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A bHLH Code for Cardiac Morphogenesis

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Abstract

Cell specification and differentiation of cardiomyocytes from mesodermal precursors is orchestrated by epigenetic and transcriptional inputs throughout heart formation. Of the many transcription factor super families that play a role in this process, the basic Helix-loop Helix (bHLH) family of proteins is well represented. The bHLH protein by design allows for dimerization—both as homodimers and heterodimers with other proteins within the family. Although DNA binding is mediated via a short variable *cis*-element termed an E-box, it is clear that DNA affinity for these elements as well as the transcriptional input conveyed is dictated largely by the transcriptional partners within the dimer complex. Dimer partner choice has a number of inputs requiring co-expression within a given cell nucleus and dimerization modulation by the level of protein present, and post-translational modifications that can both enhance or reduce protein–protein interactions. Due to these complex interrelationships, it has been difficult to identify *bona-fide* downstream transcriptional targets and define the molecular pathways regulated of bHLH factors within cardiogenesis, despite the clear roles suggested via loss-of-function animals models. This review focuses on the Hand bHLH proteins—key members of the Twist-family of bHLH factors. Despite over a decade of investigation, questions regarding functional redundancy, downstream targets, and biological role during heart specification and differentiation have still not been fully addressed. Our goal is to review what is currently known and address strategies for gaining further understanding of *Hand/Twist* gene dosage and functional redundancy relationships within the developing heart that may underlie congenital heart defect pathogenesis.

Keywords

Congenital heart diseases; Basic helix-loop-helix transcription factors; Gene dosage

Hand1 and Hand2 Transcription Factors and Cardiovascular Development

In the mouse, cardiac progenitors can be identified as early as E7.5 (roughly equivalent to day 18 in human embryos) and their presence in the embryo is dependent upon multiple epigenetic and transcriptional inputs from an ever growing number of essential transcription factors [2, 14,35,42]. The bHLH factors Hand1 and Hand2 are members of the Twist-family of proteins and are broadly expressed throughout development in a number of tissues including: neural crest that contribute to craniofacial structures, sympathetic and enteric neurons and cardiac outflow tract (OFT); developing limbs; extraembryonic tissues; placenta and most significantly the cardiomyocytes [12,13]. *Hand* mRNA is first detected around E7.5 throughout the cardiac crescent [8,9,22,43]. In chick embryos, both *Hand* genes mark the entire cardiac crescent and expression is completely overlapping through linear heart tube and cardiac chamber septation

[43]. However, in mouse *Hand1* expression becomes restricted to what will become the left ventricle and OFT, whereas *Hand2* expression maintains ubiquitous cardiac expression within the linear heart tube; though restricts to cardiomyocytes that form the right ventricle and OFT (Fig. 1). In antisense knockdown experiments that reduced *Hand1* and *Hand2* expression in chick embryos, no significant phenotypes were observed; however, simultaneous reduction in *Hand* mRNA resulted in morphological defects to the heart that resulted in diminished function and death [43]. Targeted mouse knockout models of *Hand1* and *Hand2* confirm a critical role in cardiogenesis but individual loss-of-function experiments display unique phenotypes [15, 40,44]. *Hand2* systemic knockout embryos die at E9.5 resulting from a hypoplastic right ventricle and OFT vascular defects—corresponding phenotype with expression domains and suggesting a cell autonomous role [44]. *Hand1* systemic knockout embryos die at E8.5–9.5 due to extraembryonic mesoderm and placental insufficiencies that involve deregulation of vascular genes [15,30,40]. Cardiac-restricted deletion of *Hand1* in conditional knockout mice circumvent this superseding extraembryonic phenotype and reveal a hypoplastic left ventricle (again matching phenotype with expression domain), and interestingly the phenotypes observed become more severe on a *Hand2* haploinsufficient background [29]. Subtle expression variation within species is not uncommon and given that Hand factors share a high amino acid identity (90%) within the functional bHLH domain, the idea of functional overlap was obvious, especially given the supporting chick data. Conversely, outside the bHLH domain identity is far less conserved (Fig. 1), suggesting that although some functions may be replaceable, it is likely that unique functions for each Hand factor may also exist. Thus, although it is clear that both Hand factors play significant roles in cardiac morphogenesis, given their broad expression profiles in other organ systems and combinatorial nature; the specific molecular networks within the developing heart that utilize Hand factor biological function remain elusive (Table 1).

Molecular Mechanism of Function: bHLH Partner Choice and Gene Dosage

The bHLH domain is a region of basic amino acids followed directly by an amphipathic α -Helix, which is separated from a second more carboxy α -Helix by a loop domain of varying length [27]. This domain enables protein–protein interactions between two bHLH factors. Dimerization then juxtaposes the two proteins basic domains to form a combined DNA-binding domain that in the majority of bHLH proteins recognizes a canonical sequence termed an E-box (CANNTG) [27]. This super-family of proteins was first identified as important transcriptional and developmental regulatory from study of skeletal muscle—specifically the myogenic regulatory factors (MRFs) MyoD, Myogenin, Myf5, and Mrf4 [27,33,34]. Functionally, the MRF proteins dimerize with the ubiquitously expressed E-proteins class of bHLH factors [27], although recent investigations have shown that these factors can interact and bind to non-traditional *cis*-elements [20]. In contrast to the myogenic bHLH factors, which are expressed exclusively in skeletal muscle and primarily function as heterodimers with E-proteins, Hand1 and Hand2 (and all Twist-family proteins) display a broad range of expression throughout the developing embryo (Fig. 2) and adult, and exhibit dimerization characteristics which include homodimerization, heterodimerization with other members of the Twist-family, dimerization with E-proteins as well as other bHLH factors such as Hey factors [16–18]. Moreover, interactions with other transcription factors and binding to non-canonical *cis*-elements can also be observed [21,24,45].

It is easy to see the convenience of participation in multiple transcriptional complexes, when one considers the board spectrum of tissues where these proteins are expressed. However, to propose this hypothesis there must accompany a mechanism that provides instruction to bHLH dimer choice. First, coexpression and the overall level of bHLH protein present within the nucleus is probably the primary factor governing dimerization. Clearly, two factors cannot interact if they are not coexpressed nor can a factor expressed at 1:100th the levels of its

potential partners compete effectively for a rate-limiting dimer partner (provided dimerization affinities are equal). The later supposition is unlikely to hold true and mechanisms that modulate dimerization affinities are in fact in place. One such post-translational mechanism that we have shown plays a role in Hand1 and Hand2 dimer regulation is phosphorylation of evolutionarily conserved residues within the Helix I of the bHLH domain [17]. Via protein kinase A (PKA) and B56d-containing protein phosphatase 2A (PP2A), a charge on Helix I is modulated and these changes can affect not only dimer affinities for various partners but also DNA-binding affinities to specific *cis*-elements (Fig. 1) [17,19]. Thus, the level of gene expression in union with post-translational modifications to the bHLH dimer pool within a cell may direct the formation of the specific bHLH complexes required to drive specific developmental transcriptional programs.

In addition, recent work in the Riley lab showed that in addition to dimer affinities, Helix I phosphoregulation of Hand1 may also regulate subcellular localization [26]. Hand1 promotes the differentiation of trophoblast giant cells. Expression Hand1T107;S109A within Rcho1 stem cell trophoblast cells [38] fails to induce differentiation compared to wild type Hand1. Examination of cell localization using GFP-fusion proteins demonstrated that Hand1T107;S109A is sequestered in the nucleoli via interactions with the inhibitor of myogenic factor, I-mfa [26]. Phosphorylation of Hand1 within Helix I is necessary for release from within the nucleoli fitting into the over riding regulation model proposed above—thereby limiting access to the factor where it functions. The idea that Hand1 (and perhaps Hand2) are regulated via sequestration from the transcriptional compartment in cardiogenesis will be interesting to investigate further.

To date, published data currently point toward post-translational modifications and cellular localization as defining the regulatory mechanisms. However, the remaining element to create the bHLH dimer pool; namely gene dosage effects, are also observed between *Twist1*, *Hand1*, and *Hand2* factors. During conditional ablation of *Hand1* within the myocardium haploinsufficiency of *Hand2* was able to exacerbate the observed *Hand1* phenotype [29]. In these studies, cardiomyocyte deletion of *Hand1* via *α MHC-Cre* results in VSDs and overriding aorta as well as thickened and shorten AV valves. Use of the *Nkx2.5-Cre* driver, which is expressed earlier and more directly upstream of *Hand1* for cardiomyocyte deletion yielded similar results suggesting that *Hand1* is being deleted early enough in cardiogenesis as *Nkx2.5* null mice fail to express *Hand1* in the left ventricle [14]. Moreover, removing a single *Hand2* allele on this background results in embryonic lethality at E10.5 with ventricular phenotypes clearly indicating that gene dosage is required to normal embryogenesis. Furthermore, less *Hand1* expressing cells (as visualized via a *Hand1*^{LacZ} allele) are observed in the left ventricle and heart morphology shows a thin and poorly trabeculated myocardium [29]. As expected *Hand1* conditional/*Hand2* systemic double knockouts display a more grossly hypoplastic heart that is looped but contains only a single ventricle and atria via morphological analysis [29]. However, gene dosage effects seem to require a minimum threshold before having any cardiovascular effects, as no phenotypes are observed within either *Hand1* or *Hand2* heterozygous or double heterozygous *Hand* mice. Future insights into the limit of dosage effects may be gained using *Hand2* conditional mice to focus upon cardiomyocytes-restricted autonomous defects.

Perhaps, the best example of Twist-Hand gene dosage effects is the autosomal dominant human disease Saethre Chotzen syndrome (SCS) that exemplifies post-translational modifications, dimer choice, and gene dosage most clearly. Craniofacial and limb phenotypes are largely observed in SCS patients, and this congenital defect is largely attributed to mutations in the *TWIST1* gene. Significantly, 73 documented null, missense and nonsense mutations have been associated with SCS in humans [23]. Recently, we identified a number of SCS-causing human mutations in *TWIST1* that alter phosphoregulation via PKA and B56 δ -containing PP2A [18].

Realizing that *Twist1* haploinsufficiency partially phenocopied specific *Hand2* gain-of-function in the developing limb [11,28] resembling SCS-like phenotypes within the mouse limb, we reasoned that this was an example of Twist-family gene dosage. To test this, we reduced gene dosage of *Hand2* to match that in *Twist1* haploinsufficient mice and were able to clearly genetically demonstrate that *Hand2* heterozygosity rescues *Twist1*-mediated SCS phenotypes in vivo. Similarly, overexpression of *Twist1* rescues *Hand2* gain-of-function limb phenotypes; however, overexpression of a phosphorylation *Twist1* mutation that models a human *TWIST1* SCS allele does not rescue the *Hand2*-mediated limb phenotypes [18]. Collectively these experiments define a paradigm for Twist-family bHLH gene regulation where the level of expression and the post-translational state of the proteins themselves set up a bHLH dimer pool that conveys defined biological function. In this example, *Twist1* and *Hand2* functionally and genetically interact in an antagonistic fashion, and we speculate that in other tissues different, and perhaps more complex relationships may be operating.

Recently, we uncovered a novel role for *Twist1* within the cardiac neural crest (cNCC) adding *Twist* as a new bHLH player within cardiac OFT development [47]. *Twist1* null mice die at E11.5 and present hypoplastic limbs along with extensive craniofacial defects associated with NCC cell populations [5]. Closer examination of the cardiac OFT of *Twist1* null mice reveals a cell adhesion defect where clumps of cells that are uncharacteristic of the loose mesenchymal phenotype of OFT cushion formation [47]. Lineage analysis identifies these “nodule” cells as NCC origin and perhaps most striking, the cNCC, which contribute to OFT nodules are specifically marked by expression of *Hand1* and *Hand2*. Also evident were cNCC emigration defects; however, proliferation and cell death of NCC cells in *Twist1* null mice was indistinguishable from wild-type littermates [47]. This emigration defect could be directly traced back to the neural tube where an anomalous expansion of *Wnt1-Cre* marked cells are encountered suggesting that the NCC cells become trapped in the neural tube and these trapped cells simply revert to a default CNS developmental program. Given that *Twist1* expression is not detectable within the neural tube but is observed in the surrounding mesenchyme, we believe this NCC trapping is a non-cell autonomous effect, which may be independent of the aggregation phenotype observed in the cNCC that do escape, emigrate and are marked by *Hand* gene expression. Experiments to explore gene dosage effects on the crest nodule phenotype are currently underway.

Roadblocks to Overcome Before We Can Understand bHLH Mode of Action In Vivo

In order to expand our understanding of the biochemical and genetic relationships of Twist-family proteins within the developing heart, the field will be required to continue to dissect the functional and genetic relationships, define true redundancy relationships (can functional heterodimers be formed and/or isolated) and define the unique roles these factors play in building the heart. The rub is that any loss-of-function or gain-of-function approach has ripple effects on unintended bHLH factors coexpressed within the cells, wherever the balance has been altered. Gaining an improved understanding of that balance, the code, is of paramount importance for us to gaining a true understanding of Twist-family proteins and their functional role in heart formation. What is lacking has been the elucidation of direct transcriptional targets, which likely reflects the importance of the aforementioned dimer-regulatory mechanisms. Conflicting data sets showing activation or repression in vitro may reflect the specific dimer complex forming (or being inhibited from forming) and biological and arti-factual interactions within the system studied. Although highly artificial in nature, the idea of using tethered bHLH factors, where translation of two bHLH factors as a single polypeptide that (in theory) could force the two linked coding regions to form a transcriptional complex, could be used to initially probe some of the aforementioned outstanding questions. In the fly for example, tethered *Twist* homodimers and *Twist*/E-protein heterodimers can indeed activate/repress mesoderm and

somatic muscle formation when expressed [4]. Work from the Spicer lab elegantly shows Twist expression using tethered gain-of-function approaches results in craniofacial development [6, 7]. In our own work using a tethered dimer approach, we can correlate observed limb phenotypes between specific Twist1 and Hand2 tethered dimers with those generated by expression of Twist1 phosphorylation mutants [19]. Expanding upon this approach via the use of the cardiac-specific *α -myosin heavy chain* (*α MHC*), promoter to drive *Hand1* dimers also yields consistent and provocative data that underscores the importance of genetic balance and dimer rules (Fig. 3). In E9.5 day F0 transgenic expressing *α MHC-Hand1-Hand1* homodimers, only pericardial bleeding is encountered in a high percentage of embryos. In contrast, expression of *α MHC-Hand1-E12* results in embryos that have severely hypoplastic right ventricles (Fig. 3). What is striking is that these embryos appear to phenocopy the *Hand2* systemic deletion defects. Thus, if there is a critical balance between Hand1 and Hand2 in the developing ventricle and loss of *Hand2* promotes the formation of additional Hand1-E12 heterodimers, one can see how analysis of a loss-of function phenotype of a bHLH factor could become overly complex. Similarly, over-expression of a monomeric bHLH factor may effectively titrate out endogenously expressed bHLH proteins, thereby creating a hidden loss-of-function requirement for unknown transcription factor(s).

Further compounding the unquantified nature of Twist-family dimer diversity in steady state is the lack of reliable specific antibodies. In conjunction with the low level of endogenous protein expression, the lack of antibody tools prohibits unbiased screens for downstream targets, although ectopic overexpression can be achieved, the obvious perturbation of the bHLH dimer pool via overexpression could inadvertently pollute the datasets of such screens. Perhaps targeting in a high affinity epitope within the coding region of *Twist1*, *Hand1*, and *Hand2* will be required for obtaining multiple bona fide downstream targets, as well as the targeting of specific tethered dimer complexes to force dimer choice, and thus avoid overexpression and ectopic domains of function.

In addition to these experimental roadblocks, genetic data linking Twist-family proteins to congenital heart disease has also been lacking. When considering the myriad of transcription factors that contribute to cardiogenesis, numerous factors such as, Gata4 and Nkx2.5 have all been identified as causative within human congenital heart disease via identification of haploinsufficiency coding mutations [32,36,37]. Although Hand factors have been shown to interact with all these key factors, specifically Gata4 [10] and Nkx2.5 [45], as yet no CHDs have been directly associated with a human *HAND* mutation. Excitingly, a study of 31 unrelated patients diagnosed with hypoplastic left heart syndrome (HLHS), found that 24 of these patients presented with mutations within *HAND1* [39]. These findings clearly support the importance that Hand factors play in heart development and more widespread analysis of CHD patients is likely to result in further identification of additional *HAND1* and *HAND2* mutations that are causative of disease.

Summary and Future Directions

To move forward in our understanding of the role of Twist-family bHLH factors during cardiogenesis and specifically how the clear functional mechanisms modulate heart formation, we need to gain additional information on the ultimate destination of Hand and Twist-expressing cells within the mature heart via lineage analysis, gain improved understanding of phosphoregulation of dimerization affinities without the complications of over-expression via point mutant-specific knockin alleles, and further dissect issues of gene dosage and functional relationships by replacement alleles where the coding domains of one bHLH factor are replaced with that of a relative. The results of such experiments are likely to fill in the current gaps of how we view this key family of transcription factors and their significance in cardiogenesis.

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Fig. 1.

a Schematic of Hand gene expression during early cardiac development. Published findings describe early expression within the anterior later plate mesoderm, which in the chick persists for both Hand genes. In mouse, Hand2 persists until E8.0 and upon rightward looping, restricts to second field lineage cells on the right ventricle and OFT. Hand1 prior to heart tube fusion already displays chamber-specific expression within the primary heart field derived left ventricle and OFT such that by E9.5 coexpression with the myocardial cuff and OFT is observed along with unique expression domains that define the left and right ventricles. b Schematic of Hand1 and Hand2 coding regions showing amino acid identities within the amino terminal, bHLH, and carboxy terminal domains. The positions of the basic DNA-binding

domain are shown in *black* and poly-histidine and polyalanine tracks in the amino terminal portion of each respective protein are indicated. The evolutionarily conserved threonine and serine within Helix I of the bHLH are phosphoregulated via PKA and B56d-containing PP2A. Regulation alters dimer partner choice and effects DNA-binding affinities to various cis-elements. Amino acid alignment of Hand1 and 2 over the basic domain and Helix I showing high level of conservation within the DNA binding and dimerization motifs

Fig. 2.

In situ hybridization of Twist1, Hand1, and Hand2 in sagittally sectioned E10.5 day mouse embryos. Expression is observed within a broad domain of mesodermally and neural crest-derived cells that make up the craniofacial, cardiac, sympathetic, and enteric nervous system as well as lateral mesoderm derived organs. Given the broad overlap of expression of Twist-family genes during development and the ability of the coded proteins to form transcriptional complexes modulation of where these genes are expressed as well as levels of overall expression are critical to normal development

Fig. 3.

Schematic of Hand1 tethered dimers expressed via the α MHC-promoter. Three F0 E9.5 day embryos expressing Hand1-Hand1 (*top row*) within the heart display pericardial bleeding cardiac morphological defects. Hand1-E12 tethered expression (*middle row*) results in loss of right ventricular structures phenocopying the Hand2 systemic knockout (*lower row*). Wild-type mice included for comparison

Table 1

Summary of known cardiac-related phenotypes in gain-of-function and loss-of-function analysis for Hand factors

Gene	Cardiovascular phenotypes	Reference
Hand1	<i>Mouse</i> : Systemic <i>Hand1</i> KO display extraembryonic and placental & vascular defects. Dies at E9-9.5.	[15,30,40]
	Conditional knockout using cardiac-specific Cre drivers show hypoplastic left ventricle and clear gene dosage with <i>Hand2</i> .	[29]
	Neural crest inactivation of <i>Hand1</i> displays no observable phenotypes; however, <i>Hand2</i> gene dosage effects are observed.	[1]
	<i>Hand1</i> inducible gain-of-function during embryogenesis increases cardiomyocyte proliferation. Expression via knockin to <i>Mlc2v</i> inhibited septum formation expanded ventricular size.	[46]
	<i>Hand1</i> gain-of-function in adult heart predisposes mice to arrhythmias.	[3,41]
Hand2	Systemic <i>Hand2</i> KO mice display right reduction in right ventricle and vascular defects. Dies at E9.5-E10.0	[44]
	Cardiac knockout of <i>Hand2</i> using <i>cTnt-Cre</i> phenocopy a reduction in right ventricle. <i>Hand1</i> gene dosage effects not evaluated	[31]
	Neural crest inactivation of <i>Hand2</i> display OFT defects. <i>Hand1</i> gene dosage effects not evaluated	[31]
	<i>Chick</i> : Antisense knockdown of either <i>Hand1</i> or 2 has no observable phenotype; however, knockdown of both genes results in defective heart development	[43]
	<i>Fish</i> : The single <i>Hand</i> gene as two mutant alleles termed <i>Hands off</i> these fish display in lower number of myocardial cell precursors and do not maintain <i>Tbx5</i> expression within the myocardium.	[48]
	<i>Fly</i> : <i>Drosophila</i> <i>Hand</i> semi lethal larva—lymph glands missing. Adult flies display abnormal dorsal vessel disorganized musculature and reduced function.	[25]
Twist1	<i>Mouse</i> : <i>Twist1</i> loss-of-function exhibits adhesion and emigration defects in cardiac neural crest cells marked by expression of <i>Hand1</i> and <i>Hand2</i>	[47]