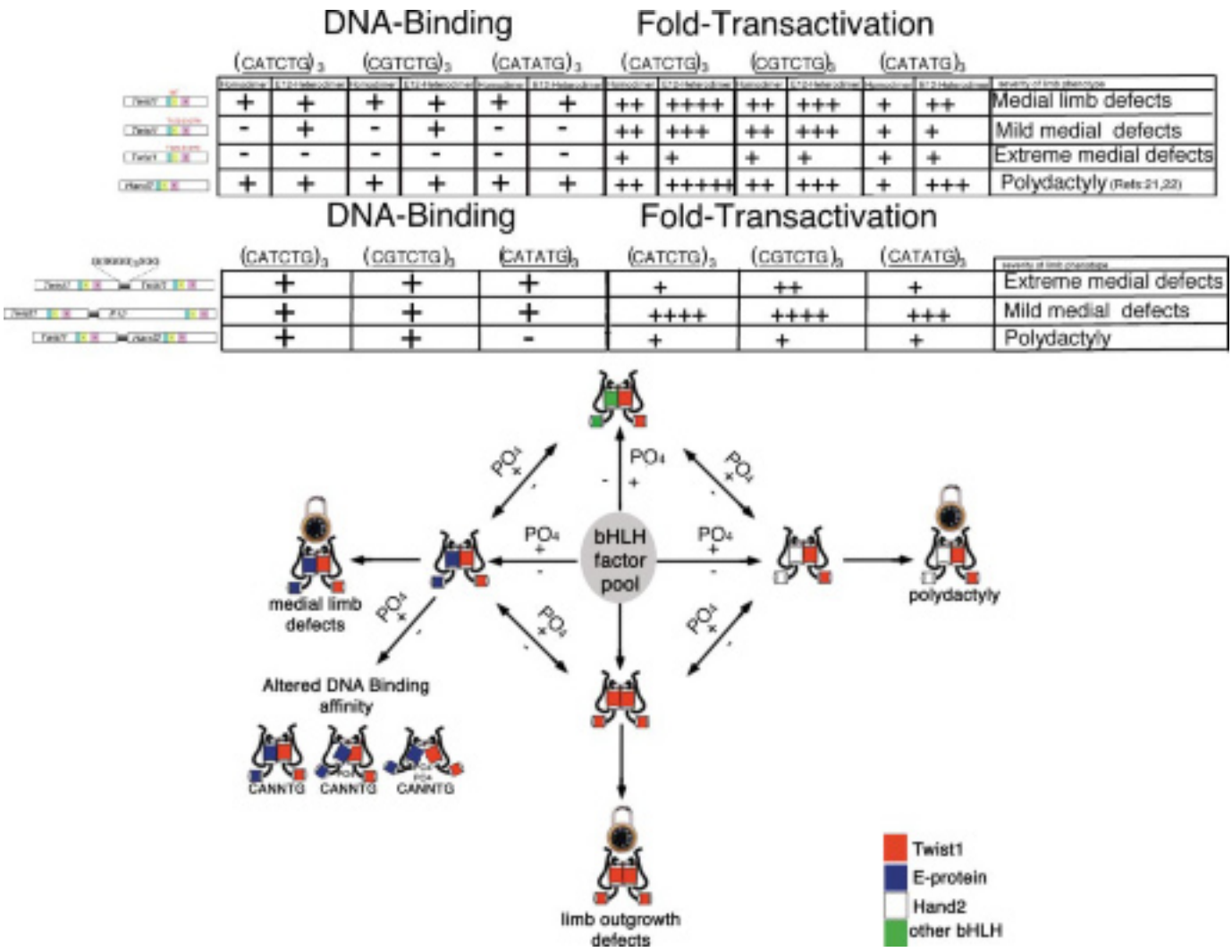

FIGURE 6. **Data summary and proposed model for Twist family phosphoregulation.** Earlier studies show that Twist family phosphoregulation of the evolutionarily conserved threonine and serine within helix I modulates changes in dimerization with various bHLH partners from the bHLH factor pool (2, 3). In this study, EMSA data from Twist wild type and mutant monomers coexpressed with E12 suggests that in addition to dimer control helix I, phosphoregulation modulates DNA binding by perhaps regulating flexibility at the junction of helix I and the basic DNA-binding domain. Considering if variations in one or both helix I sites are phosphorylated in the case of Twist family homo- and heterodimers, the "breathability" of this junction will regulate protein-DNA interactions and switch protein-DNA affinity to different *cis*-element sequences. By locking Twist1 into specific dimer complexes via the use of a flexible linker, the ability to affect the bHLH factor pool is diminished, and gain-of-function phenotypes result from direct transcriptional regulation and not from the titration of other bHLH factors. Distinct phenotypic outcomes suggest that different Twist1 dimers control specific aspects of limb morphogenesis.

(breathability) to the juxtaposition of the DNA binding basic domain (Fig. 6). This flexibility then mediates changes in the ability of the dimer to bind DNA in a *cis*-element-specific manner and has the potential to change the affinity of a Twist1 dimer complex for one gene program to another or, alternatively, to keep a functional Twist1 complex in an inactive state poised to mediate transcription upon the change in the phosphorylation of Thr^{125} and/or Ser^{127}. Interestingly, we show that the employment of multimerized *cis*-elements as EMSA probes increases the sensitivity of the DNA binding, which is necessary in order to visualize Twist1 and Hand2 homodimers bound to specific DNA sequences as well as Twist1-Hand2 tethered heterodimer. Although Hand2 homodimers and Twist1-Hand2 heterodimers were shown to form (3), this is the first demonstration of DNA binding for these complexes. The observed discrepancy in the monomer *versus* multimer probe binding may reflect that multiple *cis*-elements facilitate cooperative DNA binding, explaining why multimerized *cis*-elements are proven more efficient in transactivation assays than promoters driven by a monomeric enhancer sequence.

Given the complexity of the Twist family regulatory mechanism, where both the levels of expression and the phosphorylation state of helix I affect dimer choice and DNA binding, it is difficult to assess the functions for specific Twist1 transcriptional complexes; therefore, we employed a tethered dimer approach similar to that used for Twist and daughterless in *Drosophila* (6). In the *Drosophila* study, Twist-Twist and Twist-daughterless tethered dimers were assessed *in vivo*, showing that Twist homodimers specified mesoderm, whereas Twist-daughterless transcriptional complexes repressed gene expression required for somatic myogenesis (6). By forcing the dimer choice expressed in the developing limb via the use of tethered Twist1 transcriptional complexes, we observe distinct phenotypes that correlate well with the expression of wild type

and mutant *Twist1* monomers (Fig. 6). Twist-E12 tethered dimers produce phenotypes similar to those observed with *Twist1T125;S127A* expression, and Twist1-Twist1 phenotypes correlate to those observed with the expression of *Twist1T125; S127D*. Interestingly, Twist1-Hand2 tethered dimers in part phenocopy *Prx-Hand2* expression (21) by inducing polydactyly, suggesting that the mechanism for Hand2/Twist antagonism could be competition for third party bHLH proteins as well as forming a biologically active heterodimer.

Twist1-Twist1, Twist1-E12, and Twist1-Hand2 tethered dimers express a single fused protein that binds DNA in a manner similar to co-expressed monomer proteins; moreover, tethered dimers transactivate E-box-driven reporters in a manner similar to the monomerically expressed proteins in tissue culture (Fig. 6).

One could argue that when overexpressing monomeric Twist1 (or any bHLH factor), we will affect the dimerization choices of the endogenous bHLH factors as well as affect the direct regulation of transcription via interaction (either directly or indirectly) with various gene promoters. Indeed, we believe that both of these mechanisms hold true when expressing monomeric Twist1 and Twist1 mutant proteins and that these mechanisms are reflected in SCS (3). Until this study, we were unsure if a Twist1-Hand2 dimer exhibited any direct biological function other than serving as a mechanism of mutual inhibition via sequestration from E-proteins. Our observation of direct DNA binding as well as preaxial polydactyly in Prx-Twist1-Hand2 tethered dimer mice suggests that indeed there is a direct transcriptional function for the Twist1-Hand2 dimer. Given that the tethered proteins will not disrupt the dimerization of endogenous bHLH proteins, the phenotypes that we observed here reflect only the direct alteration of gene expression by Twist1-tethered dimers. We feel that the phenotypic differences between the tethered Twist1 and monomeric Twist1 experiments reflect this important distinction in mechanisms.

When tethered Twist1 complexes are expressed ectopically in the limb via the *Prx* promoter, distinct phenotypes are observed that reflect the functional differences that unique Twist1 dimers exert on limb morphogenesis. As stated previously, *Twist1-Hand2* expression results in polydactyly, a phenotype previously associated with ectopic *Hand2* expression (21, 22). Ectopic *Hand2* expression in the early limb induces the ectopic expression of *Shh*, which forms a secondary zone of polarizing activity, resulting in extra digits (21, 22). *Twist1* limb expression (via the regulation of the repressor Gli3) represses *Shh* expression in the anterior of the limb bud (9) restricting zone of polarizing activity expansion. *Hand2* overexpression overrides this repression and results in *Twist1* haploinsufficiency (which results in polydactyly), inadvertently allowing for an increased ratio of *Hand2* to *Twist1*; moreover, *Hand2* haploinsufficiency rescues the polydactyl phenotype seen with Twist1 haploinsufficiency (3, 8). The finding that the Twist1-Hand2 tethered dimer can result in polydactyly suggests that the induction of *Shh* by Hand2 is dominant to that of the repression of *Shh* by Twist1 perhaps by Twist1-Hand2 directly regulating *Shh* and/or by reducing expression of the *Shh* repressor *Gli3*. Additionally, the loss of the deltoid tuberosity (a phenotype not observed in *Hand2* ectopic expression (21)) suggests that this complex exhibits a Twist1-dominant function in regions proximal to the autopod.

Analysis of the transgenic mice expressing Twist1-E12 via the *Prx* promoter resulted in phenotypes that are most similar to Twist1T125;S127A (Figs. 3 and 5). This finding suggests that there is little effect on autopod development by this dimer; however, the medial bone phenotypes observed in the ulna, humerus, and clavicle from Twist1 and Twist1T125;S127A expression are recapitulated from this tethered complex. Previous interaction data show that in tissue culture the T125; S127A mutation has a lower dimerization affinity with E12 (3), and EMSA analysis suggests that the dimers that do form do not bind CATCTG or CATATG E-box sequences as robustly as wild-type Twist1 (Figs. 1 and 6). DNA binding and transcriptional activity for the D-box is indistinguishable from wild type Twist1, which suggests that the Twist1-E12 complex might be less active on some population of downstream target genes than on others.

The final dimer tested is the Twist1-Twist1 homodimer. Analysis indicates that unique phenotypes to this complex are encountered and are similar to phenotypes observed with Twist1T125;S127D, such as gross reduction in limb outgrowth and diminished ossification but relatively normal development of the autopod (Figs. 3 and 5). EMSA analysis indicates that the phosphorylation mimic does not bind to the three *cis*-elements effectively as a homodimer or when partnered with E12. Conversely, the tethered homodimer does bind to the multimerized *cis*-elements, indicating that phosphorylation at Thr^{125} and Ser^{127} may have a more profound effect on DNA-binding then on dimer stabilization. Since Twist1 function is well established to be critical for limb outgrowth via maintaining *Fgf8/Fgf10* expression within the apical ectodermal ridge (10, 23), the results observed with the Twist1 homodimer expression suggest that the function of Twist1 in maintaining the Fgf-mediated outgrowth of the apical ectodermal ridge is mediated via a Twist1 heterodimer and is perhaps antagonized by Twist1 homodimers given the reduction in limb outgrowth observed.

A remaining caveat to these studies is the inability to look at the levels of Twist1 and Twist1-tethered expression within the F_0 embryos used for skeletal preparations. Establishing stable mouse lines would allow for both phenotypic and molecular analysis, but given the severity of the limb defects encountered, this is not a practical approach. Variation in the expression levels of any monomer will clearly affect phenotypic outcome by activating/repressing genes directly and by titrating out other bHLH factors by forming either active or inactive complexes. Expression levels of tethered Twist1 complexes should not affect the balance within the endogenous bHLH factor pool; however, their artificial nature may convey novel activities or repress a required function. Ultimately, using a gene targeting strategy to express specific dimers from the Twist1 endogenous locus could resolve some (but not all) of these issues. This approach has been reported for the related factor Hand1 (24) and shows promise for deducing the role of Twist family bHLH complexes in a variety of tissues during embryonic development.

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