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.

KEYWORDS: 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, affecting protein dimer affinities with potential partners as well as dictating DNA-binding affinities in a cis-element dependent manner. Moreover, phosphorylation 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-
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 (Thosse 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; Iq 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 dme1, tinman, fag, transmembrane protein i49, 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-CIP 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 and complexly 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 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 repress 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.

The role of Twist in vertebrates

The vertebrate homologue, Twist1, shares a high degree of amino acid identity to *Drosophila* twig; 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, splanchnic mesoderm, somatopleuric mesoderm, and throughout the rostral and caudal margins of the limb bud mesenchyme underlying the apical ectodermal ridge (AER) (Wolff et al., 1991; Fuchtbauer, 1995; Stoezlet 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 *Twist1* 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 Me2 (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 osteogenitors of the mid sutureal 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-
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 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). 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.
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, then re-balancing 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 Ptx1 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, dermato me, 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 endostium (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-κB 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 uncommitted 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-
heterodimers can directly antagonize the mesodermal lineage (Castanon et al., 2001). 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 may contain multiple E-Boxes, which can be transactivated by selected dimer pairs. Dimer partner choice is particularly important since among Class B bHLH's (Massari and Murre, 2000). Once dimerized, bHLH proteins can bind tightly to E-Boxes (CANNTG) located in target genes. Passive inhibitory proteins, can sequester away E-proteins, altering the bHLH dimer pool and potentially establishing a preference for homodimers (Firulli et al., 1995). Following compartmentalization of the somitic mesoderm, within the myotome (Burgess 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). In the mature somite, Paraxis expression is downregulated with expression gone after E13.5. Paraxis 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). D 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.
such as lung bronchi, heart valves, and ligaments (Cserjesi et al., 1995a). 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 hyoid bone, cranial mesenchyme, rib primordia, 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 neomycin 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 (Kawauchi et al., 1998; Edom-Vovard et al., 2002). BMP7 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 transcription (Brent and Tabin, 2004). Additionaly, the Rho family GTPase Rac1 interacts with Paraxis and this interaction is necessary for inducing MET (Nakaya et al., 2004).

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; Thattaiyath 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

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<th>Gene</th>
<th>Expression Pattern</th>
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<td>twist</td>
<td>Presumptive mesoderm&lt;br&gt;Mesodermal segments&lt;br&gt;Thoracic light muscle progenitors</td>
<td>Required for proper gastrulation. Twist homodimers specify mesoderm. Twist-Daughterless heterodimers antagonize mesodermal differentiation, properly patterning the mesoderm. Expression persisting in adult myoblasts maintains an undifferentiated state.</td>
<td>Thirse et al., 1987; Bayles and Bate, 1996; Castanon et al., 2001; Creps and Olson, 1998; Bate et al., 1991</td>
</tr>
<tr>
<td>Twist1</td>
<td>extraembryonic mesoderm&lt;br&gt;alantois&lt;br&gt;head mesenchyme &amp; cranial sutures&lt;br&gt;pharyngeal arches&lt;br&gt;cephalic and thoracic neural crest&lt;br&gt;lateral mesoderm&lt;br&gt;distal limb bud mesenchyme</td>
<td>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</td>
<td>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; Conneye and, 2006; Firulli et al., 2007; Conneye and, 2008</td>
</tr>
<tr>
<td>Twist2</td>
<td>Sclerotome, myotome, dermatome&lt;br&gt;branchial arches&lt;br&gt;limb bud mesenchyme&lt;br&gt;osteoblasts&lt;br&gt;dermis</td>
<td>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.</td>
<td>Li et al., 1995; Maestro et al., 1999; Gong and Li, 2002; Sotic et al., 2003; Yu et al., 2003; Lee et al., 2003</td>
</tr>
<tr>
<td>Paraxis</td>
<td>caudal lateral &amp; paraxial mesoderm&lt;br&gt;dermomyotome &amp; sclerotome&lt;br&gt;fore limb buds</td>
<td>Maintains the epithelial characteristics of the somite acquired during mesenchymal-to-epithelial transition (MET). Establishes proper axial skeleton and skeletal muscle patterning.</td>
<td>Burgess et al., 1995; Burgess et al., 1996; Wilton-Rawls et al., 1999; Locascio and Nieto, 2001; Takahashi et al., 2007</td>
</tr>
<tr>
<td>Scleraxis</td>
<td>Sclerotome&lt;br&gt;Limb Bud&lt;br&gt;Cranial &amp; body wall mesenchyme&lt;br&gt;Pericardium &amp; Heart Valves&lt;br&gt;Ligaments &amp; Tendons</td>
<td>Required for tendon formation, ligaments, connective tissue, the diaphragm, and tests development. FGF signaling mediates Scleraxis signaling while BMP signaling downregulates Scleraxis expression, shifting cells away from a mesodermal towards an osteogenic lineage.</td>
<td>Cserjesi et al., 1995a; Brown et al., 1999; Schweitzer et al., 2001; Brent et al., 2003; Perez et al., 2003; Lincoln et al., 2004; Mui et al., 2005; Pryce et al., 2007; Munchison et al., 2007</td>
</tr>
<tr>
<td>Hand1</td>
<td>Extraembryonic Mesoderm&lt;br&gt;Alantois &amp; Trophoblast Cells&lt;br&gt;Pharyngeal Arches &amp; Mandible&lt;br&gt;Material Mesoderm&lt;br&gt;Cranial &amp; Cardiac Neural Crest Cells&lt;br&gt;Sympathetic Ganglia&lt;br&gt;Left Ventricle, &amp; Outflow Tract&lt;br&gt;Pericardium&lt;br&gt;Limb buds</td>
<td>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 nucleus, activating Hand1.</td>
<td>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; Riserbo et al., 2006; Martindill et al., 2007</td>
</tr>
<tr>
<td>Hand2</td>
<td>Decidua&lt;br&gt;Lateral Mesoderm&lt;br&gt;Right Ventricle &amp; Outflow Tract&lt;br&gt;Pharyngeal arches&lt;br&gt;Cranial &amp; Cardiac Neural Crest Cells&lt;br&gt;Sympathetic Ganglia&lt;br&gt;Adrenal Gland&lt;br&gt;Pericardium&lt;br&gt;Limb buds</td>
<td>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.</td>
<td>Srivastava et al., 1995; Srivastava et al., 1997; McFadden et al., 2000; Chante 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</td>
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et al., 1999; Morikawa and Cserjesi, 2004). At E12.5, Hand1 is expressed in the sympathetic 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 over-expression 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 P16 within the ectoplacental cone. P16 codes for a hormone and is expressed in developing giant-trophoblast cells (Firulli et al., 1998; Cross et al., 2002; Hughes et al., 2004). P16 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 P16 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 (Massarri 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 nucleolus and expression drives trophoblast differentiation (Martindill et al., 2008).
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 (Srivistava 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 (Srivistava 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 (Srivistava 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; Srivistava 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 (Srivistava et al., 1997). Hand2-null embryos undergo apoptosis in the region of the forming right ventricle (bulbous cords) 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 Hand2 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 upregulates 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, mesencephalic 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 Dopamineβ-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 results 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’Auteauret 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 Hand1factors (Firulli et al., 2000). GATA4 has also been shown to synergize with Hand2 to activate Ant 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 overwhelmingly broad and overlapping spatial and temporal expres-
sion. 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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