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Characterization of a dominant negative *C. elegans* Twist mutant protein with implications for human Saethre-Chotzen syndrome

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SUMMARY

Twist is a transcription factor that is required for mesodermal cell fates in all animals studied to date. Mutations of this locus in humans have been identified as the cause of the craniofacial disorder Saethre-Chotzen syndrome. The *Caenorhabditis elegans* Twist homolog is required for the development of a subset of the mesoderm. A semidominant allele of the gene that codes for CeTwist, *hlh-8*, has defects that occur earlier in the mesodermal lineage than a previously studied null allele of the gene. The semidominant allele has a charge change (E29K) in the basic DNA-binding domain of CeTwist. Surprisingly, the mutant protein retains DNA-binding activity as both a homodimer and a heterodimer with its partner E/Daughterless (CeE/DA). However, the mutant protein blocks the activation of the promoter of a target gene. Therefore, the mutant CeTwist may cause cellular defects as a dominant negative protein by binding to target promoters as a homo- or heterodimer and then blocking transcription. Similar phenotypes as those caused by the E29K mutation were observed when amino acid substitutions in the DNA-binding domain that are associated with the human Saethre-Chotzen syndrome were engineered into the *C. elegans* protein. These data suggest that Saethre-Chotzen syndrome may be caused, in some cases, by dominant negative proteins, rather than by haploinsufficiency of the locus.

Key words: CeTwist, *C. elegans*, Mesoderm, *hlh-8*, Saethre-Chotzen syndrome, Sex myoblasts, bHLH

INTRODUCTION

Understanding how basic helix-loop-helix (bHLH) transcription factors cause changes in gene expression is important for revealing how transcriptional cascades function in development. Several bHLH transcription factors such as MyoD and NeuroD have been shown to play important roles in promoting myogenesis and neurogenesis, respectively (for a review, see Massari and Murre, 2000). These transcription factors work as homo- or heterodimers through contacts made in the helix-loop-helix (HLH) domain. The dimers then bind promoter DNA at canonical sequences called E boxes by virtue of the now close proximity of their individual basic DNA-binding domains (Murre et al., 1989). In general, bHLH heterodimers are composed of one member with tissue-specific expression and another member with more broad expression throughout the organism (Lassar et al., 1991). One tissue-specific bHLH family member, Twist, plays a role in the development of the mesoderm in all organisms examined including humans, mice, *C. elegans* and *Drosophila melanogaster* (where the gene was first discovered) (Simpson, 1983; Nüsslein-Volhard, 1984; Chen and Behringer, 1995; Wang et al., 1997; Harfe et al., 1998b; Corsi et al., 2000). In several organisms, the broadly expressed bHLH family members, E (in vertebrates) and Daughterless (in invertebrates), are thought to heterodimerize with Twist (Spicer et al., 1996; El Ghouzzi et al., 2000; Castanon et al., 2001). In *C. elegans*, the E/Daughterless (DA) homolog is called CeE/DA and, although embryonic expression is ubiquitous, the protein has a restricted expression pattern postembryonically that includes mesodermal tissue expressing CeTwist (Krause et al., 1997).

We are interested in elucidating aspects of mesodermal development and have been studying the function of Twist in the model organism *C. elegans.* Of the 35 conventional bHLHs identified in the *C. elegans* genome, a single Twist homolog exists (Ruvkun and Hobert, 1998; Ledent and Vervoort, 2001). Initially, we characterized a putative null allele of the CeTwist gene, *hlh-8* and found several defects in the mesoderm of the mutant animals (Corsi et al., 2000).

The *C. elegans* mesoderm is predominantly composed of body wall muscles used for movement, macrophage-like

coelomocytes, vulval and uterine muscles used for egg laying, enteric muscles used for defecation, pharyngeal muscles used for eating, and somatic gonad used for reproduction (Waterston, 1988; Moerman and Fire, 1997). CeTwist is required for the formation of three out of 126 mesodermal cells that differentiate in the embryo (notably, striated body wall muscles were formed in the absence of CeTwist) (Corsi et al., 2000). The three cells that require CeTwist for proper differentiation in the embryo are the non-striated enteric muscles: two intestinal muscles and the anal depressor (Corsi et al., 2000). In postembryonic development, CeTwist is important for patterning of the mesoderm lineage and is necessary for the proper formation of the vulval and uterine muscles (Corsi et al., 2000).

In humans, mutations in the *TWIST* gene are associated with an autosomal dominant developmental syndrome called Saethre-Chotzen syndrome (for a review, see Wilkie, 1997). The incidence of this disease is 1/25,000-1/50,000 live births (Paznekas et al., 1998). Individuals with this syndrome have craniofacial and digit defects, including drooping eyelids, short fingers, the presence of soft webbing between the fingers and premature fusion of the cranial sutures (causing an abnormally shaped head) (Reardon and Winter, 1994; Cohen, 2000). Greater than 40 unique mutations in the human *TWIST* gene have been identified, with the majority being stop codons or mis-sense mutations in the highly-conserved bHLH domains (reviewed by Gripp et al., 2000). Most of these mutations are considered to be haploinsufficient. The best evidence for haploinsufficiency is that humans with a heterozygous deletion of the *TWIST* locus exhibit Saethre-Chotzen phenotypes (Johnson et al., 1998).

We have been studying an interesting allele in *C. elegans hlh-8* called *n2170* that may have parallels to the human Saethre-Chotzen disease. This allele behaves as a semidominant mutation. Surprisingly, although we found that the mutation was an amino acid substitution in the basic DNA-binding domain, the mutant CeTwist protein still bound DNA in vitro but could not activate transcription in vivo. Overexpression of the mutant CeTwist protein caused a phenotype similar to the *n2170* allele, suggesting the mutant could act as a dominant-negative protein. Cellular characterization of the mesodermal defects in mutant *hlh-8 (n2170)* animals provided an assay to test for dominant-negative function of several basic domain mutations in the human *TWIST* gene of some individuals with Saethre-Chotzen syndrome.

MATERIALS AND METHODS

Sequencing of the *hlh-8* locus in *n2170sd* animals

We determined the location of the point mutation in the *hlh-8* locus by amplifying the 2 kb genomic DNA of the *hlh-8* locus in two fragments using single animal PCR (Williams, 1995) and sequencing the five exons and splice junctions (Corsi et al., 2000). The G to A transition at base pair 85 was found in four independent animals and the remainder of the *hlh-8* locus was wild-type in the *n2170sd* animals.

Reporter strain constructions

We used standard genetic methods to introduce a set of characterized, integrated *gfp* fusion transgenes into *n2170sd* and *nr2061 (–)* genetic backgrounds (Corsi et al., 2000). Analysis was carried out with animals that were homozygous for both the *gfp* transgene and *hlh-8* mutations. *gfp* reporter strains used were the following: *hlh-8::gfp ayIs7(IV)* (Harfe et al., 1998b), *egl-15::gfp ayIs2(IV)* (Harfe et al., 1998a), from the *ceh-24* promoter *NdEbox::gfp ccIs4656(IV)* (Harfe and Fire, 1998), *arg-1::gfp ccIs4443* (A. Melendez, I. Greenwald, S. Kostas, J. Liu and A. Fire, personal communication), *rgs-2::gfp LX354* (Dong et al., 2000), *intrinsic cc:: gfp* (J. Fares and S. Kostas, personal communication) and *myo-3::gfp ccIs4251(I)* (Fire et al., 1998). We confirmed that the *egl-15::gfp* and *NdEbox::gfp* reporters were present and homozygous, but not expressed, in *n2170sd* strains by outcrossing the strains to wild-type (N2) males and observing that all of the cross progeny expressed *gfp*. For *egl-15::gfp*, we cloned the heterozygous animals from the N2 cross and observed whether each plate had wild-type animals expressing the *gfp* to determine whether the parent was homozygous for *egl-15::gfp*. Rescue experiments of *n2170sd* animals were performed as described elsewhere (Corsi et al., 2000).

Constructing plasmids containing *hlh-8* basic domain mutations for gel shift assays

An expression plasmid (pRSETA with CeTwist cDNA) (Harfe et al., 1998b) was used to construct new plasmids containing the *n2170sd* point mutation or missing the basic domain of CeTwist using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) and appropriate primers (sequences available upon request). cDNA inserts from plasmids containing mutations identified in individuals with Saethre-Chotzen syndrome (described in the next section) were PCR-amplified to create *Bam*HI/*Eco*RI fragments. The PCR fragments were then cloned into pRSETA and the inserts were sequenced. The mutant proteins along with wild-type CeTwist and CeE/DA were expressed in *E. coli* and purified under denaturing conditions using Ni^{2+} affinity column chromatography according to Harfe et al. (Harfe et al., 1998b). Gel shift assays using a CAGGTG E box-based oligo were performed as previously described (Krause et al., 1997; Harfe et al., 1998b).

Assaying dominant mutant CeTwist function

Plasmids were constructed that would express wild-type and CeTwist mutant proteins in the M lineage in order to evaluate their ability to cause a phenotype in the presence of wild-type chromosomal *hlh-8*. Wild-type and *n2170sd* cDNA from the pRSETA vectors described above were PCR-amplified to create *Xba*I/*Eco*RI fragments. After restriction digest, these fragments were moved into the plasmid pBH56.55 (gift from B. Harfe and A. Fire, Carnegie Institution of Washington). The plasmid pBH56.55 was made by adding ~500 bp upstream of the *hlh-8* coding region into the *gfp* expression vector pPD95.69 (A. Fire, G. Seydoux, J. Ahnn and S. Q. Xu, personal communication). The cDNA fragments replaced the *gfp* in the vector. The final wild-type plasmid was expressed in the M lineage based on the criteria that the plasmid rescued the egg-laying defective phenotype of *nr2061 (–)* animals. The amino acid residues analogous to human *TWIST* mutations shown in Fig. 1 were also created in this same plasmid backbone by using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. All cDNA inserts that were subjected to PCR were sequenced to detect any PCR-induced errors. All of these constructs will potentially express full-length CeTwist with two additional amino acids (a Ser and Arg directly after the initiator methionine). The mutant and wild-type plasmids were injected using standard techniques (Mello and Fire, 1995) at 50 μg/ml into animals that already had integrated *hlh-8::gfp* (Harfe et al., 1998b). The *hlh-8::gfp* contains only *hlh-8* promoter sequences and is not a rescuing construct. The dominant marker *rol-6* (pRF4) (Mello et al., 1991) was used to follow the presence of injected DNA.

GFP reporter activation by the *n2170sd* CeTwist protein

In order to observe the ability of the mutant CeTwist E29K protein to

activate various promoters, we constructed strains containing the *n2170sd* mutation and either *egl-15::gfp* (Harfe et al., 1998a), *rgs-2::gfp* (Dong et al., 2000), *NdEbox::gfp* (Harfe et al., 1998b) or *arg-1::gfp* (Mello et al., 1994) (A. Melendez, I. Greenwald, S. Kostas, J. Liu and A. Fire, personal communication). These reporter lines were observed for *gfp* expression in the mutant animals. In the case of strains containing *egl-15::gfp* and *NdEbox::gfp*, no expression was seen in *n2170sd* animals. Expression was restored in transgenic lines (two lines for each reporter) with extrachromosomal arrays containing genomic *hlh-8* DNA.

The ability of the mutant CeTwist E29K protein to activate *egl-15::gfp* in conjunction with CeE/DA was assayed by expressing the proteins singly or in combination from cDNAs using a heat shock promoter from the vector pPD49.83 (Mello and Fire, 1995). Embryos and larvae from stable lines were subjected to a 33°C heat shock for 4 hours followed by a 2-4 hour room temperature recovery period. Ability to activate the reporter was assayed by observing the level of *gfp* expressed using a GFP dissecting scope to scan a population of animals. The embryos were then processed for antibody staining with polyclonal anti-CeTwist and anti-CeE/DA as previously described (Krause et al., 1990; Krause et al., 1997). The same methods were used to stain *n2170sd* animals with anti-CeTwist in Fig. 3. The antiserum was raised against a peptide of the last 14 amino acids of CeTwist and was affinity purified.

RESULTS

Genetic characterization of an *hlh-8* semidominant allele

We have been taking a genetic approach to understand CeTwist function in *C. elegans* development. Previously, we showed that animals with a deletion mutation in *hlh-8*, called *nr2061*, that removes the HLH domain of CeTwist are viable and fertile (Corsi et al., 2000). The *nr2061* mutant animals lack nonstriated muscle function required for egg-laying and for defecation and are, therefore, egg-laying defective and constipated (Corsi et al., 2000). Because the *nr2061* animals are most probably null for *hlh-8* function, we were interested in finding non-null alleles that might provide further insight into CeTwist function. A potential allele, *n2170*, has been isolated by G. Garriga and H. R. Horvitz (personal communication) in a screen for egg-laying defective animals. The *n2170* animals are also constipated. We have characterized this allele in detail.

n2170 and *nr2061* homozygous animals appeared identical under the dissecting microscope: they were visibly constipated with an expanded intestine and failed to lay eggs. Both strains were fertile with embryos developing inside the hermaphrodite uterus causing a 'bag-of-worms' phenotype. The two alleles failed to complement. Trans-heterozygous animals (*n2170*/*nr2061*) were constipated and egg-laying defective (data not shown). Heterozygous *nr2061*/+ animals were wild type, indicating that *nr2061* is recessive (Corsi et al., 2000). By contrast, *n2170*/+ heterozygous animals were egg-laying defective; they could lay their eggs but not at a wild-type rate and hermaphrodites eventually bloated with unlaid embryos. However, *n2170*/+ heterozygotes were not visibly constipated, thus we considered the allele semidominant rather than dominant. For the remainder of this paper, *hlh-8 (n2170)* will be referred to as *n2170sd* and *hlh-8 (nr2061)* as *nr2061(–)*.

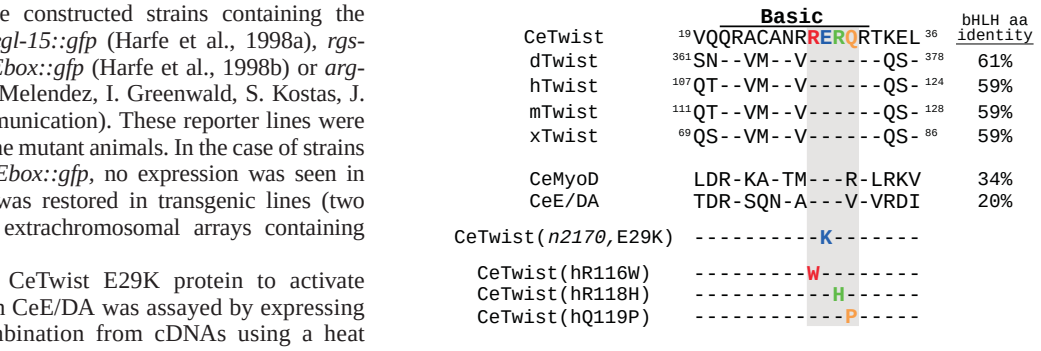

Fig. 1. Comparison of the basic domain amino acid sequence of CeTwist and several other bHLH homologs. The amino acids from the basic region and surrounding sequence are shown. Sequences were obtained from the following references: CeTwist, *C. elegans* (Harfe et al., 1998b); dTwist, *D. melanogaster* (Thisse et al., 1988); hTwist, *H. sapiens* (Wang et al., 1997); mTwist, *M. musculus* (Wolf et al., 1991); xTwist, *X. laevis* (Hopwood et al., 1989); CeMyoD (Krause et al., 1990); CeE/DA (Krause et al., 1997). Percent values of bHLH identity include the comparison of amino acids across the basic region that is shown and the HLH region that is not shown. Numbers surrounding the amino acids correspond to amino acid numbers in each Twist homolog. The location of the *n2170sd* point mutation is indicated in blue. The bottom three lines indicate the single amino acid changes made in the CeTwist protein (in Fig. 4) corresponding to known mutations found in hTwist of individuals with Saethre-Chotzen syndrome (Howard et al., 1997; Rose et al., 1997; Paznekas et al., 1998).

n2170sd is a point mutation that results in a conserved amino acid substitution in the CeTwist basic DNA-binding domain

We mapped the *n2170sd* mutation to the X chromosome and found that the allele is covered by the small duplication, stDp2, in the region of the *hlh-8* locus. The *hlh-8*-coding sequence in *n2170sd* mutant animals contained a single base pair change resulting in a glutamic acid to lysine (E29K) alteration in the basic domain (Fig. 1). The affected amino acid is conserved among Twist family members and less-related bHLH family members such as *C. elegans* MyoD and E/Daughterless homologs (Fig. 1). An independently identified allele, *n1897* (M. Stern and H. R. Horvitz, personal communication) had the identical point mutation in the *hlh-8* locus, and *n1897* was not characterized further. The egg-laying and constipation phenotypes of *n2170sd* animals were fully rescued by an extrachromosomal array containing wild-type CeTwist expressed from a 15 kb genomic DNA fragment (with approximately 9 kb of 5′-UTR and 3 kb of 3′-UTR from the *hlh-8* locus) (Corsi et al., 2000). It has previously been shown that the expression of wild-type CeTwist is necessary for the activation of two target gene reporters, an FGF receptor homolog, *egl-15*, and an NK homeodomain homolog, *ceh-24* (Harfe et al., 1998b; Corsi et al., 2000). Expression of *egl-15* and *ceh-24* reporters was not detected in *n2170sd* animals, but was restored to wild-type pattern in *n2170sd* animals that carried the extrachromosomal array containing wild-type genomic CeTwist DNA (data not shown). Altogether, these data indicate the phenotype of the *n2170sd* animals was due to the point mutation we found in *hlh-8*.

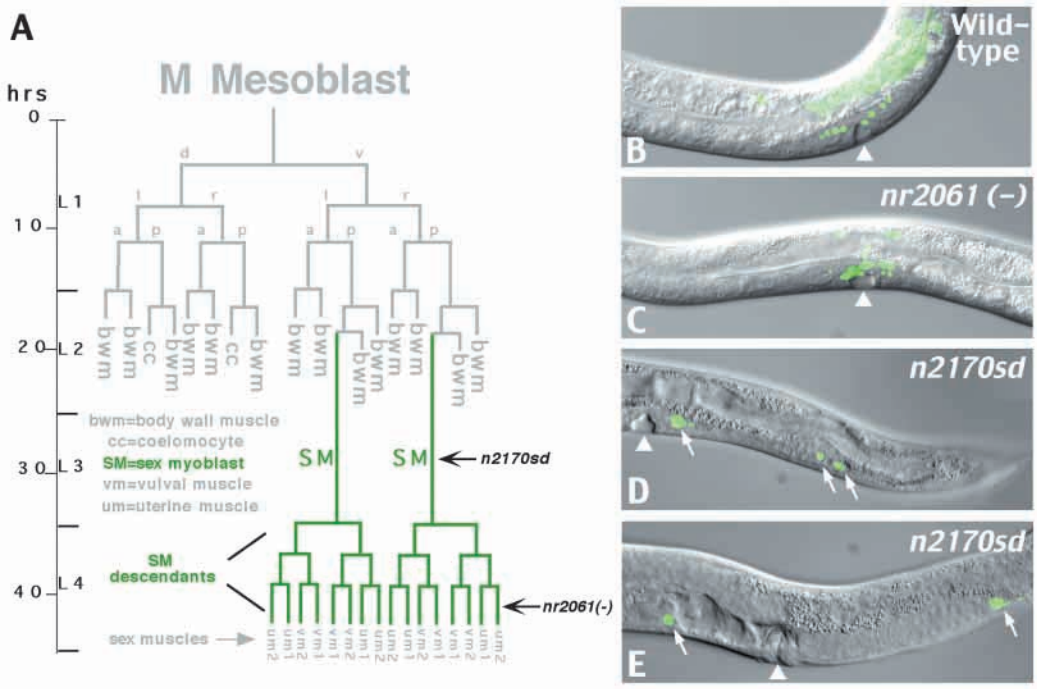

Fig. 2. The SMs are underdivided in *n2170sd* animals. The M lineage for the hermaphrodite is shown in A [adapted from Sulston and Horvitz (Sulston and Horvitz, 1977)]. The time of postembryonic development is indicated on the left in hours and larval stages (L1-L4). The M blast cell divides in characteristic planes: d, dorsal; v, ventral; l, left; r, right; a, anterior; p, posterior. Subsequent divisions occur on the a-p axis. *gfp* reporters exist that mark each tissue type that differentiates from M and the green lines shown represent gfp expression from *hlh-8::gfp* in SM and its descendants. Vertical lines represent cells, and horizontal lines represent cell divisions. (B-E) Images are Nomarski/GFP merged micrographs of animals with integrated *hlh-8::gfp*. Dorsal is upwards, and anterior is leftwards. (B) Wild-type animals have 16 SM descendants (eight can be seen in this focal plane) surrounding the developing vulval opening (white arrowhead). The green on the dorsal side of the animal is due to intestinal autofluorescence and not GFP. (C) The SMs divide in *nr2061 (–)* animals and this image shows greater than the wild-type number of cells near the vulval opening. (D,E) The undivided SMs in *n2170sd* animals can be seen along the ventral length of the animal (white arrows). The animal in D is the same age as in B and C, based on vulval development, and has three undivided SMs. In older animals (E), the SMs still have not divided and continue to express *hlh-8::gfp*.

n2170sd animals have many of the same mesodermal phenotypes as *nr2061 (–)* animals

We wanted to understand the cellular cause of the defecation and egg-laying defects observed in homozygous *n2170sd* animals. We previously performed an extensive characterization of mesodermal specification and patterning in *nr2061 (–)* putative null animals (Corsi et al., 2000). We observed similar cellular defects in mesodermal patterning in *n2170sd* and *nr2061 (–)* animals, with one notable exception (see next section). *n2170sd* animals, like *nr2061 (–)* animals, lack three out of four non-striated enteric muscles. The loss of enteric muscles in the *n2170sd* animals accounted for their constipation phenotype, as is true for the *nr2061 (–)* animals (data not shown) (Corsi et al., 2000).

The major nongonadal components of the wild-type *C. elegans* postembryonic mesoderm develop from a single blast cell, M (Sulston and Horvitz, 1977) (Fig. 2A). The M mesoblast, located in the posterior of the newly hatched animal, consistently goes through 4 sets of cell divisions in a stereotypical spatial and temporal manner to form 18 descendants that will become two coelomocytes (cc), 14 body wall muscles (bwm) and two sex myoblasts (SMs). The two SMs migrate to the center of the animal, where the vulva is forming, and divide three times each to form 16 descendants that will become eight vulval and eight uterine muscles (collectively called sex muscles). All of these events can be followed in live animals using the *hlh-8::gfp* reporter that contains only promoter sequence and is not a rescuing construct (Harfe et al., 1998b). As observed in *nr2061 (–)* animals, three major defects were found in the M lineage in *n2170sd* animals: the initial cell divisions of M did not occur in the proper division planes, an incorrect number of M descendants were formed (e.g. extra SMs in some animals

Table 1. M lineage descendants in CeTwist mutants

Early M division planes*,†	Wild type (%)‡	n2170sd (%)	nr2061 (–) (%)‡
D/V	100	56	21
V	0 (*n*=19)	44 (*n*=41)	75 (*n*=28)
D	0	0	4
Number of sex myoblast-like cells per animal†			
1	0	14	5
2	100	48	38
3	0	17	26
4	0 (*n*=21)	17 (*n*=29)	19 (*n*=42)
5	0	3	7
6	0	0	5
Divided SMs†			
Yes	100	0	100
No	0 (*n*=15)	100 (*n*=27)	0 (*n*=26)

*After the first division of M, wild-type (N2) animals have one dorsal (D) and one ventral (V) M daughter. D/V, one D and one V cell; V, two V cells; D, two D cells.

†Observations made with animals that were expressing integrated *hlh-8::gfp*.

‡Data for wild-type and *hlh-8 (nr2061)* animals have been previously reported by Corsi et al. (2000).

Table 2. Differentiated M descendants in CeTwist mutants

Normal vulval muscles (polarized light)*	Wild type (%)†	n2170sd (%)	nr2061 (–) (%)†
Yes	100	0	0‡
No	0 (*n*=21)	100 (*n*=12)	100 (*n*=29)
Number of coelomocytes per animal (*intrinsic cc::gfp*)*			
4	1.5	16	4
5	1.5	9	9.5
6	97 (*n*=59)	73 (*n*=194)	86 (*n*=232)
7	0	1	1
8	0	0.5	0
Number of adult body wall muscles (*myo-3::gfp*)*,§			
84-94	0	62	80
95	100 (*n*=25)	15 (*n*=13)	5 (*n*=40)
96-100	0	23	15

*Method used to evaluate the phenotype.
†Data for wild-type and *hlh-8 (nr2061)* animals, except for the coelomocyte counts, have been previously reported by Corsi et al. (2000).
‡Under higher resolution analysis, 30% of animals (*n*=23) had one to three birefringent muscle remnants at the vulva that were *myo-3::gfp* positive but failed to differentiate fully as vulval muscles.
§These muscle counts also involved Nomarski inspection of muscle quadrants.

possibly due to bwm to SM fate transformations), and non-striated sex muscles were not formed properly (Corsi et al., 2000) (Tables 1, 2). The lack of vulval muscles explained why the *n2170sd* animals were unable to lay any eggs.

Sex myoblasts frequently do not divide in *n2170sd* animals

Although many of the phenotypes observed in *nr2061 (–)* animals were also apparent in *n2170sd* animals, a striking difference existed in the postembryonic development of the two strains. The SMs in *n2170sd* animals had a different phenotype from *nr2061 (–)* or wild-type animals. In wild-type animals, the SMs were born in the posterior of the animal and migrated to the center of hermaphrodites where they proliferated and differentiated into vulval and uterine muscles by the end of the fourth larval stage (Fig. 2A,B) (Sulston and Horvitz, 1977). In both *n2170sd* and *nr2061 (–)* animals, the SM number may differ from 2 (Table 1). In *n2170sd* animals, the SMs often failed to divide, whereas the SMs in *nr2061 (–)* animals divided but sex muscles did not differentiate properly (Table 1; Fig. 2C,D). Some of the SMs in *n2170sd* animals migrated to, or past, the region of the developing vulva where they could receive nonautonomous signals required for division, yet they still did not divide (Fig. 2D,E). These undivided SMs sometimes persisted in *n2170sd* young adults (Fig. 2E). Initially, wild-type and *hlh-8* mutant animals were observed at the L4 stage, where, in wild-type animals, 16 SM descendants were apparent by *hlh-8::gfp*. Small GFP-expressing cells characteristic of SM descendants were found in *nr2061 (–)* animals and large SM-blast like cells were found in *n2170sd* animals at this stage and later (Table 1; Fig. 2C,D). The cells with SM morphology in *n2170sd* animals could result from underdivided or undivided SM blast cells. These possibilities were tested by observing single animals at stages younger than L4. We observed *hlh-8::gfp* in single wild type (*n*=6), *nr2061(–)* (*n*=7) and *n2170sd* (*n*=25) animals at 15-30 minute intervals over a 3.5-4 hour time period. During this time period, wild-type and *nr2061(–)* SMs divided at least one time in every animal (data not shown). By contrast, the SMs divided at least once in only 36% of *n2170sd* animals (data not shown). Only those animals showing evidence of vulval development by the end of the experiment were reported to ensure that the time interval included the period when the SMs could possibly be dividing. Although the inability to lay eggs because of the loss of properly formed sex muscles was the same in both *hlh-8* mutant strains, the underlying cellular defects were distinct in the two strains.

The mutant E29K CeTwist protein remains nuclear localized

To examine whether the defects in *n2170sd* animals resulted from E29K CeTwist protein instability, improper localization or a loss of *hlh-8* expression, we performed immunofluorescence studies on the mutant animals. The mutant E29K protein was observed in nuclei of large cells in the center of late fourth larval stage hermaphrodites (Fig. 3). The size and position of these cells suggested they were the SM-like cells found in *n2170sd* animals at this stage. The mutant protein was localized to nuclei by the coincident staining with the DNA dye, DAPI (Fig. 3D). The subcellular localization of the E29K CeTwist was indistinguishable from that of the wild-type protein (data not shown). Therefore, the mutant protein was expressed in the appropriate cells and located in the nucleus.

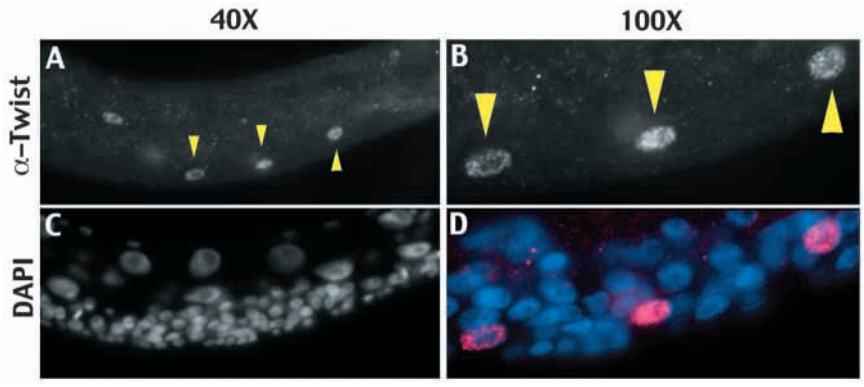

Fig. 3. A CeTwist protein product is detectable in nuclei of *n2170sd* animals. The central region of a single *n2170sd* hermaphrodite is shown at two different magnifications. Dorsal is upwards. (A,B) CeTwist antibody was used to observe cells that are expressing mutant CeTwist E29K protein (yellow arrowheads). (C,D) Nuclei are observed with DAPI staining. (D) A merged image of DAPI (blue) and α-Twist immunofluorescence (red) staining.

A

hlh-8 expression construct	# of lines	L3 stage animals: Wild type	L3 stage animals: Mutant	L3 stage animals: *n*	L4 stage animals: Wild type	L4 stage animals: Mutant phenotypes: Undivided SMs	L4 stage animals: Mutant phenotypes: Underdivided SMs	L4 stage animals: Mutant phenotypes: Extra SM desc.	L4 stage animals: *n*
none	2	100%	0%	46	95%	0%	5%	0%	21
wild type	2	96%	4%	55	88%	6%	6%	0%	35
E29K (*n2170*)	3	31%	69%	70	39%	41%	4%	16%	51
hR116W	5	90%	10%	114	68%	10%	5%	17%	81
hR118H	6	99%	1%	111	68%	22%	3%	7%	71
hQ119P	3	60%	40%	82	30%	34%	25%	11%	97

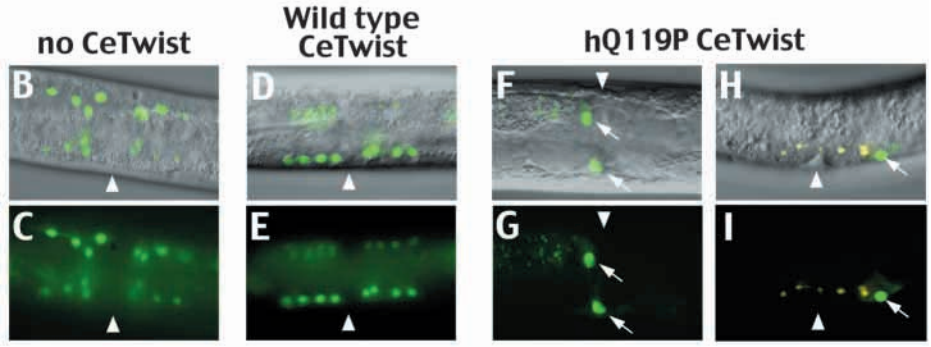

Fig. 4. Overexpression of DNA-binding domain mutations of *hlh-8* causes phenotypes in the *C. elegans* postembryonic mesoderm. Transgenic lines were made by injecting various *hlh-8* expression constructs in addition to the dominant marker, *rol-6* listed in A into animals that already contained the *hlh-8::gfp* integrated reporter construct. Stable lines were established before the phenotype of each *hlh-8* expression construct was assayed. Transgenic animals from the number of independent lines indicated were observed at either the L3 larval stage (when wild-type animals have two SMs) or the L4 stage (when wild-type animals have 16 SM descendants). We scored animals as mutant at the L3 stage if they had greater than two SMs and at the L4 stage if they had undivided SMs, underdivided SMs or greater than 16 SM descendants. Examples of phenotypes seen in L4 animals are shown in B-I. (B,C) Animals with no additional CeTwist expression. (D,E) Animals expressing wild-type CeTwist. (F-I) Animals expressing the analogous human Q119P mutation in CeTwist. (B,D,F,H) Nomarski and GFP merged images (not from identical focal planes). (C,E,G,I) GFP only images. (H,I) GFP images are merged from two different focal planes with yellow SM descendants and a green undivided SM. (B-G) Ventral views. (H,I) Lateral views with dorsal upwards. In all images anterior is towards the left. The white arrowhead indicates developing vulval opening. The white arrows indicate the position of undivided SMs. All other *gfp*-expressing cells that are green or yellow are SM descendants.

The *n2170sd* mutation and several human Saethre-Chotzen mutations are dominant in transgenic animals

To test whether the mutant CeTwist E29K acted as a dominant-negative protein, we expressed the mutant protein from a plasmid in animals that were wild type for *hlh-8* at the chromosomal locus. We looked for defects in the postembryonic mesoderm that could be observed with *hlh-8::gfp* in the transgenic animals during the third (L3) and fourth (L4) stages of larval development. Animals that were expressing extra CeTwist from a plasmid containing the wild-type CeTwist cDNA had some defects (4% of animals at L3 stage and 12% at L4 stage) compared with animals not expressing extra CeTwist (0% at the L3 stage and 5% at the L4 stage) (Fig. 4A). By contrast, at the L3 stage, almost 70% of the animals expressing the mutant CeTwist E29K protein had supernumerary sex myoblasts. This phenotype was similar to nontransgenic animals from *nr2061 (–)* or *n2170sd* strains (Table 1). At the L4 stage, 16% of animals expressing CeTwist E29K had extra SM descendants (Fig. 4A). This phenotype is observed in animals with a loss of function of CeTwist (Corsi et al., 2000). Similar to the *n2170sd* homozygotes, 41% of the transgenic animals expressing the E29K mutant CeTwist at the L4 stage had SMs that did not divide. Therefore, expressing mutant CeTwist E29K in the presence of wild-type chromosomal *hlh-8* could mimic the semidominant phenotype in the *n2170sd* animals.

As the *n2170sd* mis-sense mutation in the DNA-binding domain was able to dominantly cause a phenotype when expressed from a transgene, we reasoned that other mis-sense mutations in the same domain might also cause similar phenotypes. We performed a similar experiment using several selected alleles that had been identified in individuals diagnosed with Saethre-Chotzen syndrome [hR116W (Paznekas et al., 1998); hR118H (Rose et al., 1997); hQ119P (Howard et al., 1997)]. Although the DNA-binding domain in human Twist is not 100% identical to the same domain in CeTwist, the amino acids that have been altered in individuals with Saethre-Chotzen syndrome are conserved between humans and *C. elegans* so we made the mis-sense mutations directly in the *C. elegans hlh-8* cDNA (Fig. 1). We expressed transgenes with the engineered mutant version of the *C. elegans hlh-8* cDNA in wild-type animals and examined the post-embryonic mesodermal development. We found that expressing CeTwist with the hQ119P mutation caused phenotypes similar to CeTwist E29K (Fig. 4A,F-I). These animals also had a high number of underdivided SMs (Fig. 4A). The hR116W mutation had a slight mutant phenotype at the L3 stage (twice as many animals had extra SMs than those expressing wild-type CeTwist), and these transgenic animals had undivided SMs and extra SM descendants at the L4 stage (Fig. 4A). Expression of the third mutation, hR118H had no phenotype at the L3 stage but did have a similar phenotype to hR116W at the L4 stage (Fig. 4A). Therefore, the effect of

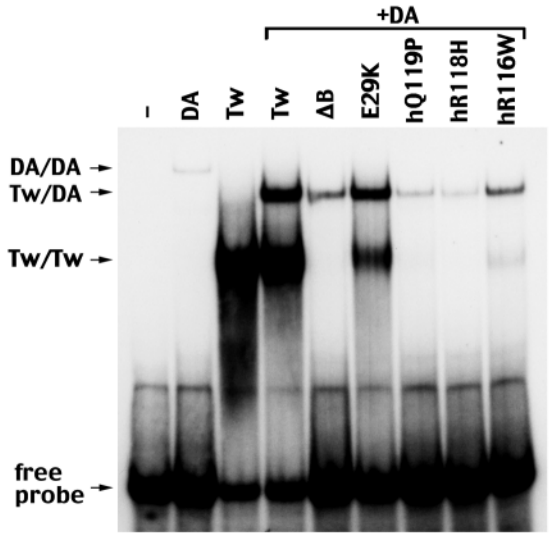

Fig. 5. The mutant CeTwist (E29K) protein binds to DNA as a homodimer and as a heterodimer with CeE/DA, whereas the human Saethre-Chotzen mutations in CeTwist bind DNA only as heterodimers with CeE/DA. In vitro gel shift assays were performed using primers containing an E box bHLH binding site. Bacterially expressed and purified proteins added to the assay are indicated across the top of the gel. DA, CeE/DA; Tw, CeTwist; ΔB, CeTwist without the basic DNA-binding domain; E29K, CeTwist with the *n2170sd* point mutation causing the E29K amino acid change; hQ119P, hR118H and hR116W, CeTwist with the human Saethre-Chotzen amino acid substitutions. Protein/DNA complexes are indicated on the left of the gel based on shifts in molecular weight.

expressing CeTwist with a mutant basic domain can be observed at the cellular level (Fig. 4B-I), providing a powerful assay for dominant negative activity of *TWIST* point mutations.

The mutant CeTwist E29K protein can still bind DNA and heterodimerize with CeE/DA to facilitate DNA binding

In order to explore the biochemical nature of the E to K mutation in *n2170sd* animals, we investigated the behavior of the mutant protein in an in vitro DNA-binding assay. Because the *n2170sd* point mutation creates a charge change in the basic domain, we predicted that DNA binding by the mutant protein might be disrupted. To test whether the protein would bind DNA, we purified a His-tagged mutant protein in parallel with wild-type His-tagged CeTwist in an *E. coli* expression system. As previously shown by Harfe et al. (Harfe et al., 1998b), CeTwist can bind DNA containing an E box sequence as a homodimer or cooperate with CeE/DA to bind as a heterodimer (Fig. 5). The mutant CeTwist E29K protein was also able to homodimerize and heterodimerize with CeE/DA to bind the same sequence in our gel shift conditions (Fig. 5). To be certain that the binding activity we observed with CeTwist E29K was not due to impurities in the protein purification procedure, we also purified His-tagged CeTwist that was missing the entire basic domain (ΔB). The ΔB mutant protein did not shift the same oligo as a homodimer (Fig. 5). Therefore, in a qualitative in vitro assay, the mutant CeTwist E29K protein behaved similar to wild-type CeTwist and could bind DNA on its own as well as with CeE/DA.

The human Twist DNA-binding domain mutations were also purified from *E. coli* as His-tagged CeTwist proteins. None of these mutants was able to appreciably bind to the E box DNA as a homodimer (Fig. 5). Like ΔB, the human mutations were able to bind DNA as a heterodimer with CeE/DA in our in vitro assay. The ability of all the mutant CeTwist proteins to form heterodimers may account for their dominant negative activity in vivo.

Mutant CeTwist E29K protein cannot activate a reporter gene in vivo

As the mutant CeTwist E29K protein could heterodimerize with CeE/DA in vitro to bind DNA, we tested whether the mutant protein could also function in an in vivo transcription assay. CeTwist and CeE/DA that are ectopically expressed from heat shock promoters can promote robust expression of an *egl-15::gfp* reporter (Harfe et al., 1998a; Harfe et al., 1998b) (Fig. 6A,C). In order to test whether the mutant CeTwist E29K protein was capable of the same coactivation, we performed an equivalent experiment expressing the mutant protein from the heat shock promoter. The CeTwist E29K protein was unable to activate the *egl-15::gfp* either on its own or with CeE/DA (data not shown) (Fig. 6E,G). We detected expression of the mutant CeTwist E29K protein from the heat shock promoter by immunostaining with polyclonal CeTwist antibodies (Fig. 6F). This observation ruled out the possibilities that the lack of expression of CeTwist E29K or

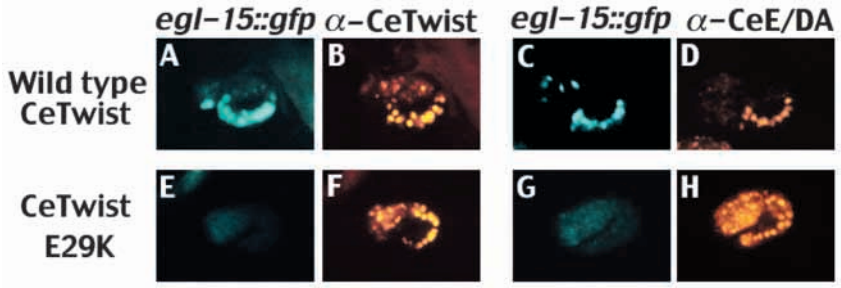

Fig. 6. The mutant CeTwist E29K protein cannot cooperate in vivo with CeE/DA to activate a reporter construct. Animals that had an integrated *egl-15::gfp* reporter construct plus extragenic heat shock expression construct(s) indicated were subjected to heat shock and after a period of recovery were scored for the amount of GFP expression observed. Two lines each of the expression constructs were scored. Embryos expressing CeTwist and CeE/DA from the heat shock promoter are labeled Wild type CeTwist. Embryos expressing CeTwist E29K and CeE/DA from heat shock promoters are labeled CeTwist E29K. Cells where either CeTwist or CeTwist E29K plus CeE/DA were overexpressed from the heat shock promoter were observed by immunostaining embryos with either the CeTwist or CeE/DA antibody (B,F or D,H, respectively). Representative embryos are shown. (A,C) Expression of *egl-15::gfp* was observed only in cells where CeTwist and CeE/DA were ectopically expressed. (E,G) Only background levels of fluorescence can be seen when mutant CeTwist E29K and CeE/DA are overexpressed in *egl-15::gfp*-containing embryos.

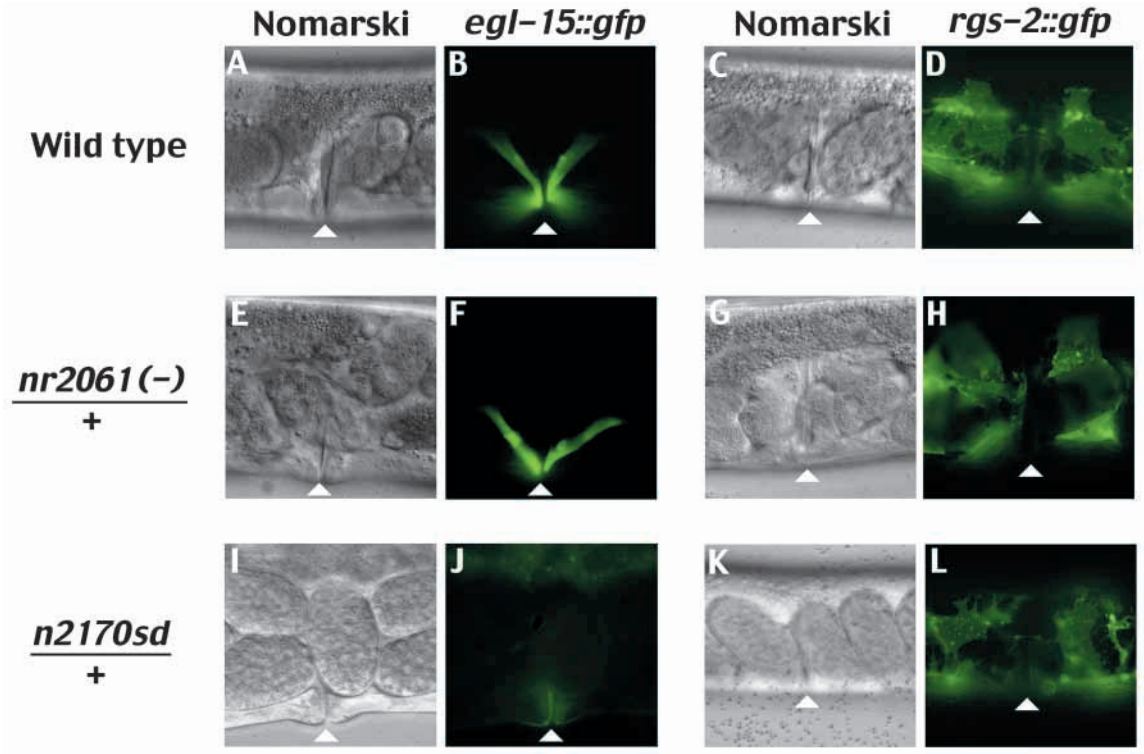

Fig. 7. Heterozygous *n2170sd*/+ animals do not turn on *egl-15::gfp*. All panels show the central region of adult hermaphrodites surrounding the vulval opening (indicated by white arrowheads). (A,C,E,G,I,K) Nomarski images. (B,D,F,H,J,L) GFP only images showing either the vulval muscles expressing *egl-15::gfp* or uterine muscles expressing *rgs-2::gfp* as indicated. (A,B,E,F,I,J) Lateral views. (C,D,G,H,K,L) Ventral views. (A-D) Wild type. (E-H,I-L) Heterozygous *nr2061 (–)*/+ and *n2170sd*/+ animals, respectively. Both wild-type and *nr2061 (–)*/+ animals express *egl-15::gfp* and *rgs-2::gfp*. (I,J) *n2170sd*/+ animals do not express *egl-15::gfp* in vulval muscles. (K,L) In these animals, uterine muscles are observed by cells expressing *rgs-2::gfp* and vulval muscles as observed by *arg-1::gfp* (not shown) are formed.

perhaps the increased degradation of the mutant protein was responsible for the inability to coactivate the *egl-15::gfp* reporter with CeE/DA. Therefore, the mutant protein is unable to cooperate in this in vivo assay with CeE/DA to promote *egl-15::gfp* transcription.

Animals heterozygous for the *n2170sd* mutation can differentially activate reporter gene expression

We have shown that the *hlh-8 (n2170)* strain is a semi-dominant allele. Because *n2170sd*/+ heterozygotes are egg-laying defective, we investigated whether a defect in vulval or uterine muscle development was causing this phenotype in heterozygous animals. We observed the uterine muscles with *rgs-2::gfp* (regulator of G protein signaling) (Dong et al., 2000), and we found wild-type, *nr2061 (–)*/+ and *n2170sd*/+ heterozygotes to be indistinguishable (Fig. 7D,H,L). In wild-type animals, the four vm1 type vulval muscles express *egl-15::gfp* (Harfe et al., 1998a). Using the *egl-15::gfp* reporter, we observed that *n2170sd*/+ heterozygotes never expressed GFP from the *egl-15* promoter in the vm1 vulval muscles (n>50), whereas wild-type and *nr2061 (–)*/+ heterozygotes always expressed the reporter in the vm1 cells (n>50) (Fig. 7B,F,J). In order to distinguish whether the vulval muscles were not formed or whether the muscles were made but the reporter gene was not expressed in that tissue, we used a third *gfp* reporter *arg-1::gfp* (*apx-1* related gene), which also is expressed in the vm1 vulval muscles (*apx-1* and *arg-1* are both Notch pathway ligands) (Mello et al., 1994) (A. Melendez, I. Greenwald, S. Kostas, J. Liu and A. Fire, personal communication). We observed wild-type expression of *arg-1::gfp* in the vm1 muscles in *n2170sd*/+ heterozygotes (data not shown). This observation indicated that the vulval muscles were made in the *n2170sd*/+ heterozygotes. Therefore, there was differential gene expression from the two *gfp* reporters that are expressed in the vm1 vulval muscles when both wild-type and mutant CeTwist E29K proteins are present in the same animal. The egg-laying defect in the *n2170sd*/+ heterozygotes does not result from a lack of vm1 muscles but was more likely to be due to a problem in their proper functioning caused by differential regulation of CeTwist target genes.

DISCUSSION

CeTwist plays a role in patterning and formation of a subset of *C. elegans* mesodermal tissues. By studying the *n2170sd* allele, more can be learned about how CeTwist functions. In *n2170sd* animals, the mutant CeTwist protein has a single glutamic acid to lysine change at a conserved position in the CeTwist basic DNA-binding domain. This charge change makes the DNA-binding region even more basic and still allows the mutant CeTwist E29K protein to bind DNA. The mutant CeTwist E29K protein binds to a canonical bHLH E box binding site in vitro as a homodimer and as a heterodimer with the

Daughterless homolog CeE/DA protein. However, the cooperation with CeE/DA does not extend to an in vivo assay because mutant CeTwist E29K cannot coactivate an in vivo reporter gene with CeE/DA. Therefore, we propose that the mutant CeTwist E29K can bind to promoters alone as a homodimer or as a heterodimer with CeE/DA but cannot activate transcription, perhaps because of the inability to interact with the transcriptional machinery or with other transcription factors important at CeTwist target promoters (see model below).

Basic DNA binding domain mutations in other bHLH proteins

How might the point mutation in the basic DNA-binding domain of E29K CeTwist affect the function of the mutant protein? Studies of basic domain mutations in other bHLH proteins suggests several possible ways the E29K mutation might interfere with function. For example, E29K could affect dissociation of the mutant bHLH from target promoters, could cause more stable DNA binding or could cause an altered protein conformation, resulting in defective transcriptional activation. The crystal structure of the bHLH domains of mouse MyoD and E47 has revealed that the amino acid corresponding to E29 in CeTwist plays an especially important role in making crucial contacts with DNA. E47 and E12 are the vertebrate E partners and are splice variants from the same locus. The glutamic acid (E118 in MyoD; E345 in E47) at the position that corresponds to E29 in CeTwist contacts DNA at the cytosine and adenine bases in the E boxes CAGCTG for MyoD or CACCTG for E47 (Ma et al., 1994; Ellenberger et al., 1994). In vitro DNA-binding studies have been performed with several E12 mutants. Sieber and Alleman (Sieber and Alleman, 1998) found that an E345Q E12 mutant protein dissociated less well from both specific and nonspecific E12 binding sites. Vitola and colleagues have found that mutations in the DNA-binding domain of E12 that cause the overall charge change of the domain to be more positive result in mutant proteins that have increased and more stable DNA-binding activity (Vitola et al., 1996). Basic domain mutations in MyoD reveal the importance of the domain for DNA binding as well as for transcriptional activation (Bengal et al., 1994). Some mutations in the DNA-binding domain of MyoD can still bind DNA as homodimers and as heterodimers with E47, but these mutants are not able to activate transcription in vitro (Bengal et al., 1994). For example, an A114N mutation in the DNA-binding domain of MyoD that still binds DNA has been proposed to alter the conformation of the activation domain to block transcription (Huang et al., 1998). Similarly, the E29K mutant CeTwist might have an altered conformation that is blocking the ability of the mutant protein to participate in transcriptional activation. Further biochemical characterization of the CeTwist E29K protein would be required to provide evidence for this possibility.

The E29K CeTwist protein may act as a dominant negative mutant

We propose that the *n2170sd* mutation created a dominant-negative CeTwist mutant protein. Because the null mutation *hlh-8 (nr2061)* is fully recessive, it is unlikely that haploinsufficiency at the *hlh-8* locus could cause a semidominant mutant. Additionally, in homozygous *hlh-8 (n2170sd)* animals, a protein that is recognized by a CeTwist antibody is present in a wild-type pattern of expression so these animals are not suffering from a lack of CeTwist caused by nonexpression of the *hlh-8* locus or protein instability caused by the point mutation. We have observed that a plasmid containing genomic CeTwist DNA can fully rescue the egg-laying and constipation phenotypes of *n2170sd* animals. As extra CeTwist can rescue, it is unlikely that the *n2170*sd allele results in too much *hlh-8* activity that would be characteristic of a strong gain-of-function mutation. The mutation is classified genetically as semidominant because we observed some but not all of the same defects in both homozygotes and heterozygotes. For example, both homozygotes and heterozygotes have egg-laying defects but the homozygotes cannot lay eggs at all, whereas the heterozygotes lay eggs at a slower than wild-type rate. The phenotypes we observed in the mesoderm of *n2170sd* animals were all in cells where wild-type CeTwist is normally found, and the phenotypes were rescued by overexpressing wild-type CeTwist, suggesting that *n2170sd* is not a neomorphic mutation. Naturally occurring dominant negative bHLH proteins exist. The vertebrate protein Id and the *Drosophila* protein Emc are HLH proteins that lack a basic domain (reviewed by Massari and Murre, 2000). These proteins can still heterodimerize with other bHLH proteins but the resulting heterodimer cannot bind DNA. In effect, these proteins sequester their partner bHLH from functioning. The mutant CeTwist E29K protein does not appear to act by the same mechanism as Id and Emc, as the protein can heterodimerize with CeE/DA and bind to DNA in vitro. Rather, the protein may be sitting unproductively at promoter sequences with CeE/DA and/or other proteins.

Why do the SMs in *n2170sd* animals fail to divide properly?

While observing *n2170sd* animals, we discovered an earlier cellular defect not seen in the *nr2061 (–)* animals. In wild-type animals, the SMs that are born in the postembryonic mesodermal lineage divide to become 16 sex muscles required for egg laying (Fig. 2A). These SM cells either did not divide at all or did not divide at the wild-type rate in the *n2170sd* animals, whereas the cells divided but did not form proper sex muscles in *nr2061 (–)* animals. In both mutant strains, the animals could not lay any eggs because the muscles that open the vulva were not present and functioning. However, the cellular defects were distinct, and we would argue are more severe in the *n2170sd* animals, suggesting an interference with, or titration of, a factor or pathway required for SMs to divide properly. In heterozygous *n2170sd*/+ animals, the SMs can divide and differentiate as we observed both vulval and uterine muscles that are the products of the SMs in adult animals (Fig. 7). This observation demonstrates that a single copy of the *n2170sd* allele is insufficient to block all SM divisions.

The decreased amount of divisions of the SMs in homozygous *n2170sd* animals cannot be explained by the lack of migration of the SMs. Although some of the undivided SMs did not migrate and remained in the posterior of *n2170sd* animals, some of the SMs migrated to the appropriate region of the developing vulva and still did not divide properly (Fig. 2D,E). If spatial signals were necessary to initiate the cell divisions, then at least those SMs near the vulva should have divided in the same way as *nr2061 (–)* SM descendants in

n2170sd animals. In wild-type animals at the young adult stage, the SM descendants no longer express *hlh-8::gfp* because the cells have differentiated into sex muscles and have turned off the reporter (Harfe et al., 1998b). In *n2170sd* young adult animals, the reporter expression often persisted in the undivided SMs (Fig. 2E). This aberrant perdurance could be due to the lack of divisions of the SMs that were now uncoupled with the age of the *n2170sd* animal or could be due to the inability of the CeTwist E29K protein to regulate *hlh-8* expression negatively.

Model for mutant CeTwist in vivo phenotypes

A working model is presented to explain the phenotypes and predict how CeTwist is influencing gene expression based on our observations with *hlh-8* mutant animals (Fig. 8). In wild-type animals, CeTwist (along with a partner protein), which in many cases will be CeE/DA, heterodimerizes to promote gene expression from promoters such as *egl-15* as indicated in Fig. 8A. A factor that physically interacts with CeTwist, i.e. the heterodimeric partner or a different protein, is responsible for allowing or promoting the SMs to divide in wild-type animals. This factor may act alone or in combination with other factors to turn on cell division targets. The factor(s) is freely available to promote or allow SM divisions when CeTwist is absent in *nr2061 (–)* homozygotes (Fig. 8B). As there is no CeTwist made in *nr2061 (–)* homozygotes, no target genes can be activated (i.e. no *egl-15::gfp*). In *nr2061 (–)*/+ heterozygotes, half of the wild-type amount of CeTwist is sufficient to turn on downstream targets resulting in animals that are indistinguishable from wild-type animals (Fig. 8C). In *n2170sd* homozygotes that are producing a dominant negative version of CeTwist, the mutant protein may bind to DNA in vivo but is unable to turn on downstream target genes (Fig. 8D). DNA binding may not be important for dominant negative activity, however, because DNA-binding domain mutations that cause phenotypes (Fig. 4) do not bind DNA efficiently in vitro (Fig. 5). The mutant CeTwist E29K protein is likely to titrate away the factor(s) that promote SM divisions, as we frequently saw SMs that did not divide or were underdivided in *n2170sd* homozygotes when compared with wild-type animals. *n2170sd*/+ heterozygotes had a subset of the defects seen in *n2170sd* homozygotes. Half the amount of defective CeTwist E29K cannot completely sequester the factor(s) required for SM divisions as SM descendants were observed in *n2170sd*/+ heterozygotes (Fig. 8E). However, some targets, such as the potential target *arg-1::gfp*, could be activated by the wild-type CeTwist present in these animals. Other promoters such as *egl-15::gfp* were not turned on, suggesting an important difference among CeTwist promoters that may be revealed in future experiments. The egg-laying defects that were observed in *n2170sd*/+ heterozygotes may be due to this lack of functional gene products from the *egl-15* and other target promoters.

In an alternative model not shown, our results could be explained by invoking CeTwist homodimers rather than heterodimers as the transcriptional regulator. In this case, the default state would be SMs dividing because of other factors, such as cell cycle proteins that have no physical or direct interaction with CeTwist. If CeTwist is not available [e.g. in *nr2061 (–)* animals] the SMs still divide with this model. The mutant CeTwist E29K protein would have to inhibit the SM divisions actively, perhaps through some neomorphic function or misexpression of the mutant protein. This model does not require a partner or other factor that would interact directly with CeTwist. Because we have been able to create multiple semidominant alleles, we do not favor this neomorphic function model.

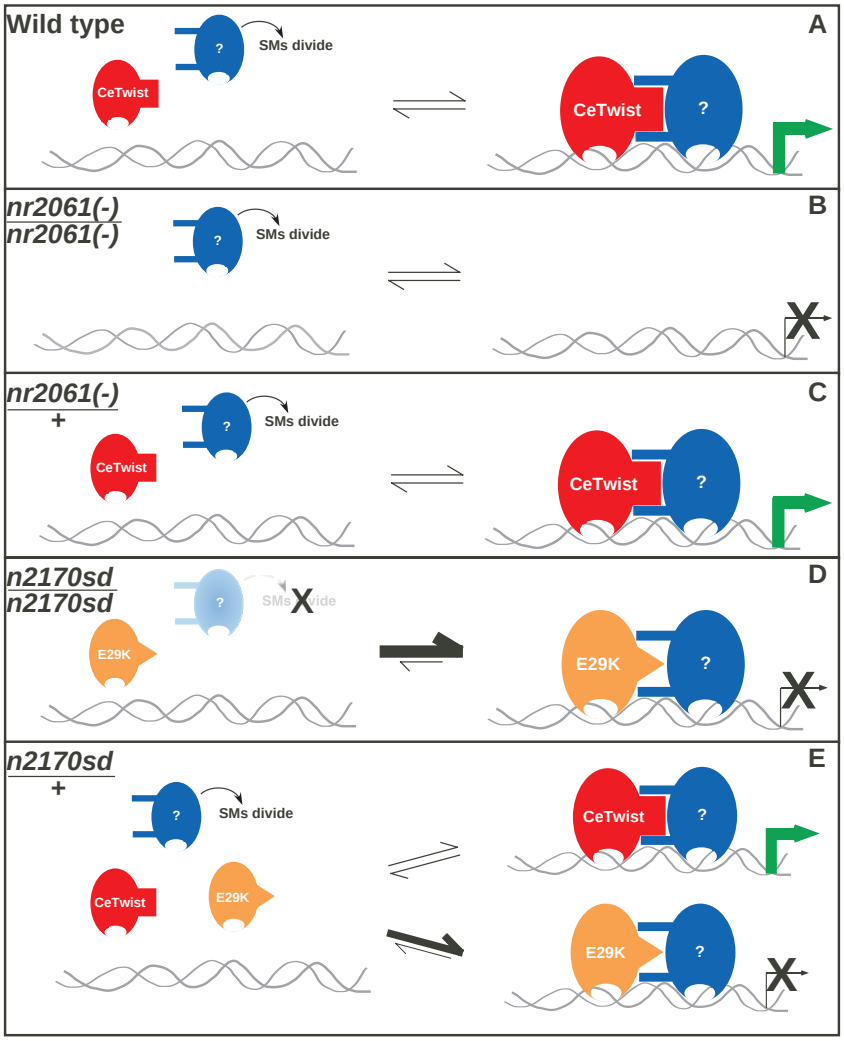

Fig. 8. One possible model to explain the phenotypes observed with *hlh-8* homozygous and heterozygous mutants. Red oval with protruding square, wild-type CeTwist. Orange oval with protruding triangle, mutant CeTwist E29K. Blue oval, CeTwist partner protein that could be CeE/DA and/or an unknown protein. Gray lines represent dsDNA from the promoter of a target gene (e.g. *egl-15)* and green arrows represent transcription proceeding from the *egl-15::gfp* promoter and other SM patterning and differentiation targets. For details of each panel, see Discussion.

It has recently been shown in *Drosophila* that Twist activity is influenced by its dimerization partner at different stages of development. Castanon and colleagues (Castanon et al., 2001) have demonstrated, using covalently linked dimers, that Twist homodimers promote specification of the mesoderm and allocation of precursors to somatic muscle fate early in development. Additionally, they have shown that linked Twist-Daughterless heterodimers are involved in repressing somatic myogenesis later in development. In *C. elegans*, overexpression of CeTwist by itself causes a less robust activation of target genes than overexpressing both CeTwist and CeE/DA (Harfe et al., 1998b), suggesting that CeTwist homodimers may not be the primary mode for transcriptional activation at least in this developmental context.

C. elegans and human Saethre-Chotzen disease

We have seen a range of phenotypes in the three human *TWIST* mutations that were examined in our *C. elegans* cellular assay. Although no genotype to phenotype correlation has been noted for any human *TWIST* mutation in the literature, our results suggest that there could be differences between individuals with different basic domain mutations or mutations elsewhere in the protein. The finding that basic domain mutations analogous to mutations found in human Saethre-Chotzen syndrome caused a phenotype in *C. elegans* is exciting for two reasons. First, although some human *TWIST* mutations are surely haploinsufficient because of the early aberrant stop codons, at least one of the human basic domain mutations is acting as a strong dominant negative in our *C. elegans* assay and thus might act similarly in humans. Second, the genetically manipulable organism *C. elegans* can provide a useful assay for evaluating proteins that might interact or be downstream from human Twist. The Saethre-Chotzen disorder belongs to a class of disorders causing craniosynostosis (premature fusion of cranial sutures). These disorders occur once in every 2500 births (Wilkie, 1997). Out of about 100 related forms of craniosynostosis about 30 are thought to arise from single gene disorders and the specific gene has been identified in less than 10 disorders (Reardon and Winter, 1995; Cohen, 2000). The two known CeTwist targets are an NK class homeodomain protein and an FGF receptor homolog (Harfe et al., 1998b; Corsi et al., 2000). In humans, other proteins known to be mutant in craniosynostotic disorders are Msx2 (an NK class homeodomain protein) and FGFR1, FGFR2 and FGFR3 (for reviews, see Wilkie, 1997; Hehr and Muenke, 1999; Lajeunie et al., 1999). These strikingly similar pathways suggest that any other downstream targets or partners of Twist that are identified in *C. elegans* will be good candidates for craniosynostotic disorders whose underlying cause has yet to be molecularly identified in humans.

Conclusions

We have characterized a semidominant mutation in the CeTwist gene called *hlh-8 (n2170sd).* We found that the *n2170sd* mutation caused a single amino acid substitution (E29K) in the basic DNA-binding domain of CeTwist. Animals with this mutation resembled a null *hlh-8* mutation, except for the improper division of blast cells that will become sex muscles in wild-type animals. This blast cell phenotype can be mimicked in otherwise wild-type animals expressing *hlh-8 (n2170sd)* or *hlh-8* with mutations analogous to those found in the DNA-binding domain of human Twist in individuals with Saethre-Chotzen syndrome who have digit and cranium developmental defects. The mutant CeTwist E29K protein could still bind DNA but could not coactivate transcription of an in vivo reporter with its potential partner CeE/DA (the Daughterless homolog). These results suggest that the defects in the *hlh-8 (n2170sd)* animals were due to either an inability to interact properly with CeE/DA or an inability to cooperate with other unknown partner proteins required for CeTwist to activate transcription. These mutant animals will be useful in future genetic screens that should identify partners and downstream target genes of CeTwist and will provide candidate genes for other human developmental syndromes.

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