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# *HLH54F* is required for the specification and migration of longitudinal gut muscle founders from the caudal mesoderm of *Drosophila*

**Afshan Ismat[1,*], Christoph Schaub[2], Ingolf Reim[1,2], Katharina Kirchner[2], Dorothea Schultheis[2] and Manfred Frasch[1,2,†]**

## SUMMARY

*HLH54F*, the *Drosophila* ortholog of the vertebrate basic helix-loop-helix domain-encoding genes *capsulin* and *musculin*, is expressed in the founder cells and developing muscle fibers of the longitudinal midgut muscles. These cells descend from the posterior-most portion of the mesoderm, termed the caudal visceral mesoderm (CVM), and migrate onto the trunk visceral mesoderm prior to undergoing myoblast fusion and muscle fiber formation. We show that *HLH54F* expression in the CVM is regulated by a combination of terminal patterning genes and *snail*. We generated *HLH54F* mutations and show that this gene is crucial for the specification, migration and survival of the CVM cells and the longitudinal midgut muscle founders. *HLH54F* mutant embryos, larvae, and adults lack all longitudinal midgut muscles, which causes defects in gut morphology and integrity. The function of HLH54F as a direct activator of gene expression is exemplified by our analysis of a CVM-specific enhancer from the Dorsocross locus, which requires combined inputs from HLH54F and Biniou in a feed-forward fashion. We conclude that *HLH54F* is the earliest specific regulator of CVM development and that it plays a pivotal role in all major aspects of development and differentiation of this largely *twist*-independent population of mesodermal cells.

**KEY WORDS: bHLH proteins, Cell migration, Mesoderm, Specification, Visceral muscles, *Drosophila***

## INTRODUCTION

The *Drosophila* mesoderm forms from the ventral-most cells of the early embryo that invaginate during gastrulation. The expression and function of two transcription factors, the basic helix-loop-helix (bHLH) protein Twist (Twi) and the zinc-finger protein Snail (Sna), in ventral cells located between ~15 and 85% egg length are essential for their invagination and for subsequent mesodermal tissue development. Although the absence of either *twi* or *sna* activity results in similar phenotypes, the molecular roles of the two genes in this pathway differ. *twi* functions in activating a variety of mesoderm-specific target genes, including several that are known to regulate the invagination, patterning and differentiation of the mesoderm. By contrast, *sna* is thought to act largely, if not exclusively, as a repressor of a number of neuroectodermal targets, permitting mesoderm formation by restricting the expression of these genes to areas outside the presumptive mesoderm (Kosman et al., 1991; Leptin, 1991; Reuter and Leptin, 1994; Hemavathy et al., 1997; Wakabayashi-Ito and Ip, 2005; Sandmann et al., 2007; Zeitlinger et al., 2007).

Notably, however, there is at least one group of mesodermal cells that requires *sna*, but not *twi*, for its initial phase of development. This cell group is located ventrally within the domain of early *twi* and *sna* expression, but is restricted to the posterior tip of the mesoderm between ~7.5 and 15% egg length. Because it is fated to develop into the longitudinal muscles of the midgut, it has been termed the caudal visceral mesoderm (CVM) primordium (Nguyen and Xu, 1998; Kusch and Reuter, 1999). In light of the apparent lack of a requirement for *twi* for the initial development of the CVM (Kusch and Reuter, 1999), it is interesting that the cells of the CVM primordium are marked by the expression of another bHLH-encoding gene, *HLH54F* (Georgias et al., 1997). This situation raises the possibility that, in the caudal-most portion of the mesoderm, *HLH54F* instead of *twi* cooperates with *sna* to control early CVM development. However, until now, specific mutants for *HLH54F* have not been available to test this possibility.

The *Drosophila* midgut musculature consists of syncytial fibers that arise through myoblast fusion between gut muscle founder cells and fusion-competent myoblasts and forms a meshwork of circular and longitudinal muscles around the endodermal layer (reviewed by Lee et al., 2005). The CVM appears to be the sole source of founder cells of the longitudinal midgut muscles (Kusch and Reuter, 1999; San Martin et al., 2001). After their ingression, these cells migrate anteriorly and spread over the future midgut, where they fuse with resident fusion-competent cells to form the multinucleated longitudinal muscle fibers of the midgut (Campos-Ortega and Hartenstein, 1997; Georgias et al., 1997; San Martin et al., 2001). The fusion-competent cells for this event come from a different source, the so-called trunk visceral mesoderm (TVM), which provides a second (and major) contribution of precursors to the musculature of the midgut (San Martin et al., 2001). The primordia of the TVM are arranged bilaterally as 11 metameric cell clusters within the dorsal mesoderm, which subsequently merge with each other into the contiguous band of the TVM. The TVM primordia are marked by the expression of the NK homeodomain gene *bagpipe* (*bap*) and the FoxF gene *biniou* (*bin*), both of which are essential for TVM formation (reviewed by Lee et al., 2005).

[1]Mount Sinai School of Medicine, Department of Molecular, Cell and Developmental Biology (currently Developmental and Regenerative Biology), Box 1020, Mount Sinai School of Medicine, New York, NY 10029, USA. [2]University of Erlangen-Nuremberg, Department of Biology, Division of Developmental Biology, Staudtstr. 5, 91058 Erlangen, Germany.

*Present address: Johns Hopkins University School of Medicine, Department of Cell Biology, 725 N. Wolfe St., Baltimore, MD 21205, USA
[†]Author for correspondence (mfrasch@biologie.uni-erlangen.de)

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DEVELOPMENT

Within the TVM, the founder cells of the circular midgut muscles are induced by Jelly belly (Jeb) signals acting through the receptor tyrosine kinase Alk (Englund et al., 2003; Lee et al., 2003). The founder myoblasts from the TVM fuse one-to-one with adjacent fusion-competent myoblasts into binucleated syncytia that form the circular midgut muscles (San Martin et al., 2001; Klapper et al., 2002). Subsequently, after the migrating CVM-derived founder cells have arrived at their destinations, each fuses with multiple fusion-competent cells from the TVM left over from the TVM founder cell fusions (San Martin et al., 2001). It is these multinucleated syncytia that will then differentiate into the longitudinal visceral muscles, which run perpendicularly to the circular muscles along the entire length of the midgut (Klapper et al., 2001). The longitudinal gut muscle fibers from the outer layer of the developing visceral musculature are tightly interwoven with the circular gut muscle fibers from the inner layer (Schröter et al., 2006).

*HLH54F* is the earliest known marker of the CVM primordia and its expression is maintained throughout the development and differentiation of the longitudinal midgut muscles (Georgias et al., 1997). To test whether *HLH54F* plays an important role in the development of the CVM and longitudinal midgut musculature, we generated loss-of-function mutations for this gene by imprecise P-excision and EMS mutagenesis screens. We demonstrate that in the absence of *HLH54F* activity, no longitudinal gut muscle founder cells are formed. The absence of all tested CVM markers and the observed apoptotic death of the cells that would normally be destined to form CVM in *HLH54F* mutants, show that *HLH54F* has an essential role in determining the CVM and in specifying the founder cells of the longitudinal gut musculature. This function includes feed-forward regulation and direct binding to target enhancers (e.g. from the Dorsocross genes). We also show that ectopic expression of *HLH54F* can interfere with normal somatic muscle, cardiac and TVM development. Further, we extend the known pathway of CVM development by showing that the initiation of *HLH54F* expression is largely independent of *twi*, but depends critically on the combined activities of *sna* and terminal patterning genes, particularly the synergistic activities of *fork head* (*fkh*) and *brachyenteron* (*byn*). Hence, the CVM primordia are determined at the intersection of the domains of these mesodermal and terminal regulators.

## MATERIALS AND METHODS

### *Drosophila* strains

The following *Drosophila* strains were used: $twi^{ID}$, $tll^{L10}$, $byn^{5}$, $hkb^{A321R1}$, $sna^{18}$, *EY06760* (all from Bloomington Stock Center, Indiana University, USA), $fkh^{XT6}$, $byn^{5}$ $fkh^{XT6}$, $zfh1^{2}$ (from R. Reuter, Tübingen University, Germany), *Df(3R)Exel9020*, *Df(2R)14H10W-21* and *Df(2R)02B10Y-12* (Mohr and Gelbart, 2002) (from the authors), *croc-lacZ* (Häcker et al., 1995) and *slp m4-lacZ #5* (Lee and Frasch, 2000). For UAS/GAL4-induced ectopic expression, embryos were grown at 28°C using the lines *UAS-p35* (Zhou et al., 1997), *UAS-bin* #35 (Zaffran et al., 2001), *byn-GAL4 UAS-GFP* (Johansen et al., 2003) (from J. Lengyel, UCLA, USA), *bap3-GAL4* (Lee et al., 2003), *2xPE-twi-GAL4; 24B-GAL4* (Reim and Frasch, 2005), *UAS-HLH54F-IR* (*P{KK103428}v103965*) and *UAS-da-IR* (*P{GD4440}v51300*) (both from VDRC, Vienna, Austria) (Dietzl et al., 2007).

### P-excision and EMS mutagenesis screens

The viable P insertion *EY06760* was used in an excision screen with *Δ2-3*-derived transposase. Balanced $w^{-}$ males in F2 were crossed individually to *Df(2R)14H10W-21* females (see Fig. 1) (Mohr and Gelbart, 2002). A total of 598 single excision lines were screened over *Df(2R)14H10W-21* for semi-lethality. Semi-lethality was confirmed over *Df(2R)02B10Y-12*. For two semi-lethal lines, $HLH54F^{\Delta 219}$ and $HLH54F^{\Delta 598}$, the molecular lesions were sequenced using genomic PCR fragments from DNA of homozygous escaper flies.

In addition, we performed an EMS mutagenesis screen for mutants with disrupted specification or migration of the CVM. *HLH54Fb-RFP* reporter males were mutagenized with 30 mM EMS (Lewis and Bacher, 1968) and balanced with *CyO*, *twi*>*EGFP*. Embryos homozygous for the mutagenized chromosome were screened for the absence, reduction or mis-migration of the RFP-labeled CVM cells. Two lines with absent RFP in the CVM failed to complement the semi-lethality of $HLH54F^{\Delta 598}$ and were semi-lethal or lethal, respectively, in trans to larger deficiencies. PCR analysis identified $HLH54F^{S0323}$ as a deficiency of *HLH54F* and flanking regions (lethal, with yet undefined breakpoints). Sequencing of genomic DNA amplified from $HLH54F^{S1750}$ identified a C-to-T transition at position 121 of the open reading frame (ORF) as compared with the parental chromosome.

### Construction of *HLH54Fb-lacZ* and *HLH54Fb-GAL4*

Genomic *HLH54F* sequences were cloned into pPelican (upstream sequences, *HLH54Fa-lacZ*) or pH-Pelican (introns, *HLH54Fb-lacZ;* downstream sequences, *HLH54Fc-lacZ*) (Barolo et al., 2000). PCR fragments from *yw* DNA were obtained with the following primers (5′ to 3′): for the upstream region, primers HLH-UPS-5′ CGGATAACCCATGGTTCAGATTCCTGATAA and HLH-UPS-3′ ACAAGTTGGTGGTATCTCGAGAATCACTTC; for the introns with exon 2, primers HLH-INTRON-5′ TCTCAAAGTGAGTTCGAATTCAATTTATAG and HLH-INTRON-3′ TAATGTGGAATGTGTGGATCCAACAGGCAT; for the downstream region, primers HLH-DS-5′ CTAGCCATGCCCATGGTACCTAAGCGGATA and HLH-DS-3′ TACTTGGGCGTTTTCCTCGAGTTTGTTTCT. Only *HLH54Fb-lacZ* expressed *lacZ*.

*HLH54Fd-GAL4* was generated from the second intron by inserting a fragment amplified with primers Gal4-2 EcoRI (ATTCGTCCGGTGGAGGGAATTCTAATTTCC) and HLH-INTRON-3′ into the *Eco*RI site of the p221-GAL4 vector (from C. Klämbt, Münster University, Germany).

### Genomic rescue experiments

HLH54F-genres, containing 4.5 kb of sequences from the *HLH54F* locus, including the intergenic regions up to the 5′ ends of both neighboring genes, was generated by genomic PCR and cloning into pCaSpeR2 (*Not*I/*Bam*H1). Sequencing confirmed the correct ORF. Rescue of CVM formation was tested in the genetic background of *w*; $HLH54F^{\Delta 598}$ *HLH54Fb-lacZ/SM6 eve-lacZ; HLH54F-genres*. Upon crossing this line with $HLH54F^{S0323}$/*CyO twi*>*EGFP*, one copy rescued the semi-lethality.

### Doc enhancer constructs

The 566 bp DocF4s1 enhancers (3L:9,022,917..9,023,482, vR5.27) were cloned into *Eco*RI/*Xho*I of a modified pH-Stinger vector, which has attB sequences inserted into its *Avr*II site (for nGFP reporters) (H. Jin and M.F., unpublished) and pH-Pelican (for *DocF4s1-lacZ*) (Barolo et al., 2000). For transformation, the landing site of M{3xP3-RFP.attP'}ZH-22A was used (Bischof et al., 2007) (Bestgene, Chino Hills, CA, USA). In DocF4s1Hmut, the E-boxes CANNTG (positions 227, 293, 417, 436, 508) were mutated to AGNNTG via de novo DNA synthesis (Mr. Gene, Regensburg, Germany). In DocF4s1Bmut, the Bin binding core motifs T/CAAACA (positions 162, 265, 289, 444, 458) were mutated to T/CAGTCA and the CAAACA at position 486 to CGGCCA.

### Staining procedures

Antibody stainings and fluorescent double stainings for proteins and mRNAs were performed as described (Azpiazu and Frasch, 1993; Knirr et al., 1999). The following antibodies were used: rabbit anti-Twi (1:2000, from S. Roth, Cologne University, Germany), guinea pig anti-Hb9 (Exex – FlyBase) (1:500, from J. Skeath, Washington University School of Medicine, USA), guinea pig anti-Eve (1:300) (Kosman et al., 1998), rabbit anti-Eve (1:3000, our own production), rabbit anti-Mef2 (1:750, from H. Nguyen, Erlangen University, Germany), rat anti-Tropomyosin (1:400, Babraham Institute, Cambridge, UK), mouse and rabbit anti-Vasa (1:1000, from A. Ephrussi, EMBL Heidelberg, Germany and Ruth Lehmann, NYU

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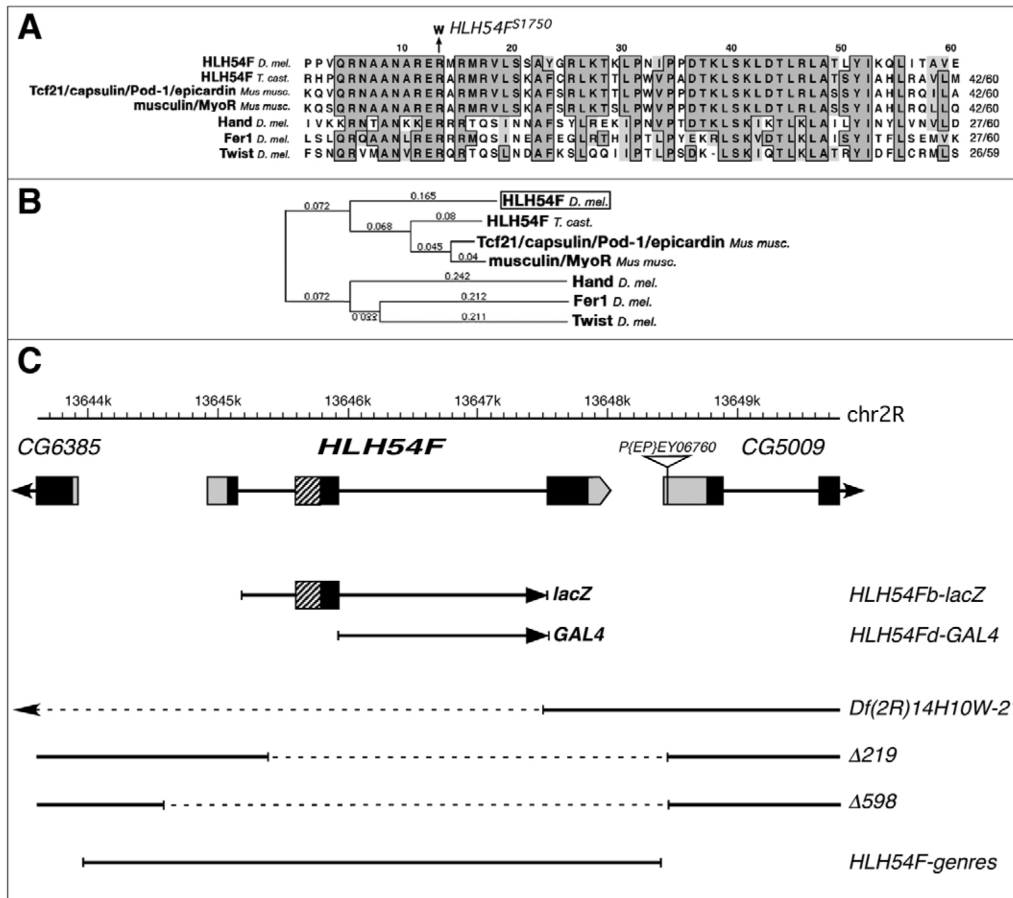


**Fig. 1. HLH54F protein sequence comparison, gene locus and mutations.** (**A**) Alignment of the bHLH domain of HLH54F with the most closely related bHLH domains from mouse, *Tribolium* and *Drosophila*. The R-to-W exchange in the predicted protein from EMS allele *HLH54F*$^{S1750}$ is indicated. (**B**) Phylogenetic analysis of data from A. (**C**) *HLH54F* gene map showing translated regions (black), the bHLH domain (hatched) and UTRs (gray). Beneath are shown the genomic regions used for the *lacZ* construct, *GAL4* construct and the genomic rescue construct *HLH54F-genres*, as well as the molecularly defined breakpoints (dashed lines) of *Df(2R)14H10W-21* and the P-excision alleles *HLH54F*$^{\Delta 219}$ and *HLH54F*$^{\Delta 598}$.

Skirball Institute, New York, USA), rabbit anti-GFP (1:2000, Molecular Probes), monoclonal mouse anti-FasIII (7G10, 1:25) and anti-Wg (4D4, 1:10) (Developmental Studies Hybridoma Bank, University of Iowa, USA), guinea pig anti-Doc2/3 (1:500) (Reim et al., 2003), rabbit anti-Zfh1 (1:1000) (Broihier et al., 1998), rabbit anti-β-gal (1:1500, Promega) and mouse anti-β-gal (1:200, Sigma). TUNEL labeling was performed as described (Reim et al., 2003). Rhodamine-conjugated phalloidin (1:50, Molecular Probes) was used as described (Swan et al., 2004). The following digoxigenin-labeled RNA probes were used: *HLH54F* and *bin* (Zaffran et al., 2001), *beat-IIa* (T7 RNA polymerase transcript of *Eco*RI/*Xba*I fragment from EST RE17794 in pBluescript KS+), *byn* [from a PCR-derived fragment using primers byn-5′ CTCATAACT-CTCCGTCGCCGACGAATGGAT and byn-3′ ATCACTGCAGCG-TGCTCTGTCGCGGAGTGA in the TOPO-II TA vector (Invitrogen)]. Mutant embryos were identified with *lacZ* or *GFP* balancers.

## RESULTS

### *HLH54F* encodes a conserved bHLH transcription factor and is driven by an intronic enhancer in the CVM

*Drosophila HLH54F* encodes a bHLH transcription factor and sequence alignments of the bHLH domain show that it is orthologous to a single gene in *Tribolium* and to the vertebrate paralogs *capsulin* (also known as *Tcf21*, *Pod-1*, *epicardin*) and *musculin* (also known as *MyoR*) (Fig. 1A,B). Within *Drosophila*, the closest bHLH domains belong to Hand, Fer1 and Twi (Fig. 1A,B). Notably, all of these *Drosophila* and vertebrate genes, with the exception of neuronally expressed *Fer1*, are prominently expressed in mesodermal tissues (Georgias et al., 1997; Hidai et al., 1998; Lu et al., 1998; Quaggin et al., 1998; Robb et al., 1998a; Robb et al., 1998b; Lu et al., 1999; Quaggin et al., 1999; Tomancak et al., 2002). To obtain a useful tool for examining the genetic function of *HLH54F* in the developing CVM of *Drosophila* embryos, which expresses *HLH54F* (Georgias et al., 1997), as well as for analyzing the development of this tissue in general, we aimed to generate *lacZ* reporter constructs that reflect this expression pattern. *HLH54Fb-lacZ*, which spans the two introns (Fig. 1C), was found to recapitulate the endogenous *HLH54F* expression pattern in the CVM. Reporter gene activity first appeared during gastrulation in a small domain abutting the posterior-most border of the gastrulation furrow, which reflects the early expression of *HLH54F* mRNA at the posterior tip of the mesoderm anlage during late blastoderm stages (Fig. 2A,B). This group of cells is known as the CVM primordium (Campos-Ortega and Hartenstein, 1997; Kusch and Reuter, 1999). During germ band elongation, the CVM cells marked by *HLH54Fb-lacZ* and *HLH54F* mRNA were internalized and occupied a position within the posterior bend formed by the posterior midgut (PMG) rudiment (stage 9; Fig. 2C,D). Subsequently, the CVM cells from this cluster migrated anteriorly along the dorsal and ventral margins of the TVM and spread along its entire length (Fig. 2E,F; see also below). Eventually the CVM cells fused with fusion-competent cells from the TVM to form multinucleated longitudinal midgut muscles (Fig. 2H; compare with Fig. 2G).

The early steps of cell migration were examined in more detail by stainings of *HLH54Fb-lacZ* embryos in the context of the neighboring tissue primordia, namely the Twi-labeled trunk mesoderm and the Hb9-labeled endoderm of the PMG rudiment (Thisse et al., 1988; Broihier and Skeath, 2002). At stage 9, the

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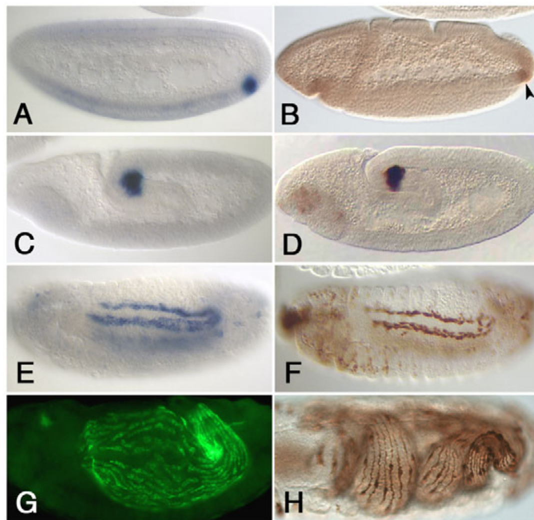


**Fig. 2. An *HLH54F-lacZ* reporter recapitulates the expression of *HLH54F.*** Wild-type *Drosophila* embryos hybridized with *HLH54F* digoxygenin RNA (A,C,E,G) and comparable *HLH54Fb-lacZ* embryos stained with β-galactosidase (β-gal) antibodies (B,D,F,H). (**A**,**B**) Blastoderm stage embryo (stage 5, A) and embryo at early gastrulation (stage 6, B). (**C**,**D**) Stage 9 embryos during germ band elongation. (**E**,**F**) In stage 13 embryos, the cells expressing *HLH54F* mRNA (E) and β-gal (F) have migrated anteriorly and spread along either side of trunk visceral mesoderm. (**G**) Stage 15 embryo with fluorescent detection of *HLH54F* mRNA, showing newly formed and elongating syncytia distributed over the midgut. (**H**) Stage 16 embryo showing *HLH54F-lacZ*-stained longitudinal muscle fibers.

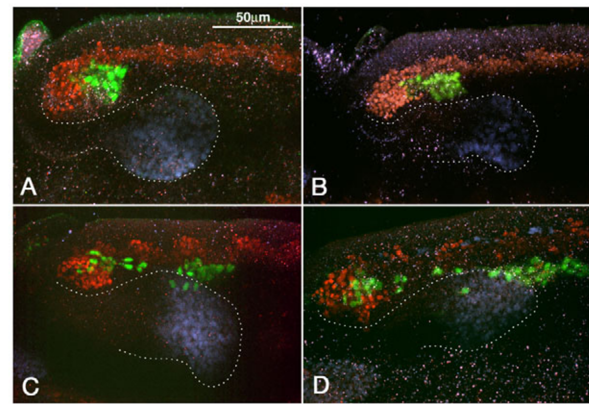


**Fig. 3. Early phase of migration of caudal visceral mesoderm cells.** Sagittal views of posterior portion of elongated germ bands of *HLH54Fb-lacZ Drosophila* embryos stained with antibodies against β-gal (green; CVM), Twist (red; trunk mesoderm) and Hb9 (blue; midgut endoderm). (**A**) In stage 9 embryos, the posterior portion of the mesoderm bends around internally such that the CVM becomes positioned internally and slightly more anteriorly. (**B**) At stage 10, the CVM separates from the remaining mesoderm. (**C**) At late stage 10, individual cells leave the CVM clusters and start migrating anteriorly between posterior midgut (dotted outline) and trunk mesoderm. (**D**) By the end of stage 11, most CVM cells have surpassed the migrating endoderm anteriorly and are associated with trunk mesoderm.

posterior portion of the mesoderm is U-shaped, perhaps because it is being bent around and drawn anteriorly on the inside by the invaginating PMG (Fig. 3A). During gastrulation *twi* and *HLH54F* were co-expressed, whereas during germ band elongation *twi* was downregulated in the *HLH54F*-expressing CVM cells, which led to mutually exclusive expression domains of the two bHLH-encoding genes after stage 8 (Fig. 3A). During stage 10, the CVM cell cluster became disconnected and moved slightly anteriorly (Fig. 3B), which was accompanied by a split into two bilaterally symmetric clusters (see Fig. 4A). At stage 10, cells became individualized and left the clusters to migrate anteriorly between the trunk mesoderm and the PMG, and ultimately towards more anterior regions of the mesoderm (Fig. 3C,D). A smaller group of CVM cells remained in the posterior portion of the mesoderm (Fig. 3D).

### *HLH54F* mutations generated by imprecise P excisions and EMS mutagenesis

To create null alleles of *HLH54F* a P element, *EY06760*, located downstream of *HLH54F* and just inside the 5′UTR of *CG5009* (Fig. 1C) was excised. Data from Mohr and Gelbart on deficiencies with defined breakpoints at 54EF indicated that loss of *HLH54F* may cause semi-lethality (Mohr and Gelbart, 2002). Indeed, two of our excision chromosomes that caused semi-lethality in trans with two of these deficiencies, *Df(2R)14H10W-21* and *Df(2R)02B10Y-12*, carried deletions of *HLH54F* coding sequences (Fig. 1C, $HLH54F^{\Delta 219}$ and $HLH54F^{\Delta 598}$). Because the deletions in both alleles also remove a small portion of the 5′UTR of *CG5009*, we generated a rescue construct encompassing the entire *HLH54F* locus (Fig. 1C). Our observation that this genomic construct can rescue the semi-lethality to full viability and also rescues the observed loss of CVM (see below) in a $HLH54F^{\Delta 598}$ background demonstrate that the phenotypes described herein with this allele are solely due to the loss of the *HLH54F* gene.

We also obtained two ethyl methane sulfate (EMS)-induced mutations in *HLH54F* from an ongoing forward genetic screen for mutations affecting the specification and/or migration of the CVM (see Materials and methods). Both alleles, $HLH54F^{S0323}$ and $HLH54F^{S1750}$, are semi-lethal in trans to the P-excision allele $HLH54F^{\Delta 598}$ and show the same CVM phenotype. Whereas $HLH54F^{S0323}$ is a deletion of *HLH54F* and flanking genes, a point mutation in $HLH54F^{S1750}$ causes a change in a conserved Arg to a Trp within the DNA-binding basic domain (Fig. 1A). The equivalent residue (Arg33) of another bHLH protein, Max, is known to contact the phosphate backbone of the DNA (Ferre-D'Amare et al., 1993). Thus, based upon the molecular and genetic data, $HLH54F^{S1750}$ and the P-excision alleles appear to be functional nulls. Transheterozygotes of the P-excision alleles with the EMS alleles or with larger deficiencies at the *HLH54F* locus, in which the effects of any second site mutations are eliminated, occur as adult escapers at less than 10% of the expected frequency and cannot be grown as a stock.

### *HLH54F* is required for proper specification and migration of the CVM

Based on its expression pattern, it was anticipated that *HLH54F* would play an important role in the development of the CVM and longitudinal midgut muscles. The genes for the Dorsocross T-box factors are normally expressed in the CVM between stages 10 and 12 (Fig. 4A,C,C′) (Reim et al., 2003), but were not turned on in the caudal mesoderm in *HLH54F* mutant backgrounds (Fig. 4B,D,D′;

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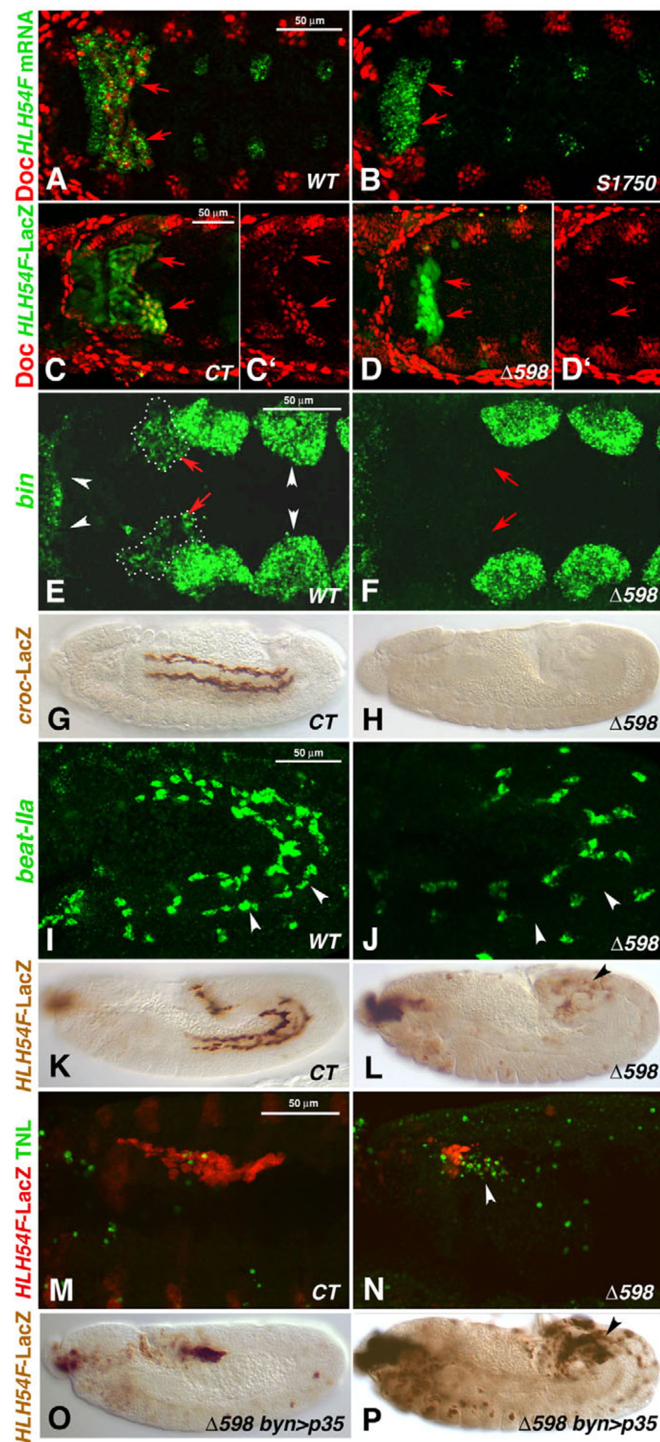


**Fig. 4. Embryonic *HLH54F* mutant phenotypes.** (A,C,C′,E,G,I,K,M) Wild-type (WT) or control (CT) *Drosophila* embryos and (B,D,D′,F,H,J,L,N-P) *HLH54F* mutant embryos (alleles as indicated). (A-F) Dorsal views; (G-P) lateral views. (**A**) Stage 10 WT embryo stained for Doc protein in bilateral CVM clusters (arrows, red) and *HLH54F* mRNA (green). (**B**) *HLH54F*$^{S1750}$ embryo stained as in A, which lacks Doc signals specifically in the CVM areas marked by mutant *HLH54F* mRNA (arrows). (**C**,**C′**) Stage 10 *HLH54Fb-lacZ* embryo stained for Doc protein (red, shown singly in C′) and β-gal (green) in CVM (arrows). (**D**,**D′**) *HLH54F*$^{\Delta 598}$ embryo with *HLH54Fb-lacZ* stained as in C is missing Doc expression in β-gal-stained caudal mesoderm. (**E**) Late stage 10 WT embryo stained for *bin* mRNA in the CVM (red arrows, dotted outlines) and in TVM and HVM primordia (white arrowheads). (**F**) *HLH54F*$^{\Delta 598}$ mutant, in which *bin* mRNA is missing in corresponding areas. (**G**) Stage 14 WT embryo expressing *croc-lacZ* in migrating CVM cells. (**H**) *HLH54F; croc-lacZ* mutant without *croc-lacZ* expression. (**I**) Stage 12 embryo showing *beat-IIa* mRNA in migrating CVM cells adjacent to the TVM (arrowheads) and in unidentified cells. (**J**) *HLH54F*$^{\Delta 598}$ mutant embryo without *beat-IIa* mRNA signals adjacent to the TVM. (**K**) Stage 12 control embryo expressing *HLH54Fb-lacZ* in the migrating CVM. (**L**) Stage 12 *HLH54F*$^{\Delta 598}$ mutant embryo expressing *HLH54Fb-lacZ*, showing a large decrease in the number of positive cells, which are not migrating (arrowhead). (**M**) Early stage 12 *HLH54Fb-lacZ* control embryo stained for β-gal (CVM, red) and by TUNEL assay (green), which shows few apoptotic cells in CVM areas. (**N**) Stage 12 *HLH54F*$^{\Delta 598}$, *HLH54Fb-lacZ* mutant embryo with many TUNEL-positive cells (arrowhead). (**O**) Stage 11 *HLH54F*$^{\Delta 598}$ mutant embryo expressing *HLH54Fb-lacZ* and *byn-GAL4*-driven anti-apoptotic p35 with normal numbers of internalized *lacZ*-positive cells (see Fig. 2D). (**P**) Stage 12 *HLH54F*$^{\Delta 598}$ mutant embryo with apoptosis inhibition as in O and an increased number of *HLH54Fb-lacZ*-containing cells as compared with mutants without forced p35 expression (see L), which fail to migrate and differentiate.

note that *HLH54F*$^{S1570}$ mRNA and *HLH54Fb-lacZ* were still expressed, which rules out autoregulation and allows identification of the cells that normally become CVM). Likewise, *bin*, a FoxF gene that is normally expressed in all three types of visceral mesoderm (Fig. 4E) (Zaffran et al., 2001), failed to be expressed in the CVM in *HLH54F* mutants, whereas it was still expressed in the TVM and hindgut visceral mesoderm (HVM) (Fig. 4F). Other markers of the migrating CVM, such as *croc-lacZ*, which reflects the CVM expression of the forkhead domain gene *crocodile* (*croc*) (Häcker et al., 1995), the Ig domain-encoding gene *beaten path IIa* (*beat-IIa*) (Tomancak et al., 2002), the enhancer trap expression of a *sloppy paired* (*slp*) reporter (*slp-m4-lacZ*) (Lee and Frasch, 2000), and expression of *byn* after stage 10, were also absent in the corresponding areas of *HLH54F* mutants (Fig. 4G-J; data not shown). The only marker of CVM primordia not affected in *HLH54F* mutants was the high-level expression of the zinc-finger homeodomain transcription factor Zfh1 (see Fig. S1 in the supplementary material), although, as for *byn*, *zfh1* expression also disappeared prematurely after stage 10. The loss of all tested CVM markers at all stages in the absence of *HLH54F* (with the exception of early Zfh1) and the observation that the cells that would normally form CVM failed to undergo any rearrangements and movements (Fig. 4B,D,D′; compare with Fig. 4A,C,C′) suggest that *HLH54F* is required for the specification of the CVM and show that development of the longitudinal visceral muscles is critically dependent on *HLH54F* function.

It has been observed that the loss of cell fate specification can cause either cell fate transformation or cell death (reviewed by Bonini and Fortini, 1999; Mann and Morata, 2000; Werz et al., 2005). Hence, we used an *HLH54Fb-lacZ* reporter to address the fate of the caudal mesodermal cells that are no longer identified as CVM in the absence of *HLH54F*. Following stage 10, we observed a dramatic decrease in the number of *HLH54Fb-lacZ*-positive cells and a total absence of migrating cells in the *HLH54F* mutants (compare Fig. 4L with 4K). Apoptosis of these particular cells was demonstrated in a TUNEL assay, which showed TUNEL-positive cells containing traces of *lacZ* signals in the vicinity of the remaining *HLH54Fb-lacZ*-positive cells in stage 12 *HLH54F* mutants (Fig. 4N). This is in contrast to the virtual lack of TUNEL signals in *HLH54Fb-lacZ*-positive cells in control embryos (Fig. 4M).

DEVELOPMENT

To examine whether any possible cell fate transformations or migratory abilities of the *HLH54Fb-lacZ*-positive cells in *HLH54F* mutant backgrounds are obscured by their death, we forced the expression of a pan-caspase inhibitor, p35 (BacA\p35), via *UAS-p35* and a *byn-GAL4* driver in these cells to prevent their apoptosis (Brand and Perrimon, 1993; Hay et al., 1994). Blocking apoptosis specifically in the CVM (along with the presumptive hindgut ectoderm) of *HLH54F* mutants resulted in a nearly normal-sized *HLH54F-lacZ*-positive domain (Fig. 4O; compare with Fig. 4C). However, these cells still did not migrate or differentiate into any kind of muscles and, instead, remained as unfused cells randomly distributed in the vicinity of the hindgut (Fig. 4P; data not shown). Thus, we infer that *HLH54F* is required not only for the survival of the CVM, but also for the initial specification and, in this context, for the correct migratory properties of these cells. In the absence of *HLH54F* activity, the failure of these cells to be specified apparently causes their entry into apoptosis.

The CVM primordia are thought to form the founder cells for all longitudinal visceral muscles. Apparently as a consequence of the absence of these muscles (see below), midgut constrictions were not formed efficiently and were present to a variable extent in the mutants, with a small fraction of mutant embryos lacking midgut constrictions altogether (Fig. 5A-C).

The larval longitudinal midgut muscles persist during metamorphosis and contribute to the longitudinal gut musculature of adult flies (Klapper, 2000). To address the question of whether the CVM is the sole source of founder cells for the longitudinal visceral muscles in larvae and adults, midguts from *HLH54F* mutant third-instar larvae and adult escapers, as well as from wild-type controls, were stained for F-actin and Tropomyosin. In wild-type animals, these stainings highlight the transverse circular midgut muscles as well as the longitudinal visceral muscles (Fig. 5D,F,H). By contrast, longitudinal visceral muscles were present neither in *HLH54F* mutant larvae nor in adult flies (Fig. 5E,G,I). At this level of resolution, we did not detect any clear alterations of the larval circular gut muscles and the midgut that would be caused by the absence of the longitudinal gut muscles (Fig. 5E). However, midguts isolated from mutant adults tended to be more curled into a spiral shape and smaller in diameter than their straighter wild-type counterparts (Fig. 5G,I). The adult mutant gut tube exhibited small bulges and frequently showed melanotic masses, similar to *hand* mutant flies (Lo et al., 2007), which suggests a tendency to rupture in vivo (Fig. 5G,I). In addition to the absence of support by longitudinal muscle fibers, this apparent fragility of adult midguts may be explained by the presence of highly disordered circular muscle fibers that showed frequent interruptions (Fig. 5G).

As HLH54F was isolated originally as a binding partner of the E-protein Daughterless (Da) (Georgias et al., 1997), we sought to obtain indications as to whether these two proteins might functionally interact in vivo during longitudinal muscle development. Indeed, RNAi knockdowns of *HLH54F* and *da* via *HLH54F-GAL4*-driven expression of the respective inverted repeat sequences caused similar phenotypes in the adult longitudinal gut musculatures, consisting of a variable reduction in the number and thickness of fibers as well as their uneven distribution around the midgut. In general, the effects appeared stronger upon knocking down both genes together (see Fig. S2 in the supplementary material). These data are indicative of functional interactions between these two bHLH proteins during longitudinal gut muscle development. Future studies need to address these interactions in more detail and compare them with the repressive effects on myogenesis reported for Da-Twi heterodimers (Castanon et al., 2001).

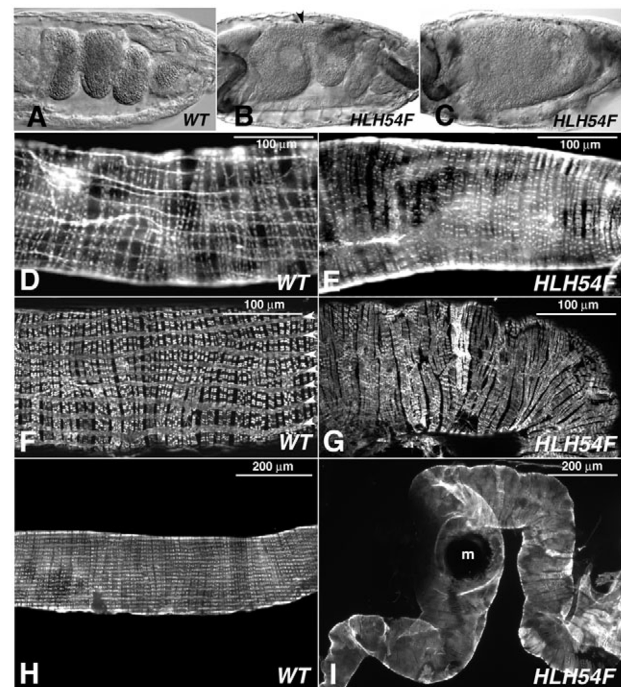


**Fig. 5. *HLH54F* mutant phenotypes of embryonic midgut and midgut musculature in larvae and adults.** (**A**) Stage 15 wild-type (WT) *Drosophila* embryo with constricted and looping midgut. (**B**) Stage 15 $HLH54F^{S1750}/HLH54F^{\Delta598}$ embryo with incomplete midgut constriction (arrowhead); also stained for GFP reporter for foregut and hindgut muscles, which are unaffected. (**C**) *HLH54F* mutant embryo as in B without any midgut constrictions. (**D**) Section of WT third-instar larval midgut stained with Rhodamine-conjugated phalloidin to visualize both the circular and the longitudinal visceral muscles. (**E**) Homozygous $HLH54F^{\Delta598}$ third-instar larval gut with absent longitudinal visceral muscles. (**F**) Section of WT adult midgut stained for Tropomyosin, showing regularly spaced longitudinal and circular muscle fibers. (**G**) Adult midgut section from a $HLH54F^{S1750}/HLH54F^{\Delta589}$ fly stained for Tropomyosin, showing the lack of longitudinal midgut muscles and circular muscles with irregular morphologies and interruptions. (**H**,**I**) WT (H, from F) and mutant (I, from G) adult midguts at lower magnification. Unlike the smooth and linear surface of WT midguts, midguts from mutants have an undulating surface and are curled. m, melanotic mass.

Based upon alterations in the mesodermal expression pattern of Even-skipped (Eve) and a disrupted Mef2 pattern in myocardial cells in *byn* mutant embryos, it has been proposed that the CVM plays a non-autonomous role in the normal development of adjacent dorsal muscle and cardiac progenitors during its migration (Kusch and Reuter, 1999). However, with our *HLH54F* mutants, which cause the specific loss of the CVM, we did not see any of the reported effects on the specification and morphogenesis of the heart and dorsal muscles, which seems to rule out a role of the CVM in the development of these tissues (see Fig. S1 in the supplementary material). It has also been proposed that germ cells require the CVM to direct their early migration from the basal surface of the PMG in anterior directions along the mesoderm (Broihier et al., 1998). Our observations in *HLH54F* mutants confirmed that the CVM does make the guidance of germ cells towards the gonadal mesoderm more reliable, although it is not absolutely required for this migration (see Fig. S3 in the supplementary material).

DEVELOPMENT

## HLH54F acts upon a CVM-specific Doc enhancer in combination with its downstream factor Bin

An exhaustive analysis of the Dorsocross (Doc) locus for enhancers (D.S., H. Jin, M.F. and I.R., unpublished) identified a 566 bp element, DocF4s1, that recapitulates the activity of the Doc genes (Fig. 6A,E,I). DocF4s1 contains five E-boxes and six forkhead domain binding motifs that are expected to bind HLH54F and Bin, respectively (Fig. 6I). Ectopic expression of *HLH54F* via *twi-GAL4* caused an expansion of DocF4s1 enhancer activity within the caudal mesoderm as well as into the head mesoderm, and analogous expression of *bin* caused similar, albeit less pronounced, effects (Fig. 6B,C). Notably, co-expression of *HLH54F* and *bin* caused much stronger effects, indicating that the products of the two genes cooperate in activating DocF4s1. The absence of ectopic expression in the trunk mesoderm points to the requirement for additional, as yet unknown activating or repressive factors.

To test whether the cooperative inputs of HLH54F and Bin upon DocF4s1 require the respective binding motifs for these proteins, we tested mutated DocF4s1 derivatives. Mutation of all five E-boxes caused a delay in the onset of enhancer activity until mid stage 11 (instead of late stage 10; data not shown) and a mild reduction in the number of CVM cells expressing the reporter (Fig. 6F). Likewise, upon mutation of all six Bin binding motifs, reporter gene activity in the CVM was delayed and occurred in even fewer cells and at lower levels (Fig. 6G). Importantly, simultaneous mutation of the binding motifs for both HLH54F and Bin led to a total loss of enhancer activity in the CVM, whereas the unrelated activity in bilateral clusters within the procephalic neurogenic ectoderm was still present (Fig. 6H). Altogether, these data provide strong evidence for a mechanism in which HLH54F and Bin cooperate in activating CVM-specific Doc expression via direct binding to this enhancer in a partially redundant fashion. Because *bin* activation in these cells requires HLH54F (Fig. 4F), this mechanism represents a feed-forward mode of regulation that might be responsible for the postponed onset of Doc expression in the CVM as compared with *HLH54F*.

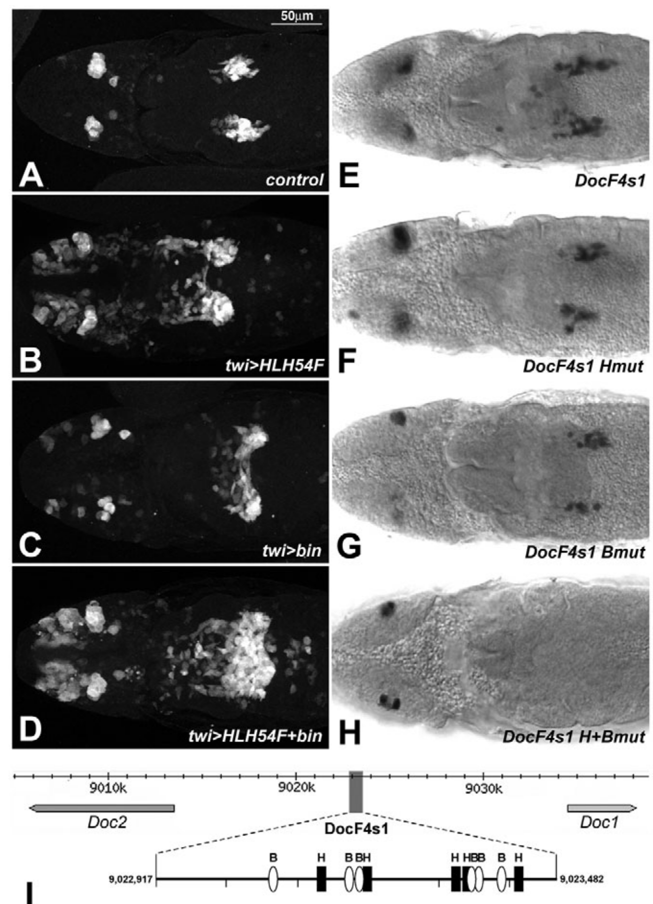


**Fig. 6. HLH54F and Bin as co-regulators of a caudal visceral mesoderm-specific enhancer from the Doc locus.** (**A-H**) *lacZ* (A-D) or nuclear *GFP* (E-H) reporter gene expression from DocF4s1 and derivatives is shown in early stage 12 *Drosophila* embryos as detected with anti-β-gal (diaminobenzidine) and anti-GFP (fluorescence) antibodies (dorsal views). (A) *DocF4s1-lacZ* in a wild-type background showing expression in bilateral CVM clusters at the beginning of cell migration (right). Additional activity is seen in bilateral clusters of the procephalic neuroectoderm (left). (B-D) *DocF4s1-lacZ* in embryos with *UAS-HLH54F* (B), *UAS-bin* (C) and *UAS-HLH54F* + *UAS-bin* (D), driven pan-mesodermally by *twi-GAL4*. (E) Control *DocF4s1-GFP* expression. (F) *DocF4s1 Hmut-GFP* expression (all E-boxes mutated). (G) *DocF4s1 Bmut-GFP* expression (all Bin binding motifs mutated). (H) *DocF4s1 H+Bmut-GFP* (all HLH54F and Bin binding motifs mutated). (**I**) Map of the DocF4s1 enhancer within the Doc locus and of HLH54F (H) and Bin (B) binding motifs within the enhancer.

## Ectopic expression of *HLH54F* is insufficient to drive CVM development

To test whether *HLH54F* is sufficient to turn other mesodermal derivatives into longitudinal midgut muscles or their progenitors, we expressed the gene ectopically under the control of the enhancers of *twi* and *held out wings* (*how*) in all myogenic cells (confirmed by in situ hybridization). However, we did not detect any ectopic longitudinal gut muscles in these embryos and in situ hybridizations for *croc*, a CVM marker downstream of *HLH54F*, failed to detect any ectopic *croc* expression. Notably, there was a reduction in the number of cardioblasts, and most of the somatic muscles were more rounded and often not properly attached to their tendon cells (see Fig. S4 in the supplementary material). In addition, the midguts failed to form any constrictions, which can in part be explained by the loss of Wingless expression in the TVM (see Fig. S4 in the supplementary material). Hence, although *HLH54F* is not sufficient to transform other mesodermal derivatives into longitudinal midgut muscles, its ectopic presence interferes with the normal development of various muscle tissues.

## *HLH54F* expression is regulated through a combination of posterior and ventral patterning genes

How is the expression of *HLH54F* in a small quadrant of cells established in the posterior portion of the presumptive mesoderm? Several genes are expressed in overlapping domains in the posterior region of the blastoderm embryo and are essential for the specification of various terminal structures (reviewed by Bronner and Jäckle, 1991; Lengyel and Iwaki, 2002). At the same time, Twi and Sna are expressed in the ventral-most region of the embryo and are required for mesoderm development (Kosman et al., 1991; Leptin, 1991; Ip et al., 1992). There are also regulatory interactions between the posterior gap genes and these ventrally expressed genes; in particular, the repression of *sna* by the terminal gap gene *huckebein* (*hkb*) defines the posterior border of the future mesoderm (Reuter and Leptin, 1994). As all these genes are known to play roles in the development of the CVM (Kusch and Reuter, 1999), we examined whether they act in this pathway via regulating the expression of *HLH54F*.

DEVELOPMENT

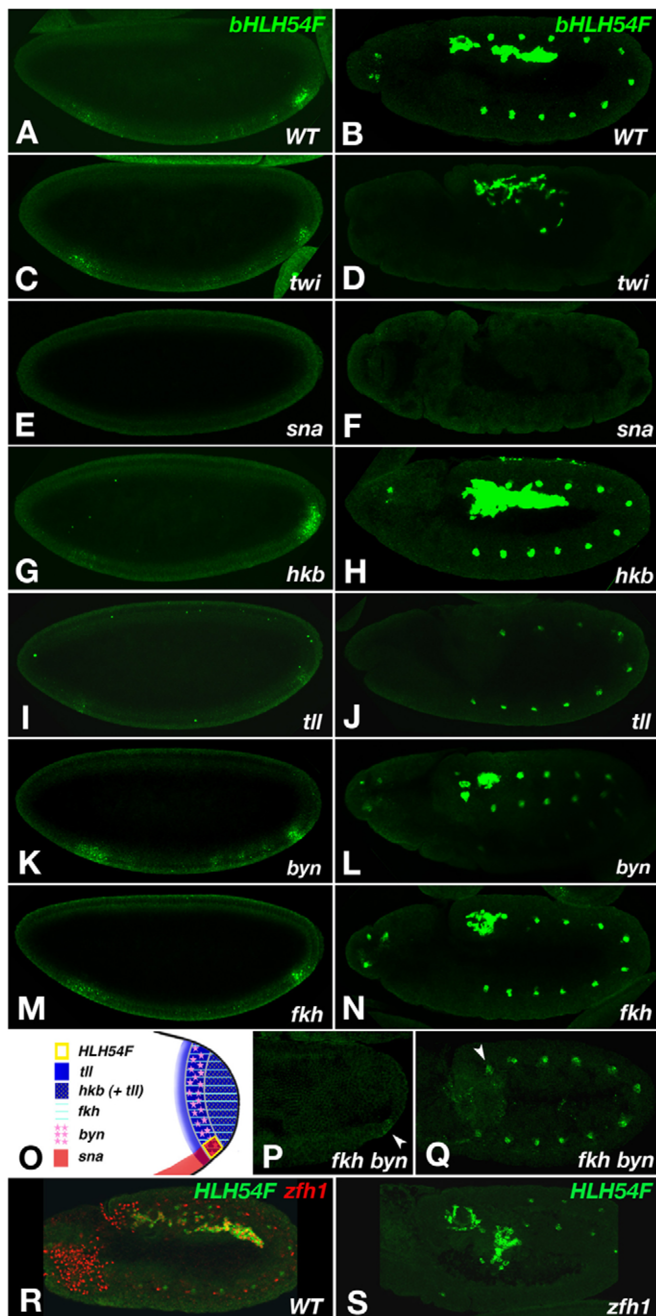


**Fig. 7. Regulation of early *HLH54F* expression.** *HLH54F* mRNA staining of late blastoderm stage *Drosophila* embryos (A,C,E,G,I,K,M,P) and in stage 11 embryos (B,D,F,H,J,L,N,Q,S,R) of wild type and mutants as indicated. (**A**,**B**) Wild-type *HLH54F* expression. (**C**,**D**) Slightly reduced *HLH54F* expression and mis-migrating CVM cells at stage 11 in *twi* mutants. (**E**,**F**) Complete absence of *HLH54F* expression in *sna* mutants. (**G**,**H**) Posterior expansion of *HLH54F* expression at blastoderm stage and increased CVM at stage 11 in *hkb* mutants. (**I**,**J**) Almost complete absence of *HLH54F* expression at blastoderm and absence of *HLH54F*-expressing CVM at stage 11 in *tll* mutants. (**K-N**) Reduced *HLH54F* expression domains in *byn* and *fkh* mutant blastoderm embryos and reduced *HLH54F*-labeled CVM at stage 11. (**O**) Schematic of the expression domains of the tested genes at late blastoderm stage. (**P**) Early gastrulation stage *fkh byn* double mutant showing only low, residual levels of *HLH54F* expression (arrowhead). (**Q**) Stage 11 *fkh byn* double mutant with very few *HLH54F*-expressing cells (arrowhead). (**R**) Early stage 12 wild-type embryo co-stained for *HLH54F* mRNA (green) and Zfh1 protein (red) in the migrating CVM. (**S**) Stage 12 *zfh1*-deficient embryo stained as in R. *HLH54F* is expressed in CVM cells, but cell migration is disrupted.

Initially, at the blastoderm stage, *twi* extends to the poles of the embryo and, therefore, overlaps with the CVM primordium. As shown in Fig. 7C, *HLH54F* mRNA was still expressed in the absence of *twi* function, albeit at reduced levels (see Fig. 7A). Furthermore, *HLH54F* expression was still observed in the migrating CVM in the *twi* mutant embryos (Fig. 7D; compare with Fig. 7B). These results indicate that *HLH54F* expression is not directly dependent on *twi* and confirm that the CVM does not require *twi* for ingression and initial migration [although the trunk mesoderm is required for proper pathfinding (Kusch and Reuter, 1999)]. *HLH54F* is also expressed in specific somatic muscle progenitors (where we have not detected any phenotype to date) (Fig. 7B) (Georgias et al., 1997). As expected, this expression was missing in *twi* mutants (Fig. 7D). In contrast to *twi* mutants, in the absence of *sna* there was no *HLH54F* mRNA expression in the CVM primordium at any time (Fig. 7E,F).

In *hkb* mutants, *HLH54F* expression was expanded towards the posterior (Fig. 7G), leading to an increased number of CVM cells as they started to migrate (Fig. 7H; compare with Fig. 7B). These data are in agreement with the known repressive activity of *hkb* on posterior *sna* expression and the involvement of *sna* in the activation of *HLH54F* expression, as shown above (see Fig. 7O for *hkb* and *sna* expression domains).

*tailless* (*tll*) is expressed in the posterior ~18% of the embryo (Fig. 7O) and is required for various structures derived from this expression domain, including the anal pads, hindgut, Malpighian tubules and the CVM (Pignoni et al., 1990; Kusch and Reuter, 1999; Lengyel and Iwaki, 2002). Accordingly, in $tll^{L10}$ (a strong hypomorph), only trace amounts of *HLH54F* mRNA expression were observed at blastoderm stage, and during elongated germ band stages caudal mesodermal *HLH54F* expression was lost completely (Fig. 7I,J). *tll* has been shown to act upstream of *byn* and *fkh*. *byn* encodes a T-box transcription factor that is expressed in a posterior stripe and is required for development of the hindgut, anal pads and Malpighian tubules (Fig. 7O) (Kispert et al., 1994; Singer et al., 1996). This domain of *byn* expression is established through activation by *tll* and repression by *hkb* at the posterior pole, which confines *byn* expression to within ~7.5-15% egg length (Pignoni et al., 1990; Singer et al., 1996). *fkh* encodes a winged helix transcription factor that is expressed in a slightly smaller, more posterior domain than *tll* (0 to ~15% egg length) that will form the future PMG and hindgut (Weigel et al., 1989). *byn* and *fkh* mutants showed a similar reduction in size of the domain of *HLH54F* expression during blastoderm and elongated germ band stages (Fig. 7K-N). In double mutants for *byn* and *fkh*, the reduction of *HLH54F* was much more severe than with the single mutants, indicating that these two genes synergize in activating *HLH54F* (Fig. 7P,Q).

The zinc-finger homeodomain-encoding gene *zfh1* is expressed preferentially in the CVM (and hematopoietic mesoderm) during early mesoderm development (Fig. 7R) (Lai et al., 1991; Broihier et al., 1998). In embryos lacking *zfh1*, *HLH54F* was expressed normally, but the migration of the CVM cells was aberrant (Fig. 7S).

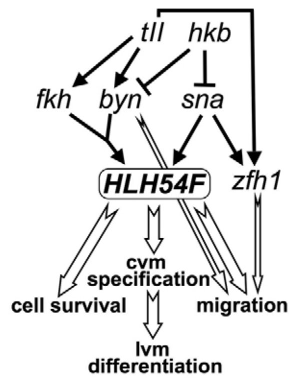


**Fig. 8. Regulatory interactions during caudal visceral mesoderm development.** There is high-level expression of *zfh1* in presumptive caudal visceral mesoderm. Although shown separately, the developmental outputs downstream of *HLH54F* are intimately connected. Black arrows, gene regulation; white arrows, developmental regulation. cvm, caudal visceral mesoderm; lvm, longitudinal visceral muscle.

Altogether, these data show that *HLH54F* expression is positively regulated by ventrally active *sna* (which itself is excluded from the posterior ~7.5% of the embryo by *hkb*) as well as by the synergistic action of the terminal genes *byn* and *fkh*.

## DISCUSSION

*HLH54F* is a key regulator in the CVM, a population of cells in which the bHLH gene *twi* appears to have only minor functions. Although *twi* is initially co-expressed with *HLH54F* in these cells, it makes only a small contribution to activating *HLH54F* expression, and the expression of both bHLH genes rapidly becomes mutually exclusive. Instead of *twi*, the activation of *HLH54F* in the CVM primarily involves the combined activities of mesodermal *sna* and the terminal genes *fkh* and *byn* (Fig. 8). As *sna* is generally thought to act as a ventral repressor of non-mesodermal genes in early mesoderm development, it will be interesting to determine whether the positive requirement for *sna* in the activation of *HLH54F* expression is direct, which would be unique to date. Alternatively, *HLH54F* might be activated by high levels of nuclear Dorsal and repressed by lateral genes that are repressed by *sna* ventrally. Along the anteroposterior axis, the posterior border of *HLH54F* expression is apparently defined by the posterior expression border of *sna*, which is delineated by the repressive action from *hkb*. We propose that the anterior border of *HLH54F* is determined by near-maximal threshold levels of *tll*, the expression of which declines steeply in the area anterior to the *HLH54F* domain (Pisarev et al., 2009). However, *tll* acts largely indirectly, through the combined activities of its downstream genes *byn* and *fkh*, in activating *HLH54F* (Fig. 8). The low residual levels of *HLH54F* mRNA in *fkh byn* double mutants suggest the involvement of direct inputs from additional posterior activities, possibly *tll* or maternal *torso*. From the data shown herein and elsewhere (Hemavathy et al., 1997; Kusch and Reuter, 1999), it appears that high-level expression of *zfh1* in the CVM largely depends on *tll* and *sna*, whereas *HLH54F* and *zfh1* do not depend on one another.

Notably, neither *twi* nor *HLH54F* is required individually for the internalization of the CVM cells during gastrulation (Kusch and Reuter, 1999) (this report), although we cannot exclude a redundant function. We have shown that the posterior portion of the mesoderm, which includes the CVM and portions of the presumptive HVM, bends around during gastrulation to form a second, internal mesodermal layer. It is conceivable that this movement is a passive process brought about by the invagination of the PMG rudiment. However, for subsequent migrations of CVM cells from these positions, the activity of *HLH54F*, but not *twi*, is crucial. In addition, *byn*, *zfh1* and *fkh* are required for normal migration after stage 10 (Kusch and Reuter, 1999) (this report). Whereas their respective functions are likely to be cell-autonomous, the observed requirement of *twi* for normal pathfinding of CVM cells is likely to be due to the absence of the migration substrate normally formed from the trunk mesoderm.

Our genetic data show that, after the caudal mesodermal cells have ingressed in this manner, they do not develop any further in the absence of *HLH54F* activity and undergo apoptosis. In the normal situation, *HLH54F* is needed for the activation of several transcription factor-encoding genes at this stage, including *bin*, *croc* and the Doc genes. Although the functions of these genes in CVM development have not been defined, it is likely that they regulate specific aspects of CVM development downstream of, and perhaps in combination with, *HLH54F*. The data from loss-of-function and ectopic expression analyses of *HLH54F* show that this gene is essential, but not sufficient, for specification of longitudinal gut muscle founders. Parallel inputs, albeit less pervasive, appear to come from high-level *zfh1*, which like *HLH54F* is required for *croc*/*croc-lacZ* expression (Kusch and Reuter, 1999). Altogether, we propose that *HLH54F* is necessary for activating the vast majority of early CVM-specific genes, with one known exception being high-level *zfh1*, and that *zfh1*, *byn* and *fkh* in various combinations act together with *HLH54F* to activate certain targets during the specification and early migration of CVM cells.

The continuous expression of *HLH54F* in the CVM and longitudinal gut muscles suggests that this gene is not only required for specification, but is also directly involved in many other developmental processes, including the continued migration, myoblast fusion and differentiation of the CVM cells. Possible downstream targets of *HLH54F* in the promotion of proper cell migration include *beat-IIa*, which encodes an as yet uncharacterized membrane-anchored Ig domain protein, and the FGF receptor-encoding gene *heartless* (*htl*), which is known to be required for normal migration (Pipes et al., 2001; Mandal et al., 2004). In this context, it is interesting that the vertebrate orthologs of *HLH54F* are expressed prominently in specific migrating populations of mesodermal cells as well. For example, *musculin* is expressed in myoblasts at the myotomal lips that migrate into the developing limbs, and *capsulin* is expressed in the migrating pro-epicardial cells (e.g. von Scheven et al., 2006). Therefore, it is possible that parts of the regulatory circuit in the control of cell migration have been conserved, even though they occur in different mesodermal cell types. *capsulin* is also expressed prominently in the splanchnopleura and tissues derived from it, including the developing smooth muscles of the stomach and gut (Hidai et al., 1998; Robb et al., 1998b; von Scheven et al., 2006). Therefore, *HLH54F* and *capsulin* might share some functions in the terminal differentiation of the respective gut musculatures in the different systems. Both Capsulin and Musculin have been characterized largely as repressors. However, their activity (and likewise that of HLH54F) as repressors versus activators might well be context specific with respect to the particular enhancer, tissue or developmental stage in question and might depend on the relative concentrations of particular heterodimerization partners (Lu et al., 1999; Miyagishi et al., 2000; Castanon et al., 2001).

Based on the phenotype of *byn* mutants, a role of the CVM in promoting midgut constrictions has also been proposed (Kusch and Reuter, 1999). The phenotype of *HLH54F* mutants confirms this effect, although we find that partial constrictions can frequently occur and that the effect is variable. We infer that the physical interactions between developing longitudinal and circular muscle fibers are necessary to provide the full force required for the efficient constriction of the midgut endoderm at the well-defined signaling centers. In the fully developed midgut, scanning electron microscopy images have revealed that the longitudinal fibers are tightly interwoven with the web-shaped circular fibers, which may explain the mechanical strength of this meshwork. Indeed, we find that the mechanical stability and integrity of the midgut, particularly in *HLH54F* mutant adults, are severely compromised.

In summary, *HLH54F* appears to sit at the top of the regulatory hierarchy of CVM development and is likely to fulfil additional key roles during the course of development of the CVM and the longitudinal gut muscles. Future efforts need to be directed towards dissecting additional downstream events that regulate the different steps of cell migration, myoblast fusion, morphogenesis and terminal differentiation of the longitudinal midgut musculature.

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
Supplementary material for this article is available at
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DEVELOPMENT

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DEVELOPMENT