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A twist of insight - the role of Twist-family bHLH factors in development

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ABSTRACT Members of the Twist-family of bHLH proteins play a pivotal role in a number of essential developmental programs. Twist-family bHLH proteins function by dimerizing with other bHLH members and binding to *cis*- regulatory elements, called E-boxes. While Twist-family members may simply exhibit a preference in terms of high-affinity binding partners, a complex, multilevel cascade of regulation creates a dynamic role for these bHLH proteins. We summarize in this review information on each Twist-family member concerning expression pattern, function, regulation, downstream targets, and interactions with other bHLH proteins. Additionally, we focus on the phospho-regulatory mechanisms that tightly control posttranslational modification of Twist-family member bHLH proteins.

KEY WORDS: *Twist1, Twist2, Hand1, Hand2, Paraxis, Scleraxis, limb, heart, trophoblast, phosphoregulation, dimerization*

The Twist-family of basic helix-loop-helix (bHLH) factors is an evolutionarily conserved family of proteins that play diverse roles in both embryonic development and pathological disease. These diverse roles are reflected in the functional mechanisms that govern Twist-family biological activity. The requirement of bHLH factors to form a dimer complex is well established and recently a number of groups have shown that Twist family proteins have broad sets of potential dimer partners. In addition to Class A bHLH factors (E-proteins), homodimerization and heterodimerization between Twist-family members form important and functional dimer complexes that are required for proper development. Given the broad partially overlapping expression patterns of Twist factors observed within the developing embryo and the potential of these factors to form numerous unique transcriptional complexes, the spatio-temporal transcriptional regulation of Twist-family members is a key component of the functional regulation defining specification and differentiation. Additionally, phosphorylation of conserved threonine and serine residues within the first amphipathic α-helix of Twist-family proteins exerts a second level of control, effecting protein dimer affinities with potential partners as well as dictating DNA-binding affinities in a *cis*-element dependent manner. Moreover, phosphoregulation can regulate protein localization within the cell thereby positioning the Twist protein (and perhaps its partner) in a functional or non-functional environment. Given the recent new insights into the developmental and functional understanding of Twist factors this review strives to integrate what is known about function and mechanism.

The bHLH domain

The bHLH secondary structure is a protein dimerization-DNA binding domain that represents an evolutionarily conserved super family of near 100 transcription factors (Massari and Murre, 2000). Structurally, the motif consists of a short stretch of basic amino acids followed by an amphipathic α-helix, a loop of varying length, ending in a second amphipathic α-helix. The hydrophobic face of the helix makes protein-protein contacts with another bHLH factor creating a dimer. The two basic domains are then juxtaposed creating a complete DNA-binding domain. bHLH factors play essential roles in myogenesis, neurogenesis, and B-cell development to name several examples. *Twist* was discov-

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Abbreviations used in this paper: AER, apical ectodermal ridge; Anf, atrial natriuretic factor; bHLH, basic helix-loop-helix; D-V, dorsal-ventral; DBH, dopamine β-hydroxylase; Edn1, endothelin-1; FRET, fluorescent resonance energy transfer; HDAC, histone deacetylase; EMSA, electrophoretic mobility shift assay; MET, mesenchymal-to-epithelial transition; Pl1, placental lactogen 1; PKA/PKC, protein kinase A/C; PP2A, protein phosphatase 2A; SCS, saethre-chotzen syndrome; Shh, sonic hedgehog; ZPA, zone of polarizing activity.

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ered in *Drosophila* and is the founding member of a subfamily of bHLH proteins based on amino acid identity and conservation in the bHLH domain (Castanon and Baylies, 2002). We focus here on the Twist subclass of bHLH proteins and their known roles and the molecular mechanisms known to orchestrate these roles.

Twist is a master regulator of cell fate in *Drosophila*

Twist encodes a tissue-restricted bHLH protein originally identified in *Drosophila*. *Twist* was first implicated in the Dorsal-Ventral (D-V) patterning of the early embryo when Twist mutants were shown to undergo abnormal gastrulation (Thisse *et al.*, 1987). Additionally, it was later revealed that *twist* is required during multiple stages of embryonic development serving as a mesodermal-determining factor (Bate *et al.*, 1991; Baylies and Bate, 1996; Cripps and Olson, 1998; Castanon and Baylies, 2002,). Immediately obvious from these studies was that *twist* expression is regulated in a dosage dependent manner.

During early gastrulation in *Drosophila*, *twist* is the first gene detected throughout the presumptive mesoderm, which is initially defined by a nuclear gradient of the *dorsal* transcription factor (Zinzen *et al.*, 2006). High levels of *dorsal* results in the upregulation of *twist* as well as the gene coding for the Zn-finger protein Snail*,* which together cooperate with *dorsal* to pattern ventral cell invagination to form the mesoderm (Leptin, 1999; Ip and Gridley, 2002; Stathopoulos *et al.*, 2002). Mutants for either *twist* or *snail* fail to undergo a ventral invagination and lack mesodermal tissue (Ganguly *et al.*, 2005; Seher *et al.*, 2007). Despite their inability to form any mesoderm, *twist* mutant embryos briefly manifest the invagination indicative of the mesodermal primordium (Seher *et al.*, 2007) although this has been attributed to a transient activation of *snail* in a narrow band of mesodermal progenitors.

In addition to *snail*, critical *twist* targets include the mesodermal transcription factors d*mef2, tinman*, *fog*, transmembrane protein *t48,* microRNA *mir-1*, and the FGF receptor *heartless* (Cripps *et al.*, 1998; Sokol and Ambros, 2005; Kolsch *et al.*, 2007; Laursen *et al.*, 2007). Recently, ChIP-on-CHIP experiments have identified over 500 target genes for *twist*, including genes required for muscle development, cell proliferation, morphogenesis, and cell migration, illustrating an essential role for *twist* in establishing multiple transcriptional networks (Sandmann *et al.*, 2007). Furthermore, the finding that nearly fifty percent of *twist* target genes have two or more *twist* enhancer binding sites potentiates that multiple and possibly different Twist dimers participate in combinatorial binding of these enhancers and together participate in essential and complex transcriptional regulation of target genes (Sandmann *et al.*, 2007).

Following mesodermal induction, *twist* expression is dynamically regulated throughout the prospective mesoderm where *twist* becomes differentially expressed in both high and low dosage in the mesodermal segments (Baylies and Bate, 1996). The disparity in gene dosage of *twist* throughout these mesodermal segments is essential for normal development. *Twist* maintains a wide network of regulation that is particularly influenced by the potential dimer partners available, thus modulation of its expression will alter its availability for dimerization with its potential bHLH partners. One important dimer partner is the class I E-protein *daughterless*. *Daughterless* is capable of dimerizing with numerous bHLH factors (Van Doren *et al.*, 1991; Giebel *et al.*, 1997; Castanon *et al.*, 2001; Jafar-Nejad *et al.*, 2006) and is expressed in a uniform pattern throughout the presumptive mesoderm while *twist* expression is dynamically modulated. Electrophoretic mobility shift assays (EMSA) showed that Twist binds to DNA as either a homodimer or as a Twist-Daughterless heterodimer; opening the possibility for these two Twist transcriptional complexes to convey different developmental impacts.

A series of gain-of-function experiments that employ forced dimerization of Twist with itself or with Daughterless by utilizing a glycine-serine linker, which effectively “tethers” two bHLH proteins as a single head-to-tail polypeptide, were carried out to investigate the role of potential Twist and Daughterless dimers (Castanon *et al.*, 2001; Castanon and Baylies, 2002). Experiments in which Twist-Daughterless heterodimers were ectopically expressed in the mesoderm of transgenic flies leads to a severe reduction in somatic musculature coupled with patterning defects (Castanon *et al.*, 2001). Conversely, over-expression of Twist-Twist homodimers leads to the formation of ectopic somatic muscle (Castanon *et al.*, 2001). These antagonistic results, suggest that Twist homodimers and Twist-Daughterless heterodimers modulate distinct developmental programs. In *twist* mutants, expression of Twist-Twist homodimers rescues only the early mesodermal and migratory defects associated with *Twist* loss-of-function (Castanon *et al.*, 2001). Twist-homodimers failed to rescue the later onset patterning defects (Castanon *et al.*, 2001). These data show that although Twist-homodimers are essential for mesodermal induction they are not sufficient for mediation of all Twist developmental functions, supporting the idea that Twist dimer regulation is a critical regulatory mechanism that modulates biological function.

When considering that *twist* expression is dynamically modulated within regions of the embryo (Baylies and Bate, 1996) while Daughterless is ubiquitously expressed at a uniform level (Cronmiller and Cummings, 1993), one would predict that in regions where *twist* is expressed at high levels (such as somatic mesoderm) Twist homodimers would prevail whereas in regions where *twist* is expression is less robust, there would be a higher probability of forming a Daughterless heterodimer, thus repressing somatic muscle development.

Validation for such a gene dosage model is observed by crossing flies heterozygous for either *daughterless* or *twist* concurrent with over expression of the other bHLH factor (Castanon *et al.*, 2001). Heterozygous *daughterless* and *twist* flies do not exhibit any mutant phenotypes. As predicted, ectopic expression of *twist* on a heterozygous *daughterless* background results in an increase of ectopic somatic muscle then what is observed from Twist expression in wild type flies (Castanon *et al.*, 2001). Ectopic expression of *daughterless* on a *twist* heterozygous background resulted in an increased suppression of mesoderm development, compared to that observed at normal *twist* gene dosage (Castanon *et al.*, 2001). Thus, the overall expression of *twist* and *daughterless* (and perhaps other bHLH factors) within a given cell can act as a molecular switch that modulates transcriptional program.

While the gene dosage model illustrates *daughterless* and *twist* genetically interact, promoter analysis reveals some functional mechanism for the twist-twist and twist-daughterless dimers. Coexpression of a *mef2* mesoderm-specific luciferase reporter with either daughterless-twist or twist-twist tethers shows that twist tethered homodimers robustly activates the *mef2* reporter whereas twist-daughterless heterodimers repressed transcription (Castanon *et al.*, 2001), thus refined dimer regulation appears critical for normal expression of the desired gene program.

While *twist* expression declines during the later stages of embryonic muscle development, *twist* persists in a select group of myogenic progenitors fated to contribute to adult muscles during metamorphosis (Bate and Arias, 1991; Bate *et al.*, 1991; Currie and Bate, 1991). *Twist* is maintained in these larval progenitors until myoblasts fuse and differentiate into adult thoracic flight muscles. Though differentiation of these larval myoblasts can initiate in *twist* knockouts, loss of *twist* function results in an inability of these myoblast progenitors to undergo differentiation (Cripps and Olson, 1998). The requirement for *twist* in these larval myoblasts is strikingly similar to the function of *twist* during early embryonic development, where it serves as an essential cofactor and mesodermal regulator of downstream factor controlling myogenesis.

Twist1 can regulate EMT during cancer

In addition to its role in embryogenesis, *Twist1* plays important functions in cancer (Ma *et al.*, 2007; Yang *et al.*, 2004; Yang *et al.*, 2008). *Twist1* is expressed in mesenchymal cell populations, a transitional population present during development, which strikingly possesses many of the same migratory characteristics that cancer cells acquire during metastasis (Acloque *et al.*, 2008). Epithelial-to-mesenchymal transition (EMT) is the process whereby structured epithelial cells lose polarity and acquire mesenchymal features, allowing for cell movement (Zavadil and Bottinger, 2005). While EMT is essential during embryonic developmental, EMT is deleterious in neoplastic disease as it allows cancer cells to emigrate away from the primary tumor and form metastatic tumors (Thiery, 2002). Increased *Twist1* expression directly corresponds to cell EMT, and it is not surprising that *Twist1* expression is an indicator of poor outcome in a number of cancers, (Hoek *et al.*, 2004; Kwok *et al.*; 2005, Kyo *et al.*, 2006).

Subtractive screens utilizing four clonal breast cancer cell lines isolated from a single mouse mammary tumor (Aslakson and Miller, 1992), which exhibit varying degrees of metastasis formation (Yang *et al.*, 2004), revealed differential expression of *Twist1* mRNA (Yang *et al.*, 2004). *Twist1* expression correlated with loss of epithelia markers such as E-cadherin induced an up regulation of mesenchymal marker genes and consequently mesenchymal cell phenotype (Yang *et al.*, 2004). Most striking, RNAi mediated knockdown of *Twist1* expression within the highly metastatic 4T1 cell line greatly reduced metastasis formation in the lung, suggesting that *Twist1* acts as master regulator of EMT and if properly regulated could inhibit secondary tumor formation.

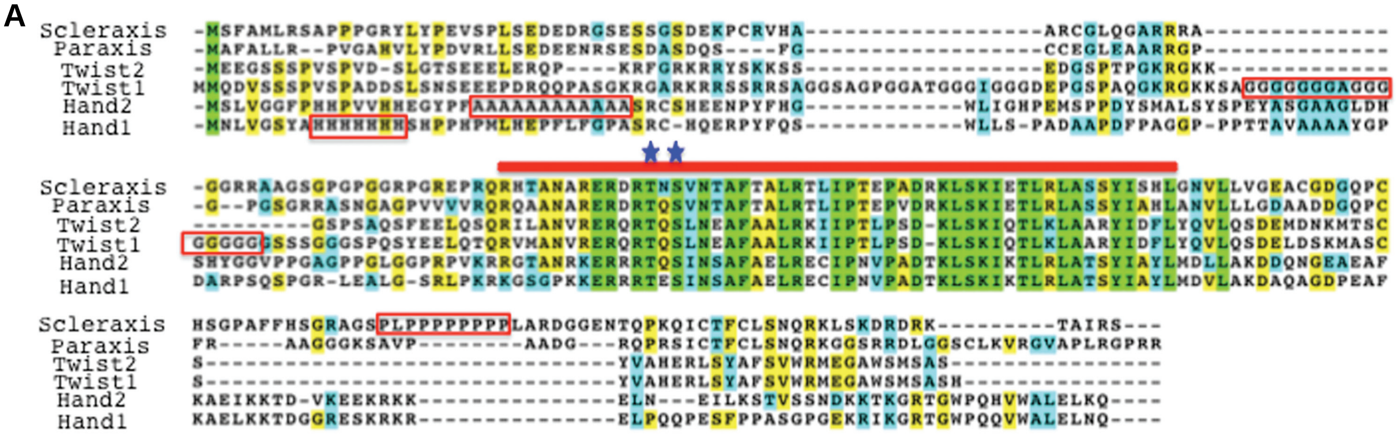
A potential mechanism that accounts for Twist1 upregulation in tumors comes from a study showing that Hypoxia inducible factor-alpha (HIF1-α) binds to a hypoxia responsive *cis*-element (HRE) within the *Twist1* proximal promoter (Yang *et al.*, 2008). Intratumoural hypoxia is an important mechanism responsible for promoting metastasis (Iyer *et al.*, 1998; Ryan *et al.*; 1998; Yang *et al.*, 2008). Downstream targets for *Twist1* also mediate tumor progression. MicroRNA mir-10b is highly expressed in cancer, exclusively restricted to metastatic cells (Ma *et al.*, 2007). Chromatin immunoprecipitation (ChIP) assays demonstrate that *Twist1* directly regulates mir-10b via binding to the promoter of microRNA mir-10b, located in an intron of the Hox D cluster (Ma and Weinberg, 2007). Mir-10b expression represses translation of HoxD10, permitting the expression of the pro-metastatic gene product RhoC, which promotes cancer cell migration and invasion. Validation of this proposed mechanism is observed with siRNA silencing of miR-10b as under these conditions Twist-mediated cell migration and invasion in breast cancer cells is inhibited (Ma *et al.*, 2007; Ma and Weinberg, 2007).

The role of Twist in vertebrates

The vertebrate homologue, *Twist1*, shares a high degree of amino acid identity to *Drosophila twist;* however, its functional role in mammals reflects its expression profile. While *twist* is necessary for gastrulation and is expressed in the prospective mesoderm, *Twist1* expression in the mouse is not detected until after gastrulation initiates. At E7.0-7.5 *Twist1* expression is first detected in the extra embryonic mesoderm and is strongly expressed in the allantois (Fuchtbauer, 1995). After E7.5 *Twist1* is reported in the head mesenchyme, somites, and somatic lateral plate mesoderm (Fuchtbauer, 1995). AT E8.5 *Twist1* continues to be strongly expressed in the head mesenchyme and is also detected in the first pharyngeal arch (Fuchtbauer, 1995). Interestingly, Twist1 protein is not detected until almost E8.5 despite the earlier expression pattern, which coincides with the first reported phenotypic abnormalities in *Twist1* null mice (Chen and Behringer, 1995; Gitelman, 1997). At E9.5 *Twist1* is expressed in the cranial mesenchyme, cephalic and thoracic neural crest, pharyngeal arches, sclerotomal mesoderm, somatopleuric mesoderm, and throughout the rostral and caudal margins of the limb bud mesenchyme underlying the apical ectodermal ridge (AER) (Wolf *et al.*, 1991; Fuchtbauer, 1995; Stoetzel *et al.*, 1995; Gitelman, 1997). Between E16-E18 *Twist1* expression is detected in developing osteoblasts, in the cranial mesenchyme, in the primordial of the tooth mesenchyme, and in the atrioventricular cushions (Fuchtbauer, 1995; Bialek *et al.*, 2004).

Given that Twist functions as a mesodermal specifier in *Drosophila* it is surprising that *Twist1* expression follows mesodermal induction in mice. In further contrast, Twist1 functionally represses muscle development by sequestering E-proteins from forming functional myogenic complexes with the skeletal muscle specific bHLH factor, MyoD, functionally blocking both *cis*- and *trans*- MyoD elements, and by inhibiting transactivation of *Mef2* (Hebrok *et al.*, 1994; Spicer *et al.*, 1996; Hamamori *et al.*, 1997). To gain additional insight into the function of Twist1 in the mouse, *Twist1* knockouts were generated (Chen and Behringer, 1995). *Twist1* null embryos die at E11.5, displaying exencephaly, pharyngeal arch and somitic defects, as well as defects in both cranial and cardiac neural crest cell populations (Chen and Behringer, 1995; Rice *et al.*, 2000; Soo *et al.*, 2002; Ishii *et al.*, 2003; Connerney *et al.*, 2006; Connerney *et al.*, 2008; Vincentz *et al.*, 2008). It is thought that the cranial mesenchyme plays a non-cell autonomous role in neural tube formation by either shaping or cushioning the neural folds and/or acting as a source of secreted factors (Chen and Behringer, 1995). *Twist1* nulls exhibit abnormal segregation and compaction of mesenchymal cell types, cell survival and proliferation deficiencies of mesodermally specified cell populations, and hypoplastic limb buds (Chen and Behringer, 1995; O'Rourke *et al.*, 2002; O'Rourke and Tam, 2002).

Twist1 is expressed in osteoprogenitors of the mid sutural mesenchyme of the developing cranial sutures (Rice *et al.*, 2000). *Twist1*-heterozygous mice experience fusion of the coronal suture, which never occurs in wild-type mice (Bourgeois *et al.*, 1998). Unfortunately, *Twist1*-null embryos die prior to osteogen-



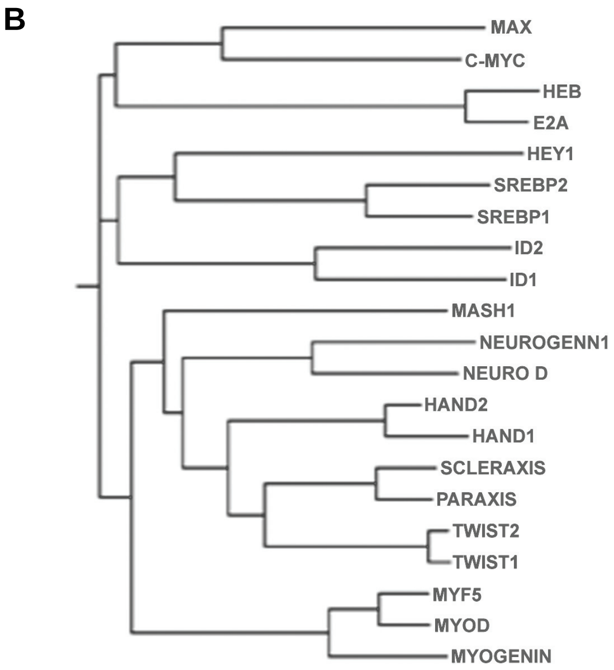


Fig. 1. Identity and conservation among Twist-family member bHLH's. **(A)** *Predicted Protein structure of all six Twist-family bHLH members with the basic-helix-loop-helix (bHLH) domain indicated (Red Bar). The entire Twist-family shares a very high degree of conservation across the functional bHLH domain, including serine and threonine residues located in the first α-helix which undergo phosphorylation, directly regulating dimerization and functionality (blue stars). Many Twist-family members have unique stretches of amino acids located outside of the bHLH domain in either the N- or C- terminus (red boxes).* **(B)** *A phylogenetic tree of a CLUSTALW alignment of bHLH domains across Twist-family members and other bHLH and HLH domain containing proteins. Twist-family members show relatively high conservation between the functional bHLH domain when compared with other bHLH sub-families.*

esis. Potentially, use of the conditional *Twist1* mutant may allow for further LOF analysis of the role of *Twist1* in cranial suture development (Chen *et al.*, 2007).

Twist1 has been implicated in FGF-signaling in the cranial mesenchyme where *FGF2* can induce *Twist1* and inhibit *Bsp*, an osteoblast differentiation marker, in addition to modulating *FGFr2* expression, which is expressed in proliferating osteogenic tissue (Johnson *et al.*, 2000; Rice *et al.*, 2000; Yoshida *et al.*, 2005). Calvarial organ culture showed that administration of a morpholino-mediated knockdown of *Twist1* causes premature differentiation with narrow sutural spaces and premature bone fusion (Yoshida *et al.*, 2005). Additionally, Twist1 is able to interact with Runx2; a protein that regulates osteoblast development, via a Twist1 C-terminal domain whereby it represses osteogenesis (Bialek *et al.*, 2004). Mutations in the C-terminal anti-osteogenic domain of Twist1 have been linked to patients with isolated cranial synostosis (Seto *et al.*, 2007). The osteogenic repressive activity of *Twist1* has been suggested to be limited to Twist1-E protein heterodimers. Twist1-E protein heterodimers have been shown to inhibit osteoblast differentiation and *FGFR2* while Twist1 homodimers have been shown to promote osteoblast differentiation and up regulate *FGFR2* (Connerney *et al.*, 2006; Connerney *et al.*, 2008). The cranial suture abnormalities that occur in *Twist1*-heterozygous mice are possible due to alterations in the dimer balance, favoring an increase in homodimer formation throughout the sutures (Connerney *et al.*, 2008).

Twist1 haploinsufficient mice are viable, and phenotypically model the autosomal dominant, variably penetrant human disease Saethre-Chotzen Syndrome (SCS; OMIM 101400). SCS is characterized by a broad set of facial and skeletal malformations including craniosynostosis, low frontal hairline, facial asymmetry, ptosis, deviated nasal septum, brachydactyly, and polydactyly (Jabs, 2004; Lee *et al.*, 2002). Indeed, *TWIST1* mutations are present in 80% of SCS cases with 73 independent *TWIST1* mutations identified ranging from single point mutations to large chromosomal deletions (Johnson *et al.*, 1998; Gripp *et al.*, 2000; Jabs, 2004). Of note, the number of SCS alleles that are point mutations that result in amino acid substitutions within the functional bHLH domain of the protein suggesting that dimer regulation defects could in part mediate SCS.

Twist-family bHLH proteins have highly evolutionarily conserved serine and threonine residues located in the first α-helix domain directly juxtaposed to the basic DNA binding domain (Fig. 1) (Firulli *et al.*, 2003; Firulli *et al.*, 2005). Twist-family members can be phosphoregulated by the actions of protein kinase A (PKA) and PKC and the trimeric protein phosphatase 2A (PP2A) containing the β56δ regulatory subunit. Dimer partner choice of Twist-family members can be modulated by phosphoregulation of these conserved residues, as was shown initially in studies of Hand1 (see below) (Fig. 2) (Castanon *et al.*, 2001; Castanon and Baylies, 2002; Firulli *et al.*, 2003; Firulli *et al.*, 2005; Connerney *et al.*, 2006;

Firulli *et al.*, 2007; Connerney *et al.*, 2008).

A subpopulation of *TWIST1* SCS point mutant alleles code for proteins that show a potential disruption of phosphoregulation (Firulli *et al.*, 2005). Direct assessment of PKA phosphorylation within Twist1 proteins coded by these SCS alleles confirms a reduced phosphorylation by PKA. Moreover, Fluorescence Resonance Energy Transfer (FRET) analysis of both hypophosphorylated and phosphorylation mimics of Twist1 confirm that Twist1 dimer affinity is altered for itself and its potential bHLH partners in the developing limb E12 and Hand2 (Firulli *et al.*, 2005).

Since haploinsufficiency of *Twist1* results in 42% penetrance of polydactyly in mice, it is interesting to note that gain-of-function expression of the Twist-family protein Hand2 phenocopies *Twist1* loss-of-function (Charite *et al.*, 2000; Fernandez-Teran *et al.*, 2000). Given that gene dosage phenotypes for Twist are observed in the fly, this observation suggests that Twist1 and Hand2 might act antagonistically in their roles limb patterning. When *Twist1* and *Hand2* heterozygous mice are intercrossed, thereby rebalancing the gene dosage of each gene to 1 allele each, the reduction of *Hand2* gene dosage completely rescues the Twist1-mediated polydactyly (Firulli *et al.*, 2005). Moreover in gain-of-function studies where over expression of *Hand2* results in polydactyly, co expression of wild type *Twist1* partially rescues this phenotype whereas expression of a hypophosphorylation Twist1 mutant that corresponds to a *TWIST1* SCS allele fails to rescue the *Hand2* induced polydactyly. Together, these data support the hypothesis that phosphoregulation of these evolutionarily conserved Helix I residues modulates Twist1 dimer choice and that in addition to the overall level of bHLH gene expression, post-translational modifications further regulate Twist-family dimer choice and thus function during development (Firulli *et al.*, 2005).

To further explore the functional role of Twist1 dimer choice, the *Prx1* limb-specific promoter was used to drive expression of Twist1 phosphorylation mutants and specific tethered Twist1 dimer complexes. Expectantly, unique limb phenotypes were associated with phosphoregulation state and specific Twist1 transcriptional complexes (Firulli *et al.*, 2007). Expression of the wild-type Twist1 resulted in medial limb defects associated with reduced ossification. Consistent with its haploinsufficient effect in SCS, expression of hypophosphorylated Twist1 resulted in milder gain-of-function phenotypes whereas expression of a phosphorylation mimic *Twist1,* resulted in a severe reduction in ossification, distorted limb patterning, and medial limb truncation (Firulli *et al.*, 2007).

Expression of Twist1-Twist1, Twist1-E12 and Twist1-Hand2 tethered dimers in the limb mirrored the effect of expressing *Twist1* phosphorylation mutants (Firulli *et al.*, 2007). Transgenic expression of Twist1-E12 heterodimers produced limb defects similar to expression of wild type Twist1 monomer whereas, expression of *Twist1-Twist1* homodimers resulted in limb phenotypes similar to expression of hyperphosphorylated *Twist1*. Interestingly, Twist1-Hand2 dimer expression resulted in preaxial polydactyly as well as mild medial limb defects suggesting that this non-E-protein heterodimer conveys instructions observed in both Hand2 over expression and Twist1 haploinsufficiency. Whether this dimer complex plays a *bona-fide* role in development or is simply an inappropriate dimer complex resulting from abnormal regulation is a question that is currently unanswered.

Twist2 directs development of dermal and chondrogenic tissue

Twist2 (*formerly Dermo1*) is a bHLH factor identified from a Yeast two-hybrid screen using the bHLH factor E12, as bait (Staudinger *et al.*, 1993). *Twist2* shows a very high degree of identity with Twist1. In particular, there is a high degree of conservation in the C-terminus and within the bHLH domains where there is only three amino acids not conserved (Fig. 1) (Li *et al.*, 1995; Perrin-Schmitt *et al.*, 1997). Consistent with all Twist-family proteins there is low conservation of amino acids between the N-terminus and both the 5'- and 3'-UTR's.

Although spatial expression patterns of *Twist2* during mouse embryogenesis demonstrate extensive overlap with *Twist1*, *Twist2* temporally follows expression of *Twist1* (Wolf *et al.*, 1991; Li *et al.*, 1995; Lee *et al.*, 2000). *Twist2* expression is first detected at E10.5 within the mesenchymal cell populations in the sclerotome, myotome, dermatome, branchial arches, and limb bud (Li *et al.*, 1995). Expression becomes further restricted to mesodermally derived cartilage and dermal tissues by E15.5. *Twist2* expression continues to increase in dermal tissues up to E17.5 and is then downregulated in the adult (Li *et al.*, 1995). Lineage trace analysis confirms that *Twist2* is restricted to sclerotomal mesenchyme, which develops into chondrocytes in growth plate cartilage and in perichondrium, periostium, and endosteum (Yu *et al.*, 2003).

Twist2 knockout mice reveal remarkable phenotypes with abnormalities in the development of vertebrate; wound healing, and hair regeneration (Gong and Li, 2002; Sosic *et al.*, 2003). These mice display severe postnatal atrophy of multiple tissues, apoptosis, and ultimately die perinatally due to cachexia. This may be partly due to the role for Twist2 in antagonizing p53-dependent apoptosis (Maestro *et al.*, 1999). Twist2 also functions downstream of NF-$_{k}$B by directly repressing the cytokine p65 promoter (Sosic *et al.*, 2003). Consequently, *Twist2* null mice exhibit an increased proinflamatory cytokine gene expression, which likely mediates the cachectic phenotype. Cytokine inhibition appears to be sensitive to Twist-family gene dosage as *Twist1* and *Twist2* compound heterozygotes recapitulate the *Twist2*-null cachectic phenotype (Sosic *et al.*, 2003).

In osteogenesis Twist2 plays a distinct role preventing differentiation of preosteoblasts (Lee *et al.*, 2000; Gong and Li, 2002). Over expression of *BMP2* within osteoblast progenitor cells leads to a down regulation of *Twist2*, further confirming a role in maintaining the unspecified state (Tamura and Noda, 1999). *Twist2* is also an ADD1/SREBP1c interacting protein that is able to suppress transcriptional activity of ADD1/SREBP1c, thus playing a role in fatty acid synthesis (Lee *et al.*, 2003). Finally, Twist2 is able to directly repress MyoD via dimer formation and Twist2 can directly associate with Mef2 factors repressing the Mef2 transactivation domain via a mechanism that is dependent on the Twist2 HLH and C-terminal domains (Gong and Li, 2002).

Paraxis regulates somite morphogenesis

The Twist family member *Paraxis* has been shown to regulate morphogenic events during somitogenesis. *Paraxis* is first ex-

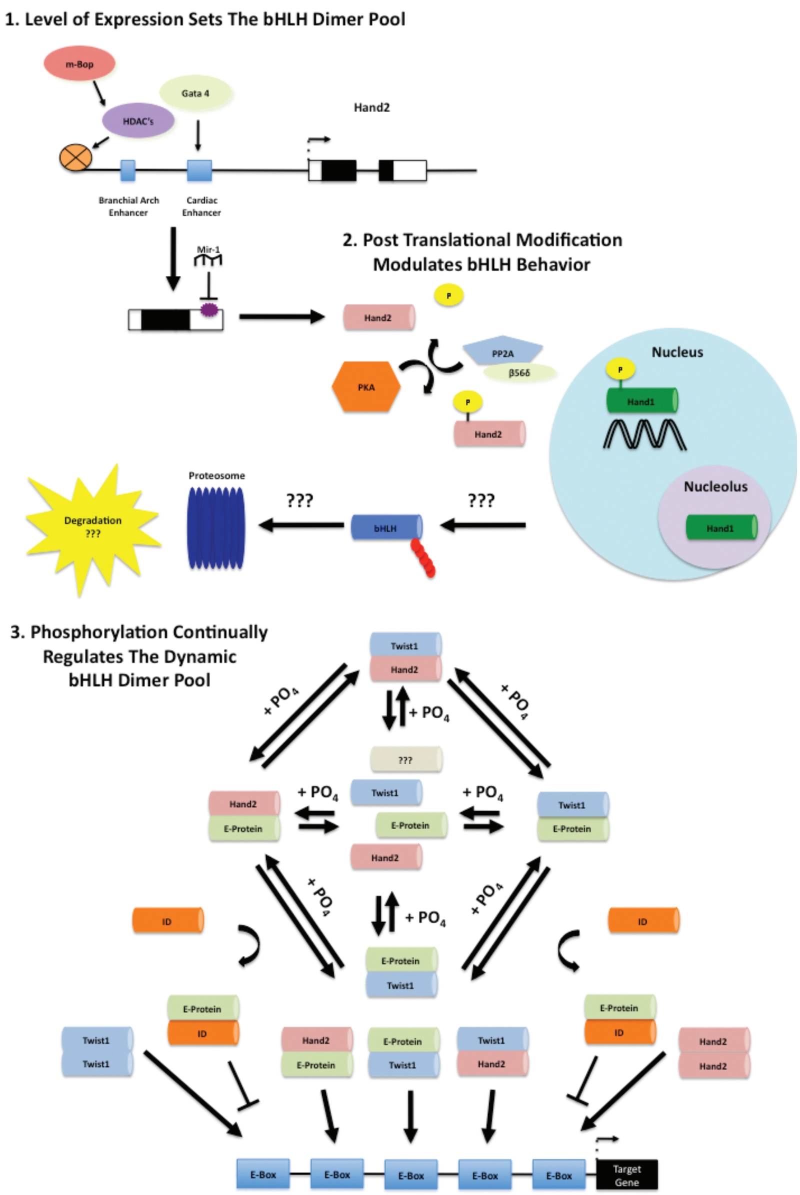


Fig. 2. Regulation and function of Twist-family bHLH members. *Twist-family bHLH's undergo several layers of regulation to tightly regulate their function. Principally, transcriptional regulation establishes the spatial and temporal expression within the cell, thereby establishing the potential bHLH dimer pool within each cell* **(1)**. Hand2 *transcriptional regulation has been well documented and serves as a prime example of the degree of transcriptional regulation associated with Twist-family members. Multiple tissue specific enhancer elements, such as the separate branchial arch enhancer and the heart enhancer, directly regulate spatial and temporal transcription of* Hand2. *The* Hand2 *cardiac enhancer is directly bound by Gata4, regulating ventricular expression (McFadden* et al., *2000). Availability of these enhancers is dependent on their accessibility, which is tightly regulated by chromatin conformation. m-Bop, recruits HDACs which deacytlate specific lysine residues promoting chromatin condensation and gene silencing of* Hand2 *(Gottlieb* et al., *2002).* Hand2 *mRNA can be regulated post transcriptionally by microRNAs. Mir-1 is capable of binding to the 3'UTR of the* Hand2 *mRNA, resulting in the silencing of the transcript and having a direct impact on the Hand2 dosage (Zhao* et al., *2007; Zhao* et al., *2005). Following translation, modifications can occur to the protein resulting in changes to the behavior of bHLH's* **(2)**. *Protein kinase's, including PKA (pictured) and PKC, and the β56δ subunit of Protein Phosphatase 2A (PP2A) are capable of regulating the phosphorylation state of conserved serine and threonine residues of Twist-family bHLH's (Firulli* et al., *2003). Phosphorylation of* Hand1 *has been shown to result in alterations of cellular localization and directly affecting the activity and accessibility of the bHLH protein (Martindill* et al., *2007). Dephosphorylated* Hand1 *is sequestered away in the nucleolus, remaining inactive. Upon phosphorylation,* Hand1 *is released from the nucleolus allowing it to dimerize and bind to* cis- *regulatory units on target genes. Although they have not been explored, multiple forms of posttranslational modification, such as ubiquitination and protein degradation, are possible and likely regulate both protein behavior and the equilibrium of the bHLH dimer pool. Once the pool of bHLH proteins is established, an antagonistic competition for dimer partners is closely regulated by the transitional phosphorylation state of the protein* **(3)**. *Phosphorylation state has a direct affect on dimer partner choice of bHLH proteins within the bHLH dimer pool (Firulli* et al., *2003). FRET analysis shows that a phosphorylated form of Twist1 has the highest affinity for Hand2 while having a low affinity for Twist1-homodimerization (Firulli* et al., *2005). In contrast, a dephosphorylated form of Twist1 has the lowest affinity for dimerization with Hand2 and instead has a preference for Twist1-homodimers (Firulli* et al., *2005). While the phosphoregulatory circuitry closely regulates bHLH dimer choice, ID-proteins, a class of HLH passive inhibitory proteins, can sequester away E-proteins, altering the bHLH dimer pool and potentially establishing a preference for homodimers among Class B bHLH's (Massari and Murre, 2000). Once dimerized, bHLH proteins can bind tightly to E-Boxes (CANNTG) located in target genes. Target genes may contain multiple E-Boxes, which can be transactivated by select dimer pairs. Dimer partner choice is particularly important since it can directly alter the function of bHLH proteins. In the fly, Twist-homodimers are capable of directing a mesodermal fate while Twist-E-Protein-heterodimers can directly antagonize the mesodermal lineage (Castanon* et al., *2001).*

pressed at E7.5 in a subdomain of primitive mesoderm located caudal to both the cranial and cardiac mesodermal primordia (Burgess *et al.*, 1995). Between E8.0 to E12.5, *Paraxis* expression proceeds in a rostral to caudal wave within the paraxial mesoderm, in newly formed epithelial somites (Burgess *et al.*, 1995). Following compartmentalization of the somitic mesoderm, *Paraxis* is expressed in the dermomyotome, sclerotome, & briefly within the myotome (Burgess *et al.*, 1995). In the mature somite, *Paraxis* expression is downregulated with expression gone after E13.5. *Paraxis* is also expressed in the fore limb buds at E10.5 in cell that are potentially myogenic progenitors that originated from the ventral edge of the dermomyotome as shown by chick-quail grafting experiments (Olson and Rosenthal, 1994; Burgess *et al.*, 1995).

Paraxis is critical to direct and maintain the epithelial characteristics of the somite, in particular the dermomyotome, acquired during the mesenchymal-to-epithelial transition (MET) necessary for somite maturation (Wilson-Rawls *et al.*, 1999;

Locascio and Nieto, 2001). *Paraxis* is capable of forming heterodimers with E-proteins and directly regulating the *Scleraxis* promoter, where *Paraxis* expression precedes *Scleraxis* in the somite (Wilson-Rawls *et al.*, 2004). Mice that are homozygous for a *Paraxis*-null allele have somites that are segmentally and molecularly specified but ultimately fail to undergo MET and properly compartmentalize (Burgess *et al.*, 1996). These mice die hours following birth due to complications attributed to axial skeleton and skeletal muscle patterning defects.

Several factors have been implicated in regulating *Paraxis* expression. *Paraxis* has been shown to be the target of β-Catenin signaling (Wagner *et al.*, 2000; Schmidt *et al.*, 2004; Geetha-Loganathan *et al.*, 2005; Linker *et al.*, 2005). *Frizzled7* transduces *Wnt6* signaling, which is expressed in the ectoderm overlying the somites. Experiments utilizing a dominant-negative form of β-Catenin show that *Paraxis* is a mediator of β-Catenin signaling initiated by *Wnt6* (Linker *et al.*, 2005). Misexpression experiments show that both *Foxc1* and *Foxc2* are capable of inducing *Paraxis* while mutant mice display defects in dermomyotome patterning (Wilm *et al.*, 2004). *Snail2* over expression prevents cells from activating *Paraxis,* thus locking them in a mesenchymal state (Dale *et al.*, 2006). Additionally, the Rho family GTPase Rac1 interacts with Paraxis and this interaction is necessary for inducing MET (Nakaya *et al.*, 2004).

The most notable Paraxis transcriptional downstream targets are Pax family members. Detailed analysis of Paraxis; Mesp2 double-null embryos reveals that Paraxis directly regulates *Pax3,* which lies upstream of *MyoD*, redundantly with *Mesp1* (Wilson-Rawls *et al.*, 1999; Takahashi *et al.*, 2007). Furthermore, Paraxis directly regulates *Pax1* expression in the ventral sclerotome as revealed by analysis of *Paraxis*-null embryos (Wilson-Rawls *et al.*, 2004; Takahashi *et al.*, 2007). Also, the Iroquois class factor *Mohawk* is a downstream *Paraxis* target as *Mohawk* expression is downregulated within the somites in *Paraxis*-null embryos (Anderson *et al.*, 2006).

Scleraxis functions during mesoderm formation, tendon formation and Sertoli cell development

Scleraxis is observed throughout the early embryo at E6.0 making it one of the earliest Twist-family members to be expressed (Brown *et al.*, 1999). High levels of the *Scleraxis* transcript then become detectable between E9.5 and E10.5 in the lateral region of the sclerotome and in mesenchymal cells of the limb buds and body wall (Cserjesi *et al.*, 1995a). *Scleraxis* expression is detected in mesenchymal progenitors of the thyroid cartilage and hyroid bone, cranial mesenchyme, rib primordial, and pericardium by E11.0 (Cserjesi *et al.*, 1995a). At E12.5 *Scleraxis* expression is also detectable in the diaphragm, connective tissue of the heart valves, and tongue (Cserjesi *et al.*, 1995a; Lincoln *et al.*, 2004). After E12.5 *Scleraxis* expression declines in all tissue except for the diaphragm but becomes apparent in regions of future dense connective tissue such as lung bronchi, heart valves, and ligaments (Cserjesi *et al.*, 1995a; Lincoln *et al.*, 2004). *Scleraxis* expression becomes restricted to the dense connective tissue of ligaments and cartilage by E14.5 (Cserjesi *et al.*, 1995a; Perez *et al.*, 2003; Pryce *et al.*, 2007). Though missed in the initial studies, *Scleraxis* is also expressed in the dense connective tissue comprising tendons of the trunk and limb at E14.5 (Schweitzer *et al.*, 2001). Further studies have gone on to describe *Scleraxis* expression in tendon progenitors of the axial skeleton, which are derived from the syndetomal somitic compartment (Brent *et al.*, 2003). Postnatally, *Scleraxis* is expressed in Sertoli cells following the onset of puberty in response to FSH (Muir *et al.*, 2005).

The original *Scleraxis*-null mice fail to gastrulate and are ultimately unable to form mesoderm, suggesting an essential role for Scleraxis in early mesoderm formation (Brown *et al.*, 1999). This observed lethality is largely due to the transcriptional effects of the neomycin-resistance cassette on neighboring genes, principally *Bop1*, a housekeeping gene that contains the entire *Scleraxis* gene within its third intron (Murchison *et al.*, 2007). Using an *FRT*-flanked neomycin cassette allowed for removal of neomyosin from the *Scleraxis* locus, where *Scleraxis* -null mice survived to term (Murchison *et al.*, 2007). These *Scleraxis* null mice display severe disruption of tendon formation by E13.5 (Murchison *et al.*, 2007). *Scleraxis*-null mice also have defects in testis development where mice that are homozygous for the null allele suffer from a range of spermatogenic cell defects (Muir *et al.*, 2008).

Several molecules are thought to regulate *Scleraxis expression*. FGF signaling has been implicated in directly regulating *Scleraxis* (Kawa-uchi *et al.*, 1998; Edom-Vovard *et al.*, 2002). *FGF8* is expressed during limb tendon development and is sufficient to induce *Scleraxis* expression (Edom-Vovard *et al.*, 2001). *Pea3* and *Erm*, transcriptional effectors of FGF signaling, are necessary to induce *Scleraxis* transcription (Brent and Tabin, 2004). Within the Sclerotome, *Scleraxis* expression has also been shown to be supported by a MKP3-ERK-MAP feedback loop (Smith *et al.*, 2005). *BMP2*, *BMP4*, and *BMP7* are expressed in a mutually exclusive domain from *Scleraxis* within the limb (Schweitzer *et al.*, 2001). Using *noggin* soaked beads reveals an upregulation of *Scleraxis* in digits, supporting the idea that BMP's down regulate *Scleraxis* in the distal most parts of the limb (Schweitzer *et al.*, 2001). *In vitro* studies confirm these results where application of *BMP2* or *CDMP*, a member of the BMP-family, were sufficient to down regulate *Scleraxis* and shift cells away from a mesodermal lineage and towards an osteogenic lineage (Liu *et al.*, 1997a; Kramer *et al.*, 2000; Yeh *et al.*, 2005).

Downstream targets of *Scleraxis* also support a role in regulating tendon development. *In vitro* experiments show that over expression of *Scleraxis* supported differentiation towards a cartilage fate by increasing levels of the aggrecan gene, type II collagen, and osteopontin while repressing expression of genes implicated in osteoblast differentiation, namely type I collagen and alkaline phosphatase (Liu *et al.*, 1997b). Expression of *Scleraxis* positively regulates *Tenulomodulin*, a late marker of tendon formation, in tendon fibroblasts (Shukunami *et al.*, 2006). Heterodimers of *Scleraxis* and *E47* have also been shown to regulate a type I collagen gene, *COL1a1* in tendon fibroblast cells (Lejard *et al.*, 2007). Thus far only *Collagen 14* and *Tenulomodulin* have been demonstrated to be downstream of *Scleraxis* based on analysis of *Scleraxis*-null embryos (Murchison *et al.*, 2007).

Hand1 is required for proper cardiac morphogenesis and is essential for extra-embryonic and trophoblast-cell differentiation

Hand1 was cloned from a yeast-2-hybrid screen using an E12 bait (Cserjesi *et al.*, 1995b). *Hand1* shares the highest degree of sequence identity with *Hand2* and to a lesser extent with Twist1 and other family members (Srivastava *et al.*, 1995). *In situ* hybridization shows *Hand1* is expressed within the trophoblast cells of the ectoplacental cone prior to E7.5 with expression throughout the yolk sac, chorion & extra embryonic mesoderm (Cserjesi *et al.*, 1995b). Extra-embryonic expression of *Hand1* is maintained throughout later stages of embryonic development (Cserjesi *et al.*, 1995b).

In the embryo, *Hand1* expression is first observed at embryonic day E7.5 in the lateral plate mesoderm that contributes to form the primitive heart tube (Srivastava *et al.*, 1997). At E8.5 *Hand1* is detected in the developing heart tube, pericardium, & the distal regions of lateral mesoderm (Cserjesi *et al.*, 1995a; Srivastava *et al.*, 1995; Biben and Harvey, 1997). During rightward looping of the heart, *Hand1* becomes restricted to the outer curvature of the myocardium contributing to the presumptive left ventricle, the septum transversum, and the pericardium where it persists thru E13.5 (Cserjesi *et al.*, 1995b; Firulli *et al.*, 1998; Thomas *et al.*, 1998b). *Hand1* expression continues to accumulate throughout the lateral mesoderm where it persists in the developing gut distal to the duodenum (Morikawa and Cserjesi, 2004). *Hand1* is also expressed throughout the umbilical and vitelline vein/artery by E9.5 (Firulli *et al.*, 1998). *Hand1* is also detected in the distal portions of the limb. At E11.5 it is expressed in the anteroventral domain of the limb bud where it is maintained thru E13.5 (Fernandez-Teran *et al.*, 2003). *Hand1* is expressed in adult-rodent and human hearts as well, where they are thought to play a role in preventing hypertrophy (Natarajan *et al.*, 2001; Thattaliyath *et al.*, 2002b).

Hand1 is also expressed within the cranial and cardiac neural crest cells occupying the medial pharyngeal arches and first appears at E9.5 as they begin to populate the outflow tract where they contribute to the smooth muscle lining the pulmonary artery (Cserjesi *et al.*, 1995b; Barbosa *et al.*, 2007; Vincentz *et al.*, 2008). *Hand1* continues to accumulate in structures derived from neural crest cells where by E10.5 it is detected in the sympathetic and splanchnic ganglia of the peripheral nervous system and the first and second aortic arch (Cserjesi *et al.*, 1995b; Firulli *et al.*, 1998;

TABLE 1

TWIST-FAMILY bHLH PROTEIN EXPRESSION AND FUNCTION

Gene	Expression Pattern	Function	References
	Drosophila		
twist	Presumptive mesoderm Mesodermal segments Thorasic flight muscle progenitors	Required for proper gastrulation. Twist homodimers specify mesoderm. Twist-Daughterless heterodimers antagonize mesodermal differntiation, properly patterning the mesoderm. Expression persisting in adult myoblasts maintains an undifferentiated state.	Thisse *et al.*, 1987; Baylies and Bate, 1996; Castanon *et al.*, 2001 Cripps and Olson, 1998; Bate et al., 1991
	Mouse		
Twist1	extraembryonic mesoderm alantois head mesenchyme & cranial sutures pharyngeal arches cephalic and thorasic neural crest lateral mesoderm distal limb bud mesenchyme	Follows mesodermal induction. Required for proper cranial mesenchyme and neural crest migration and distribution, directly affecting neural tube patterning and cushioning. Patterns the limb and cranial suture where it functions in a dimer dependent manner, regulated by dosage and phosphorylation. by dosage and phosphorylation	Wolf *et al.,* 1991; Fuchtbauer, 1995 Stoetzel *et al.*, 1995; Chen and Behringer, 1995 Gitelman *et al.*, 1997; Bialek *et al.*, 2004 Firulli *et al.*, 2005; Connerney *et al*., 2006 Firulli *et al*., 2007; Connerney *et al.*, 2008
Twist2	Sclerotome, myotome, dermotome branchial arches limb bud mesenchyme osteoblasts dermis	Essential for chondrogenesis, preventing differentiation of preosteoblasts. Dermal expression is required for proper skin formation and wound healing. Downstream of NF-κB directly repressing the cytokine p65 promoter and antagonizing p53-dependent apoptosis. Helps in fatty acid synthesis.	Li *et al.*, 1995; Maestro *et al.*, 1999; Gong and Li, 2002; Sosic *et al.*, 2003 Yu *et al*., 2003; Lee *et al*., 2003
Paraxis	caudal lateral & parxial mesoderm dermomyotome & sclerotome fore limb buds	Maintains the epithelial characteristics of the somite acquired during mesenchymal-to-epithelial transition (MET). Establishes proper axial skelton and skeletal muscle patterning.	Burgess *et al.*, 1995; Burgess *et al*., 1996 Wilson-Rawls *et al.*, 1999; Locascio and Nieto, 2001 Takahashi *et al.*, 2007
Scleraxis	Sclerotome Limb Bud Cranial & body wall mesenchyme Pericardium & Heart Valves Ligaments & Tendons	Required for tendon formation, ligaments, connective tissue, the diaphragm, and testis development. FGF signaling mediates Scleraxis signaling while BMP signaling downregulates Sceraxis expression, shifting cells away from a mesodermal towards an osteogenic lineage.	Cserjesi *et al.*, 1995a; Brown *et al.*, 1999 Schweitzer *et al.*, 2001; Brent *et al*., 2003 Perez *et al.*, 2003; Lincoln *et al.*, 2004 Muir *et al.*, 2005; Pryce *et al.*, 2007 Murchison *et al.*, 2007
Hand1	Extraembryonic Mesoderm Alantois & Trophoblast Cells Pharyngeal Arches & Mandible Materal Mesoderm Cranial & Cardiac Neural Crest Cells Sympathetic Ganglia Left Ventricle, & Outflow Tract Pericardium Limb buds	Required for giant trophoblast cell development, vasculature, and mesoderm formation in extraembryonic tissue. Establishes left ventricular and outflow tract patterning. Phosphorylation regulates export from the nucleolus, activating Hand1.	Cserjesi *et al.*, 1995b; Srivastava *et al*., 1995 Firulli *et al.*, 1998; Riley *et al.*, 1998 Fernandez-Teran *et al.*, 2003; Firulli *et al.*, 2003 Morikawa and Cserjesi, 2004; McFadden *et al*., 2005 Risebro *et al.*, 2006; Martindill *et al.*, 2007
Hand2	Decidua Lateral Mesoderm Right Ventricle & Outflow Tract Pharyngeal arches Cranial & Cardiac Neural Crest Cells Sympathetic Ganglia Adrenal Gland Pericardium Limb buds	Patterns right ventricular and outflow tract. Regulates ANF in the heart. Establishes cranial and limb morphogenesis. Required for noradrenergic differentiation of the sympathetic nervous system.	Srivastava *et al.*, 1995; Srivastava et al., 1997 McFadden *et al.*, 2000; Charite et al., 2000 Fernandez-Teran *et al.*, 2000 Ruest et al., 2003; Morikawa et al., 2005 Hendershot et al., 2007; Morikawa et al., 2007 Hendershot et al., 2008

Howard *et al.*, 1999; Morikawa and Cserjesi, 2004). At E12.5, *Hand1* is expressed in the sypatho/adrenal lineage as well as the mandible, which is derived from the pharyngeal arches (Cserjesi *et al.*, 1995b; Firulli *et al.*, 1998; Morikawa and Cserjesi, 2004). *Hand1 mRNA*–continues to persist in rudiments of neural crest derived tissues until E14.5 (Cserjesi *et al.*, 1995b; Morikawa and Cserjesi, 2004).

Knock outs of *Hand1* die by E9.5 experiencing extra-embryonic and heart defects (Firulli *et al.*, 1998; Riley *et al.*, 1998; Morikawa and Cserjesi, 2004). Heart development of null mutants is arrested during formation of the heart tube where the caudal portion failed to fuse as shown by marker analysis (Firulli *et al.*, 1998). Analysis of embryos homozygous for the *Hand1*-null allele showed that early myocardial markers such as *Nkx2.5*, *Mef2C*, *Gata4*, and *Mlc2a* were unaffected (Firulli *et al.*, 1998; Riley *et al.*, 1998). *Hand1*-null embryoid bodies are capable of differentiating into cardiomyocytes (Riley *et al.*, 2000) indicating that heart defects are not due to a failure of the myocardium to differentiate but due to improper patterning of the heart (Firulli *et al.*, 1998). Tetraploid experiments using *Rosa26* derived; *Hand1*-null ES cells are underrepresented in the left ventricular chamber but are capable of differentiating into cardiomyocytes *in vitro*'indicating that *Hand1* is not necessarily essential for cardiomyocyte differentiation but is required for proper patterning of the left ventricle (Riley *et al.*, 2000). Furthermore, the reduction of the left ventricle in mice with a conditional ablation of *Hand1* in the heart substantiate this conclusion (McFadden *et al.*, 2005), though more detailed analysis pairing the conditional *Hand1*-allele with a wider range of available Cre lines would be useful to support these findings.

Hand1 is restricted to the outer wall of the ventricular chamber during rightward looping of the heart. An asymmetric expansion of cells in this outer curvature is tightly intertwined in the process, implicating a role for *Hand1* in proliferation during heart remodeling. Misexpression of *Hand1* in the myocardium of both ventricular chambers resulted in an expansion of the outer curvature of both the left and the right ventricle (Togi *et al.*, 2004). Over expression of *Hand1* specifically in *Hand1* expressing cells resulted in abnormal looping (Risebro *et al.*, 2006). Though these hearts were accompanied by a failure of ventricular expansion, thorough analysis of these hearts revealed that *Hand1* overexpression resulted in left-ventricular defects due to elevated myocyte density and reduced myocardial differentiation. Furthermore, cells over expressing *Hand1* in *Hand1*-positive neural crest cells resulted in an elongated outflow tract due to continued proliferation and a failure to commit to differentiation (Risebro *et al.*, 2006). The complimentarity of the phenotype between loss-of-function and gain-of-function mutations of *Hand1* suggest a conserved role for *Hand1* during heart morphogenesis. Additionally, they hint that proper *Hand* gene dosage is essential for proper development, which has been elucidated in further studies with *Hand2* (McFadden *et al.*, 2005; Barbosa *et al.*, 2007).

Further analysis of *Hand1*-KO mice clearly shows that *Hand1* is essential for the development of extra-embryonic tissue. *Hand1* is expressed in all subtypes of trophoblast giant cells within the ectoplacental cone and chorion (Vasicek *et al.*, 2003; Simmons *et al.*, 2008). Hand1-null embryos have a dramatic down regulation of*)* within the ectoplacental cone. *Pl1* codes for a hormone and is expressed in the developing giant-trophoblast cells (Firulli *et al.*, 1998; Cross *et al.*, 2002; Hughes *et al.*, 2004). *Pl1* was detected in only a subset of giant cells outside of the ectoplacental cone in the placenta of *Hand1*-null embryos (Riley *et al.*, 1998). The ectoplacental cone only contains an increased number of giant cell precursors, suggesting *Hand1* plays a role during giant cell differentiation (Gardner *et al.*, 1973). This conclusion gains support when considering that over-expression of *Hand1* leads to an increase of *Pl1* in giant cells (Cross *et al.*, 1995) and *Hand1* homozygous mutant trophoblast cells display deficiencies in differentiation and normal invasive behavior (Hemberger *et al.*, 2004), illustrating the critical role for *Hand1* in trophoblast cell development.

In regard to extra-embryonic tissues, *Hand1* is also required for the formation of the extra-embryonic membrane, where it is expressed in the mesodermal compartment. *Hand1*-null embryos have abnormalities of the extra-embryonic vasculature following formation of the yolk sac by E7.5 (Firulli *et al.*, 1998; Morikawa and Cserjesi, 2004). Analysis of *Hand1*-null embryos shows that the yolk sac maintains an immature vascular plexus and smooth muscle cells required for blood vessel support during vasculogenesis failed to undergo normal recruitment (Morikawa and Cserjesi, 2004).

Hand1 was initially thought to interact only with ubiquitously expressed E-proteins (Massari and Murre, 2000). Mammalian two-hybrid and pull-down assays confirmed that Hand1 could form homodimers as well as interact with other tissue restricted bHLH proteins, such as Hand2 (Firulli *et al.*, 2000). Similar to Twist1, Hand1 was shown via EMSA's to inhibit MyoD/E12 DNA-binding (Firulli *et al.*, 2000). Although the biological relevance of this is moot given Hand1 and MyoD are not co-expressed during development, it does speak to the evolutionary conservation within the Twist-family.

Dimer partner choice clearly infers differential regulation on Twist family proteins and this was first demonstrated with Hand1. The LIM domain protein FHL2 is capable of interacting with Hand1 in the nucleus and repressing function of Hand1/E12 heterodimers though it is incapable of effecting Hand1/Hand1 homodimer activity (Hill and Riley, 2004). Additionally, deletion of the basic DNA-binding domain does not inhibit Hand1's ability to induce limb polydactyly (McFadden *et al.*, 2002). When considering this result carefully, the most likely mechanism to explain these findings would be actions as a dominant negative factor antagonizing the equilibrium of the bHLH dimer pool.

Hand1 phosphoregulation at Serine 107 and Threonine 109 modulates dimer partner specificity. Protein Kinase A and C (PKA and PKC) which can phosphorylate these Hand1 residues while β56δ-containing Protein Phosphatase 2A (PP2A) can specifically dephosphorylate these residues (Firulli *et al.*, 2003). Phosphorylation of Hand1 increases during differentiation of trophoblast giant-cells and this is associated with a down regulation of β56δ (Firulli *et al.*, 2003). Recently, it has been shown in trophoblast giant-cells that Hand1 is negatively regulated by interacting with I-mfa, which sequesters it to the nucleolus (Martindill *et al.*, 2007; Martindill and Riley, 2008). Interestingly, the Hand1 hypophosphorylation mutant targets directly to the nucleolus where the protein is sequestered, preventing differentiation (Martindill *et al.*, 2007; Martindill and Riley, 2008). Conversely a Hand1 phosphorylation mimic resides solely within the nucleus and expression drives trophoblast differentiation (Martindill *et al.*, 2007; Martindill and Riley, 2008). This data demonstrates that phosphoregulation modulates dimer choice in at least two ways. First by directly effecting protein affinity and second my dictating cell localization.

To date, upstream regulators and downstream transcriptional targets of *Hand1* have been difficult to ascertain. Hand1 *in vitro* can activate the promoter of cardiac *atrial natriuretic factor*, implicating it as a potential target of *Hand1* (Morin *et al.*, 2005). *Hand1* is co expressed in the heart with *Thymosin* β*4*, which is downregulated in Hand1-null embryoid bodies, as well as *cytostatin C*, and α*CA*, which are found to be up regulated (Smart *et al.*, 2002). Ectopic expression of *Tbx5* results in enhanced *Hand1* expression while simultaneously suppressing *Hand2,* suggesting that *Tbx5* can impart left ventricular identity upon *Hand1* expressing cells found throughout this region (Takeuchi *et al.*, 2003). Regarding upstream regulation, *Nkx2.5* knockout mice, which regulates expression of a number of cardiac specific genes, results in a severe reduction of *Hand1* in the heart, implicating that *Nkx2.5* may be upstream of *Hand1* (Tanaka *et al.*, 1999).

Hand2 is required during development of the heart, limbs, autonomic nervous system, & other neural crest derived structures

Hand2 (formerly *dHand*) was identified in a low stringency cDNA library screen using a *Hand1* bHLH domain probe (Srivastava *et al.*, 1995). In the chick, *Hand2* is first detected in the lateral mesoderm, and cardiac crescent; later it is expressed throughout the developing heart tube (Srivastava *et al.*, 1995). In the mouse, *Hand2* is first expressed at E7.5 in the maternally derived decidua and is first detected in the embryo at E7.75 in the lateral mesoderm that forms the cardiac crescent and is maintained throughout the linear heart tube to E8.0 (Srivastava *et al.*, 1997). At the onset of cardiac looping, *Hand2* cardiac expression subsequently restricts to the forming right ventricle and outflow tract down regulating within the left ventricle, which expresses *Hand1* (Overbeek, 1997; Srivastava *et al.*, 1997; Firulli *et al.*, 1998; McFadden *et al.*, 2000). *Hand2* is also expressed in the pharyngeal arches and neural crest cells where they give rise to craniofacial structures, outflow tract, the sympathetic nervous system, extra-adrenal chromaffin cells, as well as the posterior portion of the limb buds, (Gestblom *et al.*, 1999; Charite *et al.*, 2000; Ruest *et al.*, 2003).

Hand2-null embryos die by E9.5 suffering with severe morphological deficiencies in the heart as they have only a single left ventricle (Srivastava *et al.*, 1997). *Hand2*-null embryos undergo apoptosis in the region of the forming right ventricle (bulbous cordis) and results in a down regulation of ventricular markers such as *Irx4*, suggesting a role for maintenance of the right ventricle progenitors and supporting ventricular expansion (Bruneau *et al.*, 2000; McFadden *et al.*, 2000; Yamagishi *et al.*, 2001). This role for *Hand2* is further supported by evidence that a conditional deletion of *Gata4* in the heart, which has been shown to directly regulate a ventricular enhancer element of *Hand2*, results in right ventricular hypoplasia (McFadden *et al.*, 2000; Zeisberg *et al.*, 2005). Over expression of *Hand2* in the ventricles results in outward expansion of the ventricular chamber as well as an absence of the interventricular septum, which is replaced by an expanded trabecular domain, further establishing a role for *Hand2* in supporting ventriculogenesis (Togi *et al.*, 2006). In mice that have a homozygous-null allele for *m-Bop*, the histone deacetylase-dependent transcriptional repressor, *Hand2* expression is down regulated and there is an associated disruption of ventricular myocardial development (Gottlieb *et al.*, 2002). Data that may partially explain the *Hand* sided expression can be seen in studies of Tbx5 (Takeuchi *et al.*, 2003). Tbx5 can suppress *Hand2* concurrent with upregulation of *Hand1*.

Hand2 has been shown to directly regulate *Anf.* In *Hand2*-null mice, *Anf* is downregulated while a *Hand2*-heterodimer has been shown to *trans*-activates the *Anf* promoter (Thattaliyath *et al.*, 2002a). Additionally, *Hand2* cooperates with *Mef2c* to activate both *Anf* and αMHC (Zang *et al.*, 2004a, Zang *et al.*, 2004b). Moreover, Hand2 can synergize with Gata4 to activate *Anf* as well, revealing a multifunctional role for *Hand2* in *Anf* regulation (Dai *et al.*, 2002).

Recently, it has been demonstrated that *Hand2* is the direct target of microRNAs. A heart conditional knock out of *Dicer*, an enzyme required for processing of precursor microRNAs, results in the upregulation of *Hand2* (Zhao *et al.*, 2007). miR*-1*, a cardiac and skeletal muscle-restricted microRNA, is negatively affected in the *Dicer* knockouts. miR-1 over expression leads to a reduction in ventricular myocardium and is also capable of directly targeting *Hand2* (Zhao *et al.*, 2005).

Hand2 is also expressed throughout the cephalic neural crest mesenchyme of the first and second pharyngeal arches and plays a role in facial morphogenesis, where expression is directed by a *Hand2* enhancer element complete and separate from the ventricular heart enhancer (McFadden *et al.*, 2000; Ruest *et al.*, 2003; Yanagisawa *et al.*, 2003). *Endothelin-1* (*Edn1)*, which is expressed in the epithelial layer of the branchial arches, regulates *Hand2* and is downregulated in the branchial arches in *Edn1*-null mice (Thomas *et al.*, 1998a, Ivey *et al.*, 2003; Li and Li, 2006). The *Edn1* downstream effectors *Dlx5* & *Dlx6* directly regulate *Hand2* transcription via a Dlx cis-element located within the Hand2 branchial arch enhancer (Charite *et al.*, 2001; Fukuhara *et al.*, 2004). Targeted deletion of the *Hand2* branchial arch enhancer confirms that *Hand2* is required for craniofacial development as mutants exhibit craniofacial abnormalities that include cleft palate, mandibular hypoplasia, as well as a range of cartilage malformations (Yanagisawa *et al.*, 2003). A small domain of *Hand2* expressing cells in the distal most portion of the pharyngeal arches appears to be *Edn1* independent and is instead thought to be regulated by GATA3 (Ruest *et al.*, 2004). A conditional neural crest cell deletion of *Mef2c* shows that *Mef2c* likely mediates Endothelin signaling in the pharyngeal arches and is required for *Dlx 5 & 6* and *Hand2* (Verzi *et al.*, 2007). Pharyngeal arch mesenchyme undergoes apoptosis in *Hand2*-null embryos by *E9.5*; however cell death is partially rescued when mice are also null for *Apaf1* (Thomas *et al.*, 1998a, Aiyer *et al.*, 2005).

Hand2 is necessary for limb morphogenesis. *Hand2* is expressed in the posterior portion of the developing limb buds in the signaling region called the zone of polarizing activity (ZPA) (Charite *et al.*, 2000; Fernandez-Teran *et al.*, 2000). It has been implicated that retinoic acid signaling first establishes *Hand2* in the ZPA (Mic *et al.*, 2004). *Hand2* can up regulate expression of *Sonic Hedge Hog* (*Shh*) in the ZPA and expression of *Shh* up regulates expression of *Hand2*. Over expression of *Hand2* in the limb buds results in polydactyly associated with expanded *Shh* expression, which results in ectopic ZPA formation (Charite *et al.*, 2000; Fernandez-Teran *et al.*, 2000; McFadden *et al.*, 2002) while *Hand2*-null embryos lack any *Shh* expression domain (Charite *et al.*, 2000). *Hand2* also up regulates the BMP antagonist *Gremlin,* which acts to maintain an *Shh*/FGF feedback loop that maintains the ZPA (Zuniga and Zeller, 1999; McFadden *et al.*, 2002). The *Shh* repressor *Gli3* helps to restrict *Hand2* expression to the ZPA, which in turn feedbacks to regulate *Gli3*, allowing *Shh* signaling (te Welscher *et al.*, 2002a; te Welscher *et al.*, 2002b; Liu *et al.*, 2005a). Additional factors that potentially regulate *Hand2* in the limb are *Tbx3* and *Hoxd13* due to their co expression Twist1 (Rallis *et al.*, 2005; Salsi *et al.*, 2008).

Hand2 is expressed in multiple derivatives of neural crest cells, including the peripheral nervous system. Specifically, *Hand2* has been implicated in specification and maintenance of the noradrenergic phenotype of the sympathetic nervous system and chromaffin cells of the sympathoadrenal lineage development (Huber *et al.*, 2002; Xu *et al.*, 2003). Ectopic expression of *Hand2* is capable of activating the noradrenergic program (Howard *et al.*, 1999; Morikawa *et al.*, 2005). BMP's have been implicated in activating the noradrenergic phenotype and several of the transcription factors regulating sympathetic differentiation, including *Hand2* (Howard *et al.*, 2000; Muller and Rohrer, 2002; Liu *et al.*, 2005b). Unlike other transcription factors expressed during sympathetic neurogenesis that are responsive to BMP's which include *Phox2a, Phox2b,* and *Mash1,* only *Hand2* is exclusive to noradrenergic differentiation. Cilliary neurons lacking *Hand2* expression become cholinergic in response to BMP (Muller and Rohrer, 2002). Additionally, mesenchephalic neural crest cells that are *Hand2*-negative cannot differentiate into catecholaminergic neurons (Lee *et al.*, 2005).

These studies suggest a role for *Hand2* specifying and maintaining the noradrenergic phenotype during catecholaminergic differentiation. Additional evidence to support this hypothesis is that *Hand2* directly transactivates *Dopamine β-Hydroxylase* (*DBH*) in conjunction with *Phox2a* (Rychlik *et al.*, 2003; Xu *et al.*, 2003). Conditional knockouts of *Hand2* in neural crest cells reveals that sympathetic precursors differentiate into neurons but fail to express noradrenergic biosynthesis enzymes, such as *DBH*, further suggesting a role in the determination of the catecholaminergic phenotype (Morikawa *et al.*, 2007; Hendershot *et al.*, 2008). In the enteric nervous system, gain-of-function of *Hand2 r*esults in an overall increase of neurogenesis, suggesting it may have the potential to drive the noradrenergic phenotype; however, *Hand2* loss of function suggests that *Hand2* neural crest migrate properly and express neurogenic markers but fail to terminally differentiate, again suggesting a role for *Hand2* in specification and maintenance of the noradrenergic phenotype (D'Autreaux *et al.*, 2007; Hendershot *et al.*, 2007). In Zebrafish, there is only a single hand gene most identical to *Hand2.* A mutation of *Hand2*, called *Hands off*, shows that sympathetic precursors migrate properly and undergo proper neurogenesis, but ultimately fail to express noradrenergic genes indicative of terminal differentiation of catecholaminergic neurons (Lucas *et al.*, 2006).

As with all *Twist*-family bHLH's, *Hand2* is capable of forming heterodimers with E proteins to regulate transcription (Dai and Cserjesi, 2002). Though E-proteins are ubiquitously expressed in embryonic tissue, they are expressed at lower levels in the heart, suggesting that *Hand2* potentially dimerizes with other bHLH proteins or other factors to regulate development in heart tissue (Murakami *et al.*, 2004). Among these potential dimer partners, it has been shown that *Hand2* can heterodimerize with *Hand1* and potentially acts as an inhibitor, imparting a multifunctional role on *Hand* factors (Firulli *et al.*, 2000). *GATA4* has also been shown to synergize with *Hand2* to activate *Anf* through a direct interaction with P300 (Dai *et al.*, 2002). The ability of *Hand2* to transactivate is enhanced through stabilization when bound to DNA by *JAB1* (Dai *et al.*, 2004).

Phosphoregulation also regulates dimerization of Hand2. As previously discussed in Twist1, phosphorylation alters the dimerization preference of Hand2, mediated by PKA, and directly influences the antagonistic relationship with Twist1 (Firulli *et al.*, 2003; Firulli *et al.*, 2005). BMP's regulate Hand2 via induction of PKA, which phosphorylates the conserved helix 1 threonine and serine promoting noradrenergic differentiation from a specified cell type (Liu *et al.*, 2005b).

Summary

Twist-family bHLH proteins share a partially overlapping and expansive domain of expression throughout many stages of embryonic development. However, as illustrated in this review, each member plays a unique role during gestation mostly due to tight, specific regulation of each gene and subsequently protein. A network of transcriptional regulation, including chromatin conformation, tissue-restricted enhancer elements, and micro-RNA regulation, ensures tightly controlled spatial and temporal expression and ultimately sets the potential bHLH dimer pool in each cell. bHLH proteins function by binding E-boxes following dimerization with other bHLH proteins. Twist-family members exhibit a wide range of potential bHLH dimers as they are capable of forming a multitude of heterodimers and homodimers, creating an environment for dynamic and complex regulation due to potential alterations in function. Recently it has been established that a phospho-regulatory circuit shared in Twist-family members due to conserved serine and threonine residues dynamically regulates posttranslational modification, altering protein behavior and function. Though many of these proteins share significant regulatory mechanisms, these mechanisms often times employ unique functional characteristics to each protein. For instance, phosphorylation regulates *Twist1* and *Hand1*, however the mechanism for each appears different as *Twist1* appears to undergo an alteration in preference for its dimer partner with other bHLH proteins while *Hand1* undergoes export from the nucleolus. Therefore, it is likely that a multitude of information regarding regulation of Twist-family members, particularly post translationally has yet to be explored.

While systemic knockouts of all Twist-family members have been completed, conditional knockout analysis is not altogether complete or in some cases has yet to be established. Comprehensive analysis of conditional knockouts for all Twist family members will serve particularly useful given their overwhelming broad and overlapping spatial and temporal expression. Additionally, future construction of mouse lines engineered to characterize specific bHLH dimer function will serve useful to pinpoint the *in vivo* function for each Twist-family member in different developmental system.

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