

SURVEY AND SUMMARY

Redundant or separate entities?—roles of Twist1 and Twist2 as molecular switches during gene transcription

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ABSTRACT

Twist1 and Twist2 are highly conserved members of the Twist subfamily of bHLH proteins responsible for the transcriptional regulation of the developmental programs in mesenchymal cell lineages. The regulation of such processes requires that Twist1 and Twist2 function as molecular switches to activate and repress target genes by employing several direct and indirect mechanisms. Modes of action by these proteins include direct DNA binding to conserved E-box sequences and recruitment of coactivators or repressors, sequestration of E-protein modulators, and interruption of proper activator/repressor function through protein–protein interactions. Regulatory outcomes of Twist1 and Twist2 are themselves controlled by spatial-temporal expression, phosphoregulation, dimer choice and cellular localization. Although these two proteins are highly conserved and exhibit similar functions *in vitro*, emerging literature have demonstrated different roles *in vivo*. The involvement of Twist1 and Twist2 in a broad spectrum of regulatory pathways highlights the importance of understanding their roles in normal development, homeostasis and disease. Here we focus on the mechanistic models of transcriptional regulation and summarize the similarities and differences between Twist1 and Twist2 in the context of myogenesis, osteogenesis, immune system development and cancer.

INTRODUCTION

The basic helix–loop–helix (bHLH) family of proteins comprises a series of transcription factors that act as master regulators on different tissues. Their complex regulatory functions make them fine-tuned machinery for controlling cell fate at the early stages of embryogenesis. The hallmark for DNA binding of bHLH transcription factors is the formation of a bipartite DNA-binding domain created when two of these factors form homo or heterodimer complexes through their HLH motifs allowing the basic stretch of amino acids to contact the DNA. This DNA-binding domain is able to recognize *cis* regulatory elements containing the consensus sequence 5'-NCANNTGN-3' (termed E-box). E-boxes are found in the regulatory regions of many lineage specific genes, which account for the numerous pathways regulated by these transcription factors (1–3).

The bHLH transcription factors are classified into three major classes: the ubiquitous Class A bHLH factors that include E2-2, HEB and the two isoforms of the E2A gene E12/E47 (also known as E proteins); the tissue-restricted Class B bHLH factors; and the inhibitory HLH proteins, constituted by the Id proteins, which lack the basic region used to contact DNA. The Twist proteins form a subfamily of the Class B bHLH factors. These include Paraxis (1), Scleraxis (4), Hand1 (5), Hand2 (6), Twist1 and Twist2. In this family of transcription factors, Twist1 and Twist2 exhibit a high degree of sequence similarity suggesting that their functions might be redundant. These proteins also exhibit bifunctional roles as activators and repressors of gene transcription making the characterization of their individual modes of action a complex task (7,8). It is therefore the focus of this review to highlight the

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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similarities between Twist1 and Twist2 and distinguish when their functions as gene regulators are unique.

Twist1

The first Twist protein to be described was the *Drosophila* Twist (DTwist) as one of the zygotic genes required for dorso-ventral patterning during embryogenesis (9). As such, it is a key regulator for gastrulation and subsequent mesoderm formation, where differential expression patterns of *DTwist* have been observed. *DTwist* was primarily thought to be an activator based on its role in defining the dorsoventral axis in parallel with the *Drosophila* NF-κB homolog *Dorsal*, and driving gastrulation synergistically with *Snail*. It is now known that *DTwist* can form both homodimers and heterodimers with the *Drosophila* E-protein *Daughterless*, making it either a transcription activator or repressor (10,11). Although *DTwist* induces cellular differentiation in *Drosophila*, the classical roles of Twist1 and Twist2 proteins in mammals are to inhibit differentiation of mesenchymal cell lineages, notably muscle and bone.

Mammalian Twist1 was identified in mouse by its high similarity with *DTwist*, having 48% amino-acid identity, and was first described as a repressor of gene transcription due to its role in the inhibition of myogenesis and osteogenesis (12–14). Mutations in Twist1 cause Saethre–Chotzen syndrome (SCS; OMIM 101400) that is characterized by premature closure of the calvarial sutures, or craniosynostosis (15,16). The various mutations found in SCS patients give rise to Twist1 loss of function, protein instability and improper localization (17). A *Twist1*-KO mouse was shown to be embryonic lethal and displayed abnormalities in neural tube closure (18). The lethality of the Twist1 knockout mouse led to the development of a Cre-mediated conditional knockout mouse to help study the regulatory functions of Twist1 post-neural crest cell migration (19). A *Twist1*-null heterozygous mouse has also been developed that presents a Saethre–Chotzen syndrome-like phenotype (20), thus providing an animal model for studying the SCS craniosynostosis phenotype.

Twist2

Twist2 was first identified from a yeast-two-hybrid screen using E12 as bait, and it was named Dermo1 for its expression pattern in the dermis of mouse embryos (21). Dermo1 was later renamed Twist2 based on its high homology and overlapping expression pattern with Twist1, and as seen with Twist1, it was found to inhibit both myogenic and osteoblast maturation (21–23). During mammalian embryogenesis, Twist2 is expressed in mesodermal tissues, although temporally expressed after Twist1. The *Twist2*-KO mouse presents relatively normal embryonic development and no notable bone abnormalities, but typically dies 2–3 days after birth due to cachexia, failure to thrive and high levels of pro-inflammatory cytokines (22). It is important to note that the *Twist2*-KO mouse was developed in an inbred 129/Sv background and that the same *Twist2*-KO mouse in a 129/C57 mixed background survives with only a mild phenotype suggesting the existence of modifier genes in these different genetic backgrounds (22). Recently it has been shown that two homozygous nonsense mutations (c.324C>T and c.486C>T) in *TWIST2* cause Setleis Syndrome (MIM 227260) (24). Setleis syndrome is an inherited developmental disorder classified as a Focal Facial Dermal Dysplasia type III (FFDD III) and is characterized by bilateral temporal marks and additional facial features, including, absent eyelashes on both lids or multiple rows on the upper lids, absent Meibomian glands, slanted eyebrows and chin clefting (24). These mutations truncate the *TWIST2* protein in glutamines 65 and 119 resulting in C-terminal domain mutants (Figure 1). Examination of the *Twist2* KO mouse developed in the 129/C57 mixed genetic background has revealed a facial phenotype similar to that of Setleis syndrome patients and has been established as a relevant mouse model for the study of FFDD's (24). Although human *TWIST1* and *TWIST2* encode bHLH transcription factors with a high degree of sequence identity, the finding that *TWIST2* recessive mutations cause an FFDD and dominant *TWIST1* mutations causes Saethre–Chotzen craniosynostosis suggests that these two genes exhibit non-redundant functions in skin and bone development and highlights

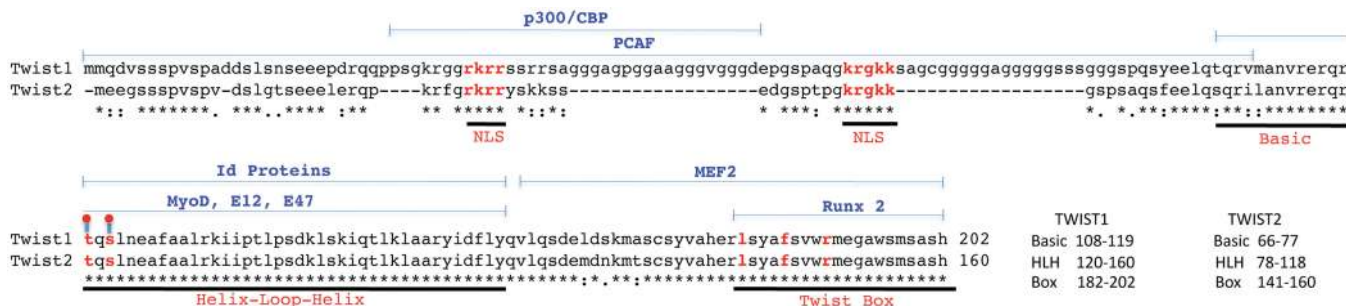


Figure 1. Amino acid sequence alignment between Twist1 and Twist2. The functional motifs are delineated with black bars. Similarity between the two proteins increases from 54% in the N-terminus to 95% in the bHLH region and 100% in the C-terminal Twist Box. Conserved threonine and serine phospho-regulated residues involved in dimer choice and DNA-binding site selection are indicated (27), and protein–protein interaction domains characterized for Twist1 are located above the alignment. It is interesting to note that the Twist box has been characterized as both an activation domain with the amino-acid motif LX3FX3R (indicated in red) (8) and as a repressor domain (25).

the importance of studying Twist1 and Twist2 as separate entities (24).

Twist2 is 66% identical to Twist1 and identity increases to 98% in the basic and HLH regions of the proteins (Figure 1). The major differences between both proteins are found in the N-terminal region, where Twist1 has two glycine-rich tracks that are absent in Twist2, making Twist1 a bigger protein than Twist2 by having 202 amino acids versus 160 amino acids respectively. The glycine-rich motifs found in Twist1 may be used to interact with proteins that are not bound by Twist2 leading to differences in protein function (Figure 1). The last 20 amino acids at the C-terminus contain a repressor domain termed 'Twist box' which is identical in both Twist1 and Twist2 and not found in other Twist subfamily members (25). A transactivation domain has also been characterized within the Twist box, containing amino-acid residues Leu-187, Phe-191 and Arg-195 (LX₃FX₃R) in Twist1, which are completely conserved throughout the animal kingdom (8). At the gene level, *Twist1* and *Twist2* share a common intron/exon organization that likely reflects an evolutionary gene duplication event (14,24).

This review will first focus on the factors that influence regulatory outcomes of Twist1 and Twist2, and the various mechanisms described for controlling gene expression. In addition, specific developmental programs regulated by Twist1 and Twist2, such as myogenesis and osteogenesis will help illustrate the diverse mechanisms of action. Finally, the role of Twist1 and Twist2 in inflammatory disorders and cancer will be discussed.

GENERAL MODES OF TRANSCRIPTIONAL ACTIVATION AND REPRESSION

There are several factors that influence the function of Twist1 and Twist2. These include dimer choice, phosphoregulation, protein-protein interactions and spatial-temporal expression. Dimer choice is mainly influenced by the availability of other bHLH proteins within the cell and the phosphorylation state of these proteins (26–28). It is known that Class B bHLH factors can form functional homodimers as well as heterodimers with Class A bHLH factors including E12/E47. However, heterodimer formation among class B bHLH proteins has also been reported as in the case with Twist1 and Hand2 dimer formation (Figure 2D) (6,29). Id proteins have great affinity for E12 and compete against class B bHLH proteins for E12 binding (26,28,30). Therefore, Id proteins actively alter dimerization pools by binding and sequestering bHLH factors, without themselves being able to bind DNA. The composition of these partner pools will ultimately determine the expression profile and therefore the cellular response. Hence, the relative stoichiometry of these factors influence whether Twist1 or Twist2 form heterodimers with E12, or homodimer formation is favored due to Id-mediated E12 sequestration.

Partner choice can also be regulated by the phosphorylation state of specific threonine and serine residues that are conserved in the first α -helix of the HLH domain of Twist proteins (Figure 1). Threonine 125 and serine 127 of

Twist1 are conserved from DTwist throughout the Twist subfamily of proteins (6). The phosphorylation state is determined by protein kinase A and C, and protein phosphatase 2A (29). Interestingly, mutations of these phosphoregulated residues not only alter partner choice but additionally alter E-box-binding affinity (Figure 2D) (31). Recently, PKB/AKT has also been linked to the phosphorylation of Twist1 at serine 42 (32). Hence, the difference in functions of Twist homodimers and Twist1/E-protein heterodimers could be in part explained by changes in E-box affinity due to phosphorylation state. The ubiquitous E-proteins can also be phosphoregulated, contributing to an additional level of regulation of these proteins. For example, phosphorylation of E47 is required for heterodimerization with the myogenic bHLH protein MyoD to occur (33). However, the phosphoregulated residue on E47 is not found in the first helix like in Twist1 and Twist2 but rather on serine 140, which is found in the N-terminus. Nonetheless, the phosphorylation status of these proteins contributes to the dynamics of bHLH protein function.

Many of the target genes of mammalian Twist1 and Twist2 have multiple E-boxes in their promoter regions. Therefore dimer choice could influence E-box selection, which, with combinatorial binding of other bHLH transcription factors, will ultimately control transcriptional outcomes. Direct mechanisms of gene activation by Twist1 and Twist2 usually require cis-binding to E-boxes found in the regulatory regions of target genes. Once bound, these factors provide a surface that can recruit other co-activators to form an open chromatin conformation for the assembly of the transcriptional machinery. The C-terminal domain described for Twist1 is required for gene activation (Figure 1). By fusing this transactivation domain to another DNA-binding protein, it was possible to demonstrate that the C-terminal domain of Twist1 was sufficient and necessary to activate transcription (8). Therefore, C-terminal domain mutants could result in dominant negative mutants by sequestering E-proteins or by non-functional binding to E-boxes. Indirect mechanisms of transcriptional activation usually involve sequestration of repressors or other bHLH proteins poised for inhibition, activation of another transcription factor, or through cross-talk with other gene regulators through protein/protein interactions (12,34).

The mechanisms of repression for Twist1 and Twist2 include E-protein titration (mimicking Id protein sequestration), blocking the DNA binding and/or the action of other transcription activators, binding E-boxes as heterodimers with E-proteins, and recruitment of histone deacetylases (HDAC) (Figure 2). Dimerization of Twist with E-proteins to form inhibitory complexes usually occurs through the HLH domain, however, the N-terminal domain as well as the C-terminal domain are known to bind and block the transactivation activity of other transcription factors, or to form multi-protein repressor complexes (22,25,35).

An attractive paradigm has emerged in the literature in which repression and activation by these proteins depends on dimer choice, and that Twist homodimers typically activate transcription while heterodimers function

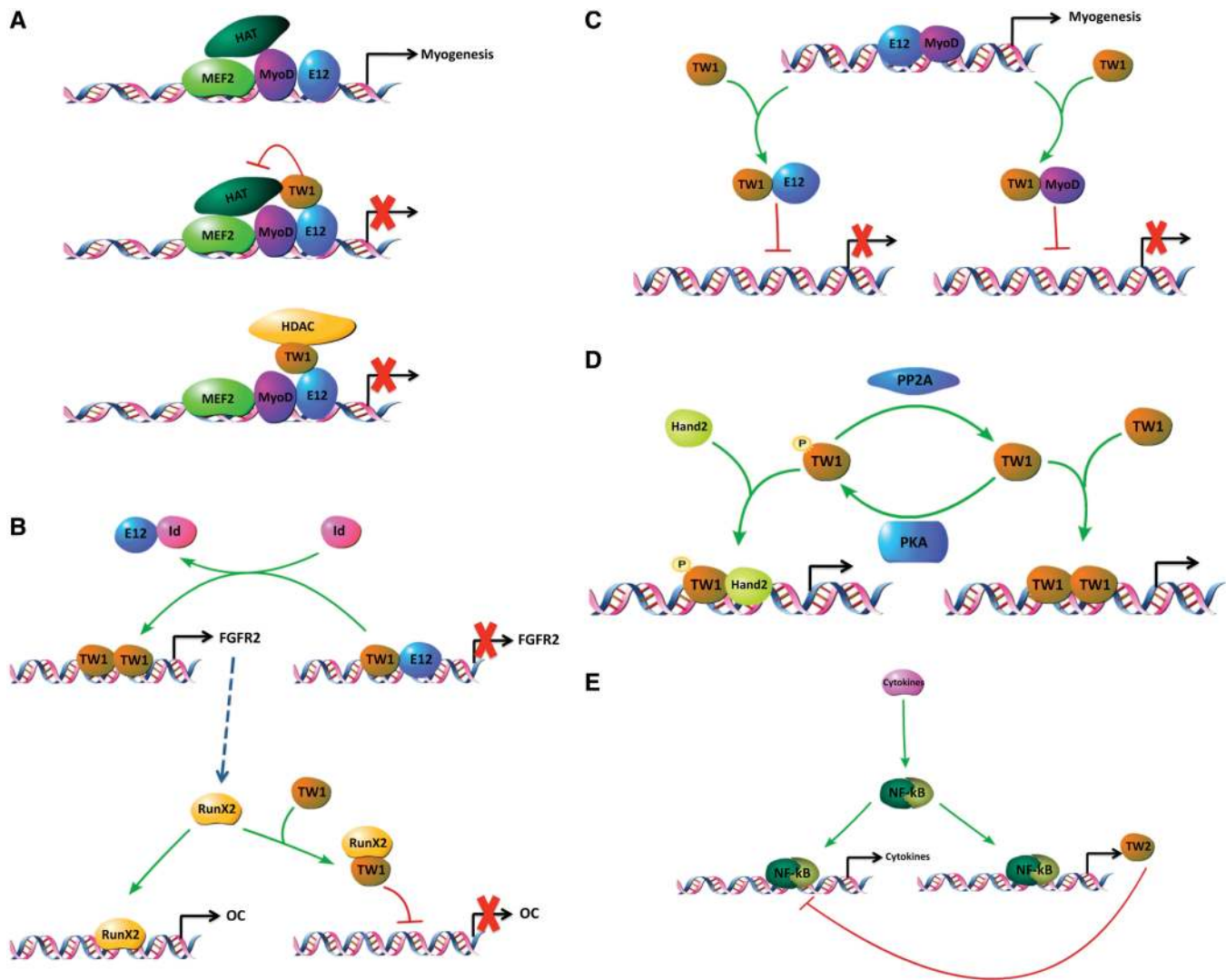


Figure 2. Modes of transcriptional regulation by Twist1 and Twist2. The Twist1 (TW1) and Twist2 (TW2) bHLH transcription factors exhibit a bifunctional role by acting as activators and repressors depending on post-translational modifications, partner choice and cellular context (6,7,28). (A) The mode of action employed by Twist1 and Twist2 was first understood in genes involved in muscle development. The myogenic bHLH factors such as MyoD bind as heterodimers with E-proteins to E-boxes found in regulatory regions of muscle-specific genes. MyoD-mediated activation typically requires the binding of MEF2 and involves the recruitment of HATs. Twist acts as a repressor by promoting deacetylation either by blocking HAT activity or recruiting HDACs (35,38). (B) The role of Twist during osteogenesis was highlighted when mutations of Twist1 were identified in SCS patients. Based on the SCS mouse model and studies in human osteoblasts, it was determined that Twist1 acted before Twist2 on osteoblast maturation (20,23). Twist1, as a heterodimer with E12, represses the *FGFR2* gene, and as Id protein levels rise, E12 gets sequestered leading to Twist1 homodimerization. As a homodimer, Twist1 activates *FGFR2* which mediates downstream activation of RunX2 (26,42). Aside of FGF signaling, both Twist1 and Twist2 can block the transactivation activity of the master regulator RunX2 (25). (C) Twist can repress genes independent of DNA binding. For example, Twist can sequester E12 or MyoD leading to the repression genes of the myogenic program. By doing so, Twist mimics the mechanism of inhibition employed by the Id proteins. (D) Twist partner choice can be determined by the phosphorylation state of these proteins leading to homodimerization, or heterodimerization with Class A and Class B bHLH factors. E-box selection can also be influenced by phosphoregulation. In this case, affinity for a class B bHLH factor, Hand2, is favored when Twist1 is phosphorylated by PKA (29,31). (E) The Twist2 KO mouse suggests a role of Twist2 in regulating cytokine gene expression (22). This work led to a proposed negative feedback loop in which inflammatory cytokines activate NF- κ B, and NF- κ B activates the expression of cytokines and both Twist1 and Twist2. In turn, Twist1 and Twist2 can block the transactivation activity of NF- κ B in a promoter-specific manner (45).

to repress transcription. This was observed during *Drosophila* development where D-Twist homodimers drove gene activation while D-Twist/Daughterless heterodimers were associated with gene repression (10). However, this paradigm has been challenged in mammalian systems where Twist1/E12 heterodimers were found to be responsible for both the activation and repression of

separate genes within the same cells (28). Interestingly, murine Twist1/E12 heterodimers were able to drive expression of a *Drosophila*-derived reporter construct that was activated by *Drosophila* Twist homodimers (8).

Although all of the regulatory mechanisms employed by Twist1 and Twist2 have yet to be elucidated, their functions have been studied in several well-characterized

model systems. The following sections will discuss how Twist1 and Twist2 function in these systems.

INHIBITION OF MUSCLE DIFFERENTIATION

The best understood example of inhibition by Twist1 and Twist2 is the repression of the muscle differentiation program, which is activated by the myogenic bHLH subfamily of proteins composed of MyoD, myogenin, Myf5 and MRF4. Muscle program inhibition integrates several repression mechanisms carried out by both Twist1 and Twist2 (Figure 2A and C). Activation of muscle-specific genes, like the muscle creatine kinase (*MCK*) gene promoter, occurs through heterodimers between myogenic bHLH proteins and E-proteins. Therefore, sequestration of E-proteins by increased Twist1 and/or Twist2 levels functions to inhibit muscle specific gene activation (Figure 2C) (35,36). Id proteins also compete for E protein binding with MyoD, Twist1 and Twist2, adding an additional level of regulation for dimer formation. This provides a specific example of how HLH protein levels at any given time can influence partner choice and therefore affect gene expression.

For promoters like *MCK*, the myocyte enhancer factor 2 (MEF2) is needed as a co-activator in addition to the myogenic bHLH proteins. There are two consensus MEF2 sites found in the *MCK* promoter, one of which is in close proximity to the *MCK* right E-box bound by MyoD/E12, allowing MEF2 and MyoD/E12 to act synergistically. Inhibition of MEF2 action by Twist1 and Twist2 is not mediated through E-protein titration, as is the case for MyoD inhibition, but rather through physical interactions that preclude the transactivation activity of MEF2 (35,36). It is currently not known whether MEF2/Twist interaction inhibits DNA binding by MEF2 or if it precludes the transactivation activity of MEF2 (35,36). Furthermore, these proteins may use different mechanisms as Twist1 requires the bHLH domain and the C-terminus for MEF2 inhibition (36), while Twist2 requires only the N-terminus and the C-terminus (35) which argues against redundancy in Twist protein function.

Direct interaction of Twist1 with MyoD occurs through the basic and the HLH domains of both proteins, and functions to inhibit MyoD transactivation (Figure 2D) (37). This mechanism of repression might be exclusive of Twist1 since Twist2 has not been shown to interact with MyoD in the same manner. Three arginine residues (R120, 122 and 124) found in the basic region of Twist1 are required for the interaction and inhibition of MyoD (37). However, mutation of these three arginine residues in the Twist2 basic region presented no effect on MyoD function indicating that only Twist1 binds MyoD (35).

Finally, chromatin modifications could be involved in MyoD-MEF2 inhibition. It is known that MyoD acts synergistically with the histone acetyltransferase (HAT) p300 to activate muscle-specific gene targets. Twist1 binds and inhibits the HAT domains of both p300 and PCAF (38) (Figure 2A). Physical interactions between Twist1 and p300/PCAF are mediated through the N-terminal region of Twist1, where the glycine rich

tracks are present (Figure 1) (38). The absence of the glycine rich tracks in Twist2 might explain its inability to inhibit HAT activity (39). The finding that Twist1 can block HAT activity suggests that it might regulate gene expression by modulating histone acetylation states and thus altering chromatin conformation.

Although Twist2 does not inhibit HATs, it can however alter chromatin conformational states by the recruitment of histone deacetylases (HDAC). For example, Twist2 requires HDAC for MyoD-MEF2 inhibition since Twist2-dependent repression can be relieved by treatment with HDAC inhibitors (35). It is now known that both Twist1 and Twist2 can interact with HDAC's to exert transcriptional repression (40,41).

REGULATION OF THE OSTEOLASTIC PROGRAM

Identification of Twist1 mutations associated with Saethre-Chotzen syndrome (SCS) and the phenotype presented by the Twist1 null heterozygous mouse has generated interest in the role of Twist1 during osteoblast differentiation (17). The study of the Twist1 null heterozygous mouse, as a model for SCS, provides an example of how bHLH partner choice influences gene regulatory outcomes. Both SCS patients and the Twist1^{+/-} mouse have low Twist1 levels, thus altering the available bHLH partner pool and contributing to the molecular pathology of SCS. The haploinsufficiency of Twist1 decreases the ability of Twist1 to compete with Id proteins for E12 dimerization, resulting in higher levels of Twist1 homodimers within the calvarial sutures (28). In this diseased state, higher levels of Id proteins in comparison to Twist1 favors the formation of Id/E12 heterodimers. Therefore, sequestration of available E12 by Id precludes the formation of Twist1/E12 heterodimers that favors Twist1 homodimerization (Figure 2B). The regulation of fibroblast growth factor receptors (Fgfrs) constitutes an important pathway in controlling cranial bone formation (42). The higher ratio of Twist1 homodimers versus heterodimers in Twist1^{+/-} mice causes abnormal activation of the *fibroblast growth factor receptor-2* (*Fgfr2*) gene (26), resulting in expansion of the osteogenic fronts and suture closure. Thus, the modulation of E-protein and/or Id protein levels in Twist1^{+/-} mice can restore the normal balance between homo and heterodimers and prevents craniosynostosis (26). These findings challenge the classical view that Twist1 merely prevents osteoblast differentiation, by suggesting that Twist1/E12 heterodimers maintain the cells in a preosteoblast state through the inhibition of Fgfr2, and as differentiation continues, a switch to increased levels of Twist1 homodimers promotes osteoblast maturation through the activation of Fgfr2 (26).

In addition to craniosynostosis, SCS patients and Twist1 haploinsufficient mice present limb abnormalities. As mentioned above, Twist1 and Hand2 can form heterodimers between themselves, and their expression patterns overlap in the developing limb (6). It was found that overexpression of Hand2 could phenocopy the limb abnormalities observed in Twist1 haploinsufficiency

suggesting a possible genetic interaction between Twist1 and Hand2 (6). The intercross of Twist1^{+/-} and Hand2^{+/-} mice resulted in normal limb patterning thus rescuing the phenotype by rebalancing the dimer pool (6). Gene dosage and phosphoregulation have been found to influence the interaction between Twist1 and Hand2. The phosphorylated form of Twist1 has higher affinity for Hand2 while the dephosphorylated form of Twist1 favors homodimerization (Figure 2D) (6). Interestingly, some mutations that cause SCS disrupt protein kinase A (PKA) recognition sites on Twist1 (6), thus altering its phosphorylation state and changing Twist1 partner choice.

Twist2 is also known to regulate osteoblast differentiation, however its involvement occurs temporally after Twist1 (23). There are no known mutations in Twist2 associated to SCS-like phenotypes, hence the Twist2 appears to have no role in cranial bone development, which is consistent with its lack of expression in developing skull (21).

The transcription factor Runx2 is considered a master regulator of the osteogenic program due to its indispensable role in the regulation of most of the genes that give rise to the mature osteoblast phenotype (25). It has been demonstrated that Twist1 and Twist2 are involved in the modulation of Runx2 function at the transcriptional level as well as at the protein level (25,34,42–44). Twist1 is proposed to be a transcriptional activator of the *RUNX2* gene when immortalized human calvarial osteoblasts from SCS patients were shown to have reduced levels of Runx2 mRNA and protein (43). EMSA analysis using nuclear extracts from SCS patient calvarial osteoblasts resulted in reduced DNA binding to a *cis* element found in the promoter of the Runx2 target gene *osteocalcin* (OC) suggesting decreased expression of Runx2 in SCS patients (43). Other groups have used chromatin immunoprecipitation (ChIP) assays to demonstrate that both Twist1 and Twist2 can bind to the Runx2 promoter, although this binding was not correlated to changes in Runx2 expression (34). The mechanisms by which Twist haploinsufficiency alters osteoblast differentiation has therefore been attributed to indirect regulation of Runx2 through the action of Fgfr2 (Figure 2B) (42). Twist1 directly binds to the promoter of Fgfr2 and overexpression of Twist1 restores expression of Fgfr2, Runx2 and Runx2-regulated genes in Twist1 mutant osteoblasts (42). Concurrently, overexpression of Fgfr2 in Twist1 mutant calvarial osteoblasts rescued expression of Runx2. In this model of indirect activation of Runx2 expression through Fgfr2, it was also concluded that Runx2 exhibits a positive feedback relationship with Fgfr2 (42). As stated above, formation of Twist1 homodimers results in different outcomes when compared to heterodimers with E12. Therefore it is critical to consider the stoichiometric relationship of all of the available dimer partners within the cell under study. To this extent, Twist1 partner choice might determine Runx2 action since Fgfr2 is activated by Twist1 homodimers and inhibited by Twist1 heterodimers (Figure 2B) (26).

Both Twist1 and Twist2 can also regulate Runx2 at the protein level by physically interacting with Runx2 and

inhibiting its ability to bind DNA (Figure 2B) (25). It was found that the antiosteogenic function of Twist1/2 is mediated through the C-terminal Twist box (Figure 1). This region was found critical for Runx2 interaction since mutation of Serine 192 to Proline within the Twist box of Twist1 resulted in poor interaction with Runx2. The mice harboring this mutation were termed Charlie Chaplin (CC/+) and displayed a craniosynostosis phenotype with limb polydactyly highlighting the importance of the Twist box for dictating proper bone formation. The same study showed that expression of Runx2 was unaffected in the *Twist1*-null heterozygous mouse and attributed premature ossification of the calvarial sutures to the inability of Twist1 to inhibit Runx2 transactivation (25). Twist1 heterozygosity reverses skull abnormalities in Runx2^{+/-} mice while Twist2 heterozygosity reverses clavicular abnormalities, indicating different genetic interactions between both Twist1/2 and Runx2 (25).

ROLE IN IMMUNE SYSTEM DEVELOPMENT AND RESPONSE

One of the most significant phenotypes of the Twist2 KO mouse is the perinatal death induced by high levels of pro-inflammatory cytokines. As mentioned above, Twist2 KO mice exhibit normal development until birth but die 2–3 days after. Failure to thrive in these mice is caused by cachexia that is manifested by severe wasting of body tissue, loss of appetite and anemia (22). It has been demonstrated that Twist1 and Twist2 modulate the expression of proinflammatory cytokines through inhibition of NF-κB mediated transactivation (Figure 2E). Immunoprecipitation assays were used to demonstrate that Twist1 and Twist2 physically interact with NF-κB suggesting a possible mechanism for this repression. The activation of Twist1/2 by NF-κB and their physical interaction is evolutionarily conserved. Based on this data, a negative feedback loop has been proposed where cytokines activate NF-κB and downstream activation of Twist1/2 results in repression of NF-κB transactivation (Figure 2E) (45). Twist1 and Twist2 are also known to bind to E boxes in the promoters of cytokines regulated by NF-κB such as TNFα and Il-1β (22). Further studies have shown that Twist2 exhibits bifunctionality in the regulation of cytokines by inhibiting the production of pro-inflammatory cytokines such as interleukin-12 (IL-12) and interferon-γ (IFNγ) while promoting the anti-inflammatory cytokine IL-10 (7).

One of the major characteristics of cachexia is severe wasting of body tissue that can be attributed to deregulation of energy homeostasis. Interestingly, Twist1 and Twist2 have been characterized as critical regulators of energy homeostasis involving adipose tissue (46,47). It has been demonstrated that Twist2 acts as a physical inhibitor of the transcription factor ADD1/SREBP1c involved in adipocyte differentiation as well as regulation of the LDL receptor gene, fatty acid and sterol biosynthesis genes (48). Also, local chronic inflammation has been linked to insulin resistance in adipose tissue (49), therefore, Twist1 and Twist2 involvement in proinflammatory cytokine regulation and inhibition of ADD1/SREBP1c by

Twist2 could contribute to the cachexic phenotype seen in the Twist2 KO mouse.

Twist1 and Twist2 are also key regulators of the development of immune cells (7,41,50,51). For example, Twist2 is a key negative regulator of myeloid lineage development by inhibiting the proliferation and differentiation of macrophage progenitors (7). Twist2 repression of macrophage proliferation is mediated by direct interaction and inhibition of the transcription factors Runx1 and C/EBP α (7). In chronic inflammatory diseases, the proliferation of human B cells and their differentiation into immunoglobulin-secreting cells is influenced by IL-17 and the B cell-activating factor (BAFF) (51). Twist1 was identified as the central mediator of the effects of IL-17 and BAFF by protecting B-cells from apoptosis through the induction of the anti-apoptotic genes Twist2 and Bcl-1 (51). It is important to note that after Twist2 induction, Twist1 levels decrease while Twist2 levels are maintained, therefore the sequential induction of Twist1 followed by Twist2 and Bcl-1 suggest that Twist2 and Bcl-1 are the primary antiapoptotic effectors in B-cell response (51). Interestingly, Twist2 was found to be overexpressed in B cells from patients with systemic lupus erythematosus compared to its expression in B cells from healthy volunteers (51). The sequential induction and maintenance of Twist2 levels after Twist1 in immune system proliferation and response suggests that Twist2 plays a more critical role in the direct regulation of inflammatory cytokines than Twist1. This may account for the immune dysfunction observed specifically in Twist2 KO mice.

ROLE IN CANCER PROGRESSION AND METASTASIS

The role of Twist proteins during embryonic development is exploited in disease by tumor cells. Twist1 is known to play a role in the migration of neural crest cells during embryogenesis (52). However, this ability to promote migration by Twist1 is known to facilitate epithelial-to-mesenchymal transitions (EMT) (53). This has been attributed to down regulation of epithelial markers such as E-cadherin, and up regulation of mesenchymal markers such as N-cadherin. In addition, the cell adhesion protein periostin (POSTN), also involved in cancer metastasis, is directly activated by Twist1 (54). Twist1/2 also function as proto-oncogenes by protecting cells from Myc- and p53-dependent apoptosis (55). Repression of p53 transactivation is thought to be mediated by Twist1 inhibition of p300/PCAF (38,55).

Direct regulation of genes involved in cancer progression has also been observed. AKT2 is a modulator of Y-box-binding protein 1 (YB-1) function, and both are central to tumor proliferation (56). Twist1 can up-regulate the proto-oncogene AKT2 by directly binding to its promoter (57). Twist1 activation of the YB-1 transcription factor, involved in cellular proliferation, has also been observed (56). Additionally, the cyclin-dependent kinase inhibitor p21, involved in growth arrest, is directly

regulated by both Twist1 and Twist2 in the presence of E12 (58,59).

Recent evidence has shown that Twist1 can directly activate miR-199a/214 and miR-10b micro RNAs by binding to their respective promoters (60,61). Activation of these micro RNAs by Twist1 leads to repression of target genes implicated in development and cancer, such as inhibition of translation of the homeobox D10 mRNA by miR-10b, resulting in increased expression of the pro-metastatic gene *RHOC* (61). The ability of Twist1/2 to inhibit apoptosis and facilitate EMT has been correlated with poor outcomes in cancer patients having elevated Twist1/2 expression (62).

A general paradigm of cancer progression is that cellular senescence and EMT are separate processes and as such, are regulated independently (63). Twist1 and Twist2 are frequently activated in human cancers and have also been shown to override oncogene-induced senescence by inhibiting p53 and Rb tumor suppressor pathways while at the same time promoting EMT (64). These findings challenge the classical view by showing that overexpression of Twist1 and Twist2 in neoplastic lesions allows for simultaneous regulation of cellular senescence and EMT (63,64).

CONCLUDING REMARKS

The complex regulatory pathways orchestrated by both Twist1 and Twist2 are testament to the importance of these two gene regulatory proteins. Modes of action by these proteins include binding to conserved E-box sequences, recruitment of coactivators or repressors, dimer pool modulation and inhibition of activator/repressor function through protein-protein interactions. Regulatory outcomes of Twist1 and Twist2 are themselves controlled by spatial-temporal expression, phosphorylation and dimer choice. Deregulation of genes that are targeted by these transcription factors, such as those involved in mesenchymal cell lineage differentiation, results in a wide array of human diseases such as Saethre Chotzen syndrome, Setleis syndrome, chronic inflammatory diseases and cancer. However, due to the paucity of information available that directly compares the mechanisms of gene regulation by Twist1 versus Twist2, the full extent to which these proteins diverge in function remains to be seen. We know that these proteins are not entirely redundant based on the contrasting phenotypes observed in knockout mice and the fact that dominant mutations in *Twist1* result in craniosynostosis while recessive mutations in *Twist2* result in facial focal dermal dysplasia with no apparent bone abnormalities (15,24). However, we cannot rule out the possibility that compensatory functionality between these two proteins may exist as part of a biological safeguard to ensure accurate regulation of certain developmental and mature tissue programs.

In order to further examine the redundancy between Twist1 and Twist2, gene replacement strategies can be used to test the extent to which these factors may be interchangeable during embryogenesis. This approach has been used with two other members of the Twist subfamily of

proteins, namely Hand1 and Hand2, to show that they indeed act via unique transcriptional mechanisms (65). Dissection of bHLH dimer pool dynamics is expected to be challenging, as experimental manipulations such as knockdown and overexpression may inadvertently alter the balance of these factors. Additionally, overlapping expression patterns of the Twist subfamily members could result in heterodimerization between themselves as seen in the case of Twist1 and Hand2 (6). The potential for dimerization between Twist1 and Twist2 further compounds the complexity of bHLH dimer partners. Therefore, genetic engineering techniques that ensure physiological expression levels of these proteins and mutant versions would aid in the elucidation of true modes of action. These techniques could also be used for the expression of Twist1- and/or Twist2-tethered dimers to help illustrate how partner choice can dictate transcriptional outcomes.

The spatial-temporal expression of Twist1 and Twist2 is critical to the composition of the bHLH dimer pool within the cell. Therefore, the signals that dictate the expression patterns of Twist1/2 must be better understood. There are several pathways that control their expression, which include NF- κ B and STAT3 mediated cytokine signaling (45,66), the WNT/ β -catenin (67) and TGF β pathways (68), and induction through hypoxia-inducible factor 1 α (69). However the specificity by which these pathways activate these proteins is not well understood.

Except in those cases where Twist1 and 2 mechanisms have been characterized within single gene contexts, there are no comprehensive genomic expression or chromatin binding profiles directly comparing these two proteins. The use of ChIP–Chip and ChIP–Seq technologies would allow the generation of comprehensive transcription factor binding maps across the entire genome that would facilitate the comparison of binding specificities between Twist1 and Twist2 (70). Comprehensive analysis of the binding locations, chromatin structural features and post-translational modifications that influence the binding of Twist1 versus Twist2 can also provide insight into the mechanisms by which each factor regulates transcription.

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