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Dimerization partners determine the activity of the Twist bHLH protein during *Drosophila* mesoderm development

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SUMMARY

The basic helix-loop-helix transcription factor Twist regulates a series of distinct cell fate decisions within the *Drosophila* mesodermal lineage. These *twist* functions are reflected in its dynamic pattern of expression, which is characterized by initial uniform expression during mesoderm induction, followed by modulated expression at high and low levels in each mesodermal segment, and finally restricted expression in adult muscle progenitors. We show two distinct partner-dependent functions for Twist that are crucial for cell fate choice. We find that Twist can form homodimers and heterodimers with the *Drosophila* E protein homologue, Daughterless, in vitro. Using tethered dimers to assess directly the function of these two particular dimers in vivo, we show that Twist homodimers specify mesoderm and the subsequent allocation of mesodermal cells to the somatic muscle fate. Misexpression of Twist-tethered homodimers in the ectoderm or mesoderm leads to ectopic somatic muscle formation overriding other developmental cell fates. In addition, expression of tethered Twist homodimers in embryos null for *twist* can rescue mesoderm induction as well as somatic muscle development.

Loss of function analyses, misexpression and dosage experiments, and biochemical studies indicate that heterodimers of Twist and Daughterless repress genes required for somatic myogenesis. We propose that these two opposing roles explain how modulated Twist levels promote the allocation of cells to the somatic muscle fate during the subdivision of the mesoderm. Moreover, this work provides a paradigm for understanding how the same protein controls a sequence of events within a single lineage.

Key words: Muscle, Myogenesis, *daughterless*, Tethered dimers, Twist, *Drosophila melanogaster*

INTRODUCTION

During development of the *Drosophila* mesoderm, the transcription factor Twist (Twi) directs several critical cell fate decisions. Yet how this transcription factor achieves different outcomes within the mesodermal lineage is unclear. Initially, Twist is required for specification of the mesoderm (Simpson, 1983; Leptin, 1991), activating a set of defined genes, notably the FGF receptor *heartless* (*htl*), which is required for mesodermal migration (Shishido, 1993; Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997), *tinman* (*tin*), which is critical for heart and gut muscle development (Azpiazu and Frasch, 1993; Bodmer, 1993), and *Mef2*, which is required for all muscle differentiation (Lilly et al., 1995; Bour et al., 1995; Taylor et al., 1995). Subsequently, in response to regulation by segmentation genes, Twist levels are modulated into stripes of low and high expression within the segment (Azpiazu et al., 1996; Riechmann et al., 1997; Bate and Rushton, 1993). Mesodermal cells expressing low Twist levels give rise to tissues such as visceral muscle, fat body and gonadal mesoderm, whereas cells expressing high Twist levels eventually develop into heart and body wall muscles (Dunin-Borokowski et al., 1995; Baylies and Bate, 1996). When Twist levels are artificially kept higher throughout the mesoderm, differentiation of visceral muscle, fat body, heart and gonadal mesoderm is blocked. Progenitors of these cell types develop into body wall muscle. Thus, high Twist levels direct cells to adopt body muscle fate and low levels are permissive for the execution of other tissue fates (Baylies and Bate, 1996). Later, Twist levels decline, first dorsally, then ventrally and finally laterally until Twist is only found in a subset of cells that mark the progenitors of the adult muscles (Bate et al., 1991; Currie and Bate, 1991; Fernandes et al., 1991). At these stages, Twist is required for patterning a subset of larval muscles and also for the correct differentiation of the adult musculature (Cox and Baylies, 2001; Cripps and Olson, 1998; Anant et al., 1998). Thus, during *Drosophila* development, Twist is a multifunctional protein that controls a sequence of important decisions within the same lineage.

Twist belongs to a family of transcription factors characterized by the basic-helix-loop-helix (bHLH) motif (Murre et al., 1989a; Thisse et al., 1988). These factors participate in many developmental decisions, including sex determination, neurogenesis, segmentation and myogenesis (for review see Jan and Jan, 1993). The HLH region mediates protein dimerization whereas the basic region is necessary for HLH dimers to contact DNA (Murre et al., 1989a; Murre et al., 1989b). Different classes of bHLH proteins act as either positive or negative transcriptional regulators; for example, in *Drosophila*, members of the *achaete-scute* complex are thought to heterodimerize with the Daughterless (Da) protein, bind to specific DNA sequences and activate target genes that promote neurogenesis (Campuzano et al., 1985; Cabrera and Alonso, 1991; Cronmiller et al., 1988; Caudy et al., 1988a; Caudy et al., 1988b), while Hairy is thought to exclusively form homodimers that repress transcription (Rushlow et al., 1989; Ohsako et al., 1994). Moreover, individual bHLH homodimers and heterodimer combinations differ in DNA binding affinity, target preference site and inferred biological activities, suggesting that partner choice is a key regulation point (Jones, 1990; Kadesch, 1993).

Several lines of experimentation suggest that Twist forms dimers that have distinct functions during development. Genetic studies have revealed two *twist* alleles ($twist^{v50}$ and $twist^{ry50}$) that are temperature sensitive only when in *trans* with one another. Neither allele is temperature sensitive by itself or over a deficiency, but $twist^{v50}/twist^{ry50}$ survive at 18ºC and die at 29ºC (Thisse et al., 1987). This suggests that the two mutant proteins form a temperature sensitive dimer. Temperature shift experiments indicate that this homodimer functions early, to direct specification of mesoderm (Thisse et al., 1987; Leptin et al., 1992), and during allocation of somatic muscle (Baylies and Bate, 1996). These results also suggest that Twist homodimers are capable of activating mesodermal and somatic muscle specific genes.

In vertebrates, the Twist protein is a negative regulator of differentiation in several lineages, including myogenesis and osteogenesis (Hopwood et al., 1989; Wolf et al., 1991; Fuchtbauer, 1995; Gitelman, 1997; Wang et al., 1997; Chen and Behringer, 1995; Howard et al., 1997; el Ghouzzi et al., 1997; Maestro et al., 1999). Mouse Twist inhibits myogenesis by titrating E proteins and by forming heterodimeric complexes that block DNA binding of E heterodimers with myogenic bHLH transcription factors like MyoD (Spicer et al., 1996; Hebrok et al., 1997).

Here, we show that, depending on dimer partner, Twist acts either as an activator or a repressor of somatic muscle development. We present evidence that Twist homodimers activate early mesoderm as well as the later allocation of mesodermal cells into the somatic muscle fate. Consistent with Da being expressed in the mesoderm (Cronmiller and Cummings, 1993), analysis of Da loss of function, dosage experiments with Twist and biochemical experiments indicate that heterodimers of Twist and Da repress somatic myogenesis. Hence dimer partners in vivo provide a key regulatory interaction which modifies Twist behavior throughout the development of the mesoderm. This work thus provides a model for understanding how a single transcription factor regulates multiple events in a single lineage in vivo.

MATERIALS AND METHODS

Drosophila stocks

Ectopic expression of bHLH proteins was achieved with the GAL4-UAS system (Brand and Perrimon, 1993) and the following stocks: *twist-GAL4; twist-GAL4* {2X twist-GAL4} (Baylies et al., 1995); *da-GAL4* (Wodarz et al., 1995); twi^{ID96}*/CyOftz-lacZ*; *twist-GAL4*, twi^{ID96}*/CyOftz-lacZ;* da^{II}*/CyOwg-lacZ*; *twist-GAL4*, da^{II}*/CyOftz-lacZ*; *UAS-twist; UAS-twist*; *UAS-twist* {2X UAS-twist} (Baylies and Bate, 1996). *UAS-twist,* twi^{ID9}*/CyOftz-lacZ; UAS-da* (a gift from J. Campos-Ortega); twi^{ID96}*/CyOftz-lacZ; UAS-twist-twist*; *UAS-twist-twist* and *UAS-twist-da*. *twist-GAL4* drives expression of UAS constructs within mesoderm, whereas *da-GAL4* drives expression ubiquitously. All crosses were performed at 25°C unless noted. The temperature sensitive da^1 allele was used to reduce both the maternal and zygotic Da levels (provided by C. Cronmiller). da^1/da^1 mutant embryos survive at 18°C and die at 25°C. Fly stocks carrying the 175 bp *Mef2* enhancer-*lacZ* construct were used to monitor Twist and Da activities (provided by R. Cripps and E. Olson). This *Mef2* enhancer contains two E-boxes, only one of which is essential for Twist activation both in tissue culture and in vivo (Cripps et al., 1998).

Transgenic lines carrying *UAS-twist-twist* and *UAS-twist-da* linked dimers were generated by injection of *yw* embryos, according to published procedures (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Six and seven independent transformant lines, respectively, were obtained and expanded into homozygous stocks.

Constructs

For in vitro transcription/translation reactions, pNB40 or pCDNA3 containing *twist* (provided by N. Brown) and *da* (provided by M. Caudy) cDNAs were used. To create a truncated Twist (bHLHTwist), *twist* cDNA was digested with *Bam*HI and then religated in frame, eliminating amino acids 141-331. To construct Twist-Twist and Twist-Da linked dimers, pCDNA3 containing a flexible polypeptide linker was used (provided by M. Markus and R. Benezra; M. Markus, 2000; Neuhold and Wold, 1993). The N-terminal Twist monomer was amplified by PCR and cloned as a blunt-ended fragment into pCDNA3, in frame with the flexible linker. The C-terminal Twist (or Da) monomer was also amplified by PCR and cloned in frame after the linker (see Figs 1B, 7C). Construct integrity was verified by sequencing. For P-element transformation, the tethered dimers were subcloned into pUAST (Brand and Perrimon, 1993). For tissue culture experiments, full-length *twist* cDNA was subcloned as a 1.7 kb *Hin*dIII-*Not*I fragment into pCDNA3, which contains the CMV promoter (Invitrogen). *da* cDNA and the linked dimers were subcloned into the same vector.

DNA binding assays

The Twist binding site from *rhomboid* (*rho*; GATCCCTCG-**CATATG**TTGAA) containing a canonical E-box (bold letters) was the probe in mobility shift experiments (Ip et al., 1992). The Da oligonucleotide (GATCCCTCG**CACCTG**TTGAA) was derived from the *rho* site and was engineered to match known Da binding sites (Ohsako et al., 1994). The mutated oligonucleotide for competition assays was GATCCCTCG**AGTATG**TTGAA. Oligonucleotides were annealed and labeled with digoxigenin, using the DIG gel shift kit (Roche Molecular Biochemicals). To test labeling efficiency, a dot blot was performed according to the manufacturer's protocol.

In vitro translated protein products (Promega TnT Kit) were incubated in 2 mM DTT at 37°C for 10 minutes (Markus, 2000). After a 5 minute equilibration to 25°C, protein products were added to 50 fmol of 3′ digoxigenin-labeled oligonucleotide probe. A typical 25 μl reaction mixture contained 1 μg poly d(I-C), 2 mM $MgCl_2$, 25 mM Hepes pH 7.5, 100 mM NaCl, 0.1% Igepal CA-630, 12% glycerol (v/v). The mixture was separated by electrophoresis at 10 Volts/cm through 5% polyacrylamide gel (acrylamide-bisacrylamide, 29:1-3.3%) in 0.25× TBE. The gel was transferred to a nylon membrane

(Roche Molecular Biochemicals) using a semi-dry transfer. The membrane was developed following the manufacturer's protocol.

Determination of apparent dissociation constants

Apparent K_ds were determined by gel shift analysis of Twist, Da and Twist/Da heterodimers in which equivalent amounts of input protein were mixed with a range of DNA concentrations of E-box oligonucleotides and binding was determined. A Hill plot [(free oligonucleotide) versus (bound oligonucleotide/1-bound oligonucleotide)] was prepared with the apparent K_d being equal to the X-intercept.

Cell culture and transfections

For transfection assays, the expression vector pCDNA3 containing *twist*, *da*, *twist-twist* or *twist-da* was used. A reporter construct containing a 175 bp *Mef2* enhancer (Cripps et al., 1998) was subcloned upstream of the *luciferase* gene in the pGL2 Basic Vector (Promega). Equal molar amounts of plasmids containing *twist*, *da*, *twist-twist* or *twist-da* were cotransfected with 3 μg reporter plasmid and 3 μg *actin-lacZ* plasmid to control for transfection efficiency (a gift from T. Lieber). The DNA concentration for each transfection was equalized by addition of pBluescript plasmid to a final concentration of 20 μg.

Schneider Line 2 cells (SL2; a gift from T. Lieber) were maintained at 25°C in Schneider's *Drosophila* medium (M3) supplemented with 12.5% fetal calf serum (FCS) and 1% penicillin/streptomycin solution. The day before transfection, cells were seeded at approx. 80% confluence into 12-well tissue culture plates (Falcon 3043). Cells were transfected with a ratio of 1:3 nucleic acid/DOSPER (Roche Molecular Biochemicals) mixture. After a 15 minute incubation at 25ºC, the mixture was added drop by drop to the cultures. 24 hours later, the medium was removed, 2 ml of fresh M3 containing 12.5% FCS and antibiotics were added, and the cells incubated for a further 24 hours prior to harvesting.

Transfected cells were harvested by scraping attached cells into culture medium and collecting all adherent and non-adherent SL2 cells by centrifugation. The cells were washed twice with 5 ml 1× PBS (phosphate-buffered saline), resuspended in 250 μl 1× lysis buffer (125 mM Tris pH 7.8, 10 mM EDTA, 10 mM DTT, 50% glycerol, 5% Triton X-100), and incubated at 25°C for 10 minutes. Cells were lysed by freezing and thawing. 30 μl of lysate from each transfection was assayed for Luciferase activity in a luminometer (LUMAT LB 9501; Berthold) using Luciferase assay reagent (Roche Molecular Biochemicals). 5 μl of lysate was measured for absorbance at 574 nm to determine the amount of β-galactosidase activity using the substrate chlorophenol-red-β-D-galactopyranoside monosodium salt. The data shown are mean values of at least three independent, triplicated transfections and are expressed as the fold activation obtained in each sample over luciferase activity generated by addition of pCDNA3 (control). Luciferase activity is normalized against β-galactosidase activity. Each construct was titrated to determine the linear range of activity. Selected points shown.

Immunocytochemistry

Embryos for immunocytochemistry were harvested at 25°C, following standard techniques for whole mounts (Rushton et al., 1995). Antibody dilutions were as follows: anti-myosin (1:1000; Keihart and Feghali, 1986); anti-Twist (1:5000; provided by S. Roth); anti-Even-skipped (1:800; provided by J. Reinitz and D. Kosman); anti-Fasciclin III (Fas III; also known as Fas3 (FlyBase); 1:100; Developmental Studies Hybridoma Bank, University of Iowa); anti-zfh1 (1:1000; provided by Z. Lai); anti-Krüppel (1:2000; provided by J. Reinitz and D. Kosman); anti-β-galactosidase (1:1000; Promega); anti-Heartless (1:2000; provided by A. Michelson); anti-S59; and anti-Bagpipe (1:500; provided by M. Frasch).

Double staining for anti-β-galactosidase and the appropriate second antibody were performed in the rescue experiment and whenever blue balancers were present. To compare intensity of staining for the da^1 experiments, wild-type embryos were mixed with the mutant embryo collection and processed together under identical conditions. Biotinylated secondary antibodies were used in combination with the Vector Elite ABC kit (Vector Laboratories, CA). For detection of Bap-antibody, we used a biotinylated secondary antibody and fluorescein (FICT)-labeled streptavidin. Specimens were embedded in Araldite. Images were captured using a DXC-970MD camera (Sony). Different focal planes were combined into one picture using Adobe Photoshop software.

RESULTS

Twist forms homodimers

To determine whether Twist acts as a homodimer, we used an electrophoretic mobility shift assay. bHLH proteins bind the E box consensus sequence, CANNTG, (Ephrussi et al., 1985) as dimers (Murre et al., 1989a; Murre et al., 1989b). For mobility shift experiments we used the E box and flanking region from the *rho* promoter, a target activated directly by Twist (Ip et al., 1992). As shown in Fig. 1A, Twist protein made by in vitro transcription/translation specifically binds oligonucleotides containing *rho* E boxes (CA**TA**TG). To determine if the bound form of Twist is a homodimer, a truncated form of Twist was constructed that lacked approximately 190 amino acids from the Twist amino terminus but retained DNA binding and dimerization domains. Fig. 1A shows that the truncated Twist (bHLH Twist) binds the *rho* E box. When full-length and truncated forms of Twist are mixed together, we detect a DNA-protein complex with intermediate mobility, which would be consistent with dimers composed of full-length and truncated forms of Twist.

Twist homodimers promote mesoderm and somatic muscle formation

We next tested whether Twist homodimers are functional in vivo. To increase formation of Twist homodimers, we physically linked two monomers by a flexible glycine-serine polylinker, which results in an increase in local concentration of these proteins and, therefore, favors dimer formation (Neuhold and Wold, 1993). This "tethered" dimer strategy has been used successfully by several groups including Neuhold and Wold (Neuhold and Wold, 1993) and Sigvardsson et al. (Sigvardsson et al., 1997) to determine the function and specificity of MyoD and E homodimers and heterodimers. Fig. 1B shows the configuration of the tethered Twist homodimer. The two Twist genes are joined in a head to tail arrangement. This tethered form of Twist bound the *rho* E-boxes and promoted activation of a *Mef2* muscle enhancer reporter construct (Cripps et al., 1998) in tissue culture, similarly to unlinked Twist dimers (Fig. 1C,D). Hence, both in in vitro assays and in tissue culture, the linked homodimers of Twist are capable of mimicking aspects of Twist function.

We then assayed whether this tethered form can replicate Twist's ability to induce mesoderm/somatic muscle in vivo. Previous studies have shown that over-expression of Twist in ectoderm and mesoderm leads to ectopic muscle formation (Baylies and Bate, 1996). Like the Twist monomer, over-expression of the tethered Twist dimer in the ectoderm led to a dramatic transformation of ectodermal cells into mesoderm and somatic muscle. We detected expression of muscle specific

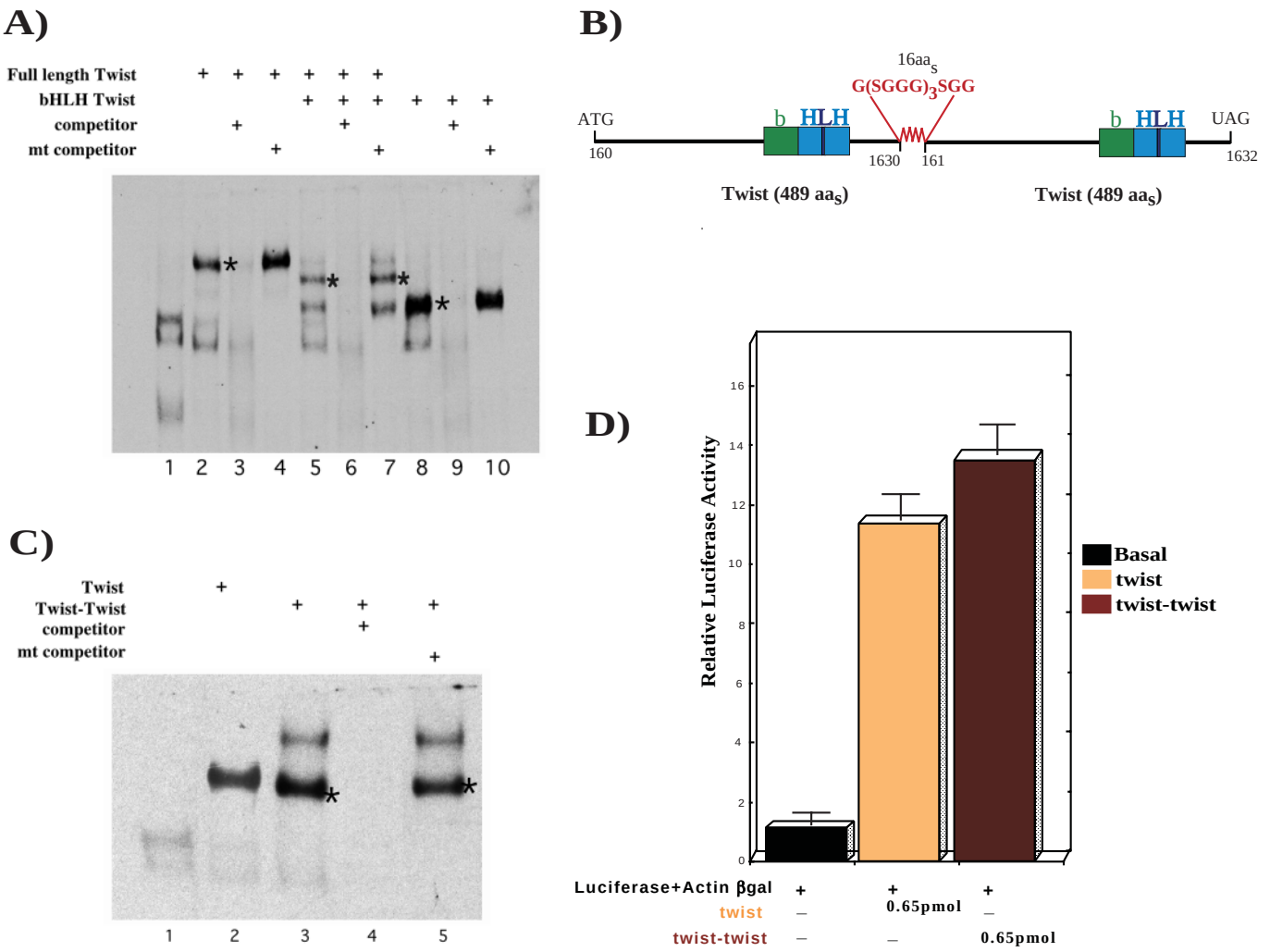

Fig. 1. Twist binds DNA and activates gene expression as a homodimer. **(**A)Mobility shift assays with *rho* E box (CA**TA**TG; Ip et al., 1992) were performed with full-length Twist (lanes 2, 3, 4), truncated Twist (bHLHTwist; lanes 8, 9, 10) or both (lanes 5, 6, 7). An * marks the full-length homodimer (lane 2) and the truncated Twist homodimer (lane 8). A mixture of full-length and the bHLH region of Twist produced an intermediate band shift (lanes 5, 7 *), corresponding to a full-length and bHLH Twist heterodimer. These bands are competed away with 200× unlabeled *rho* E box (lanes 3, 6, 9), but not with a mutated (mt) E box (AG**TG**TG) (lanes 4, 7, 10). The unprogrammed lysate is included in lane 1. Probe is in excess in all lanes in all shifts shown. (B) Schematic representation of two Twist proteins joined in-frame by a flexible 16 amino acid Gly/Ser rich linker (Markus, 2000; Neuhold and Wold, 1993). The boxes signify the basic, DNA-binding domain and the HLH dimerization domain. The linker sequence is given in single letter amino acid code. Numbers below the line refer to the nucleotide sequence of the *twist* cDNA (Thisse et al., 1988). (C) DNA binding properties of tethered Twist-Twist. In vitro translated products are assayed for binding to *rho* E box (CA**TA**TG). Twist-Twist linked dimers (*, lane 3 and 5) have the same DNA binding specificity as Twist alone (lane 2). The upper band in lanes 3 and 5 is a dimer of the linked dimers. These higher order complexes can be competed away easily upon addition of a bHLH monomer, leaving only the forced homodimer (data not shown). We have no evidence that this complex nor other complexes (the tethered dimer and another bHLH) are found in tissue culture or in vivo, yet this possibility does exist. (D) Twist and Twist-Twist linked dimers activate a 175 bp *Mef2* enhancer in *Drosophila* SL2 cells (Cripps et al., 1998). SL2 cells are transfected with indicated amounts of *actin-lacZ* plasmid, 175bp *Mef2* enhancer-luciferase reporter plasmid, equimolar amounts of Twist or Twist-Twist expression vector and carrier DNA to equalize total DNA/transfection. Activation values are expressed relative to controls (see Materials and Methods). Error bars represent the standard error of means of triplicated experiments. Transfection of Twist-Twist linked dimers results in slightly better reporter gene transactivation as compared to Twist monomers alone.

proteins such as myosin heavy chain (Mhc) in the most external cell layer. Many of the Mhc-expressing cells were multinucleate, a hallmark of somatic muscle formation (Fig. 2A,G,M; cf. Baylies and Bate, 1996). In addition, ectodermal tissues such as the nervous system and the epidermis were not produced in these embryos (not shown; cf. Baylies and Bate, 1996).

Over-expression of Twist monomers or tethered Twist homodimers in the mesoderm, using *twist-GAL4*, converted non-somatic mesoderm into somatic muscle. Ectopic multinucleated somatic muscles were detected dorsally where the heart normally forms and around the gut and central nervous system. For clarity, we show ventral views demonstrating the presence of these ectopic syncytial Mhc-positive muscles (Fig. 2B,H,N). Since individual muscles are

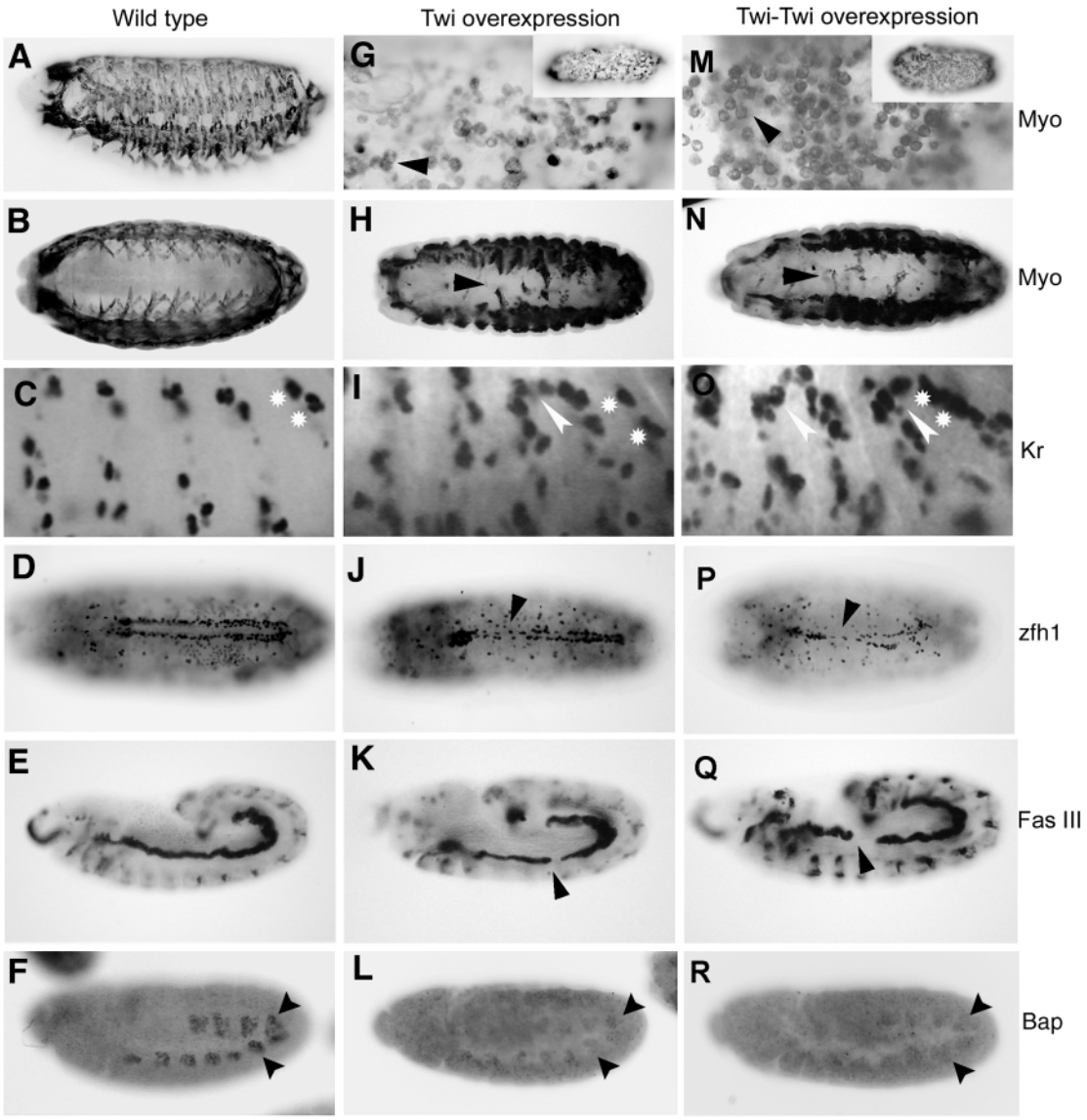

Fig. 2. Overexpression of Twist or Twist-Twist in vivo leads to ectopic muscle formation. (A-F) Wild-type embryos; (G-L) ectopic expression of *UAS-twist*; (M-R) ectopic expression of *UAS-twi-twi*. In this and the following figures, dorsal is up and anterior to the left, unless noted. Expression of Twist (Twi; G) or Twi-Twi (M) using one copy of *da-GAL4* driver led to conversion of all ectoderm into somatic muscle (cf. Baylies and Bate, 1996). G and M show high magnification of multinucleated, Myosin heavy chain (Mhc)-positive external cells (arrows). Insets show a lateral view of whole embryos. Fused di- and tri-nucleated cells, which are characteristic of somatic myogenesis, were found. Since no ectodermal derivatives were detected, these muscles did not spread out to form a pattern owing to lack of epidermis. Expression of Twist (H) or Twi-Twi (N) in the mesoderm using *twist-GAL4* driver led to ectopic formation of Mhc-positive muscle cells, shown here ventrally (arrowheads). Note that Mhc is found in cells where it is never usually expressed. Extra muscle formation was supported by the ectopic expression of founder cell markers, such as Kr (I and O; white arrow; asterisks are shown as a reference). Zfh1 expression in pericardial and cardial cells was lost in embryos overexpressing Twist (J) and Twi-Twi (P). Fas III expression in visceral muscle progenitors was also disrupted in stage 12 embryos that ectopically expressed Twist (K) and Twi-Twi (Q). At stage 10, Bap is expressed in a subset of mesodermal cells that are progenitors for visceral mesoderm and fat body. Bap is highly reduced in embryos that overexpress Twist (L) or Twi-Twi (R). Despite greatly reduced Bap, a complete loss of Fas III under these conditions was not found. Azpiazu and Frasch reported similar effects for *bap* hypomorphs (Azpiazu and Frasch, 1998).

seeded by a special set of myoblasts, the founder cells (for review see Baylies et al., 1998), we asked whether more founder cell gene expression could be detected. Consistent with the ectopic formation of somatic muscles, we found an increase in founder gene expression such as *Krüppel* (*Kr*), both dorsally (Fig. 2C,I,O) and around the gut (Fig. 3). Concomitant with the gain in cells adopting a somatic muscle fate, a loss in cells contributing to other mesodermal tissues was found. Progenitors of the visceral muscles and fat body, marked by Bagpipe (Bap) expression, were reduced (Fig. 2F,L,R) as well as the number of visceral muscle progenitors expressing Fas III (Fig. 2E,K,Q). We likewise detected a decrease in both pericardial and cardial cells that contribute to the heart (Fig. 2D,J,P). The migration of the mesoderm was normal under

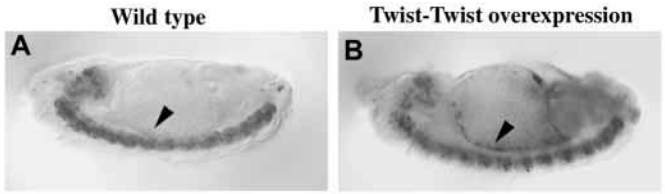

Fig. 3. Overexpression of the tethered Twist-Twist dimer in the mesoderm leads to ectopic expression of Kr in the visceral mesoderm. (A,B) Lateral view of stage 15 embryos stained for Kr. (A) Wild-type visceral muscle showing no Kr staining surrounding the gut. (B) Ectopic expression of UAS-Twi-Twi in the mesoderm induces expression of the founder cell marker Kr (arrowhead) in the mesoderm surrounding the gut, suggesting a conversion of visceral mesoderm into somatic muscles.

these conditions (data not shown). Moreover, we find enhanced activation of the *Mef2* enhancer-reporter construct when the linked dimer was expressed (data not shown). Hence over-expression of tethered Twist homodimers mimicked the effects of over-expression of Twist monomers in both the ectoderm and mesoderm.

The most stringent test for activity of Twist homodimers is whether tethered Twist dimers can substitute for endogenous Twist during mesoderm and somatic muscle development. To express tethered Twist dimers or Twist monomers in *twist* null mutant embryos, we used the GAL4/UAS system, which has been successfully used by Ranganayakulu et al. (Ranganayakulu et al., 1998) to drive the expression of *tin* in *tin* mutant embryos. Moreover, Staehling-Hampton et al. (Staehling-Hampton et al., 1994) showed *twist-GAL4* driven expression of Decapentaplegic (Dpp) led to the induction of a target gene, *bap*, as the ventral furrow forms. Expression of Twist monomers in embryos null for *twist* (e.g., *twist-GAL4, twist^{ID96}/twist^{ID96}; UAS-Twist* or *twist-GAL4; twist-GAL4 twist^{ID96}/twist^{ID96};UAS-twist*) completely rescued mesodermal development. The rescued embryos developed, hatched and subsequently gave rise to fertile adults. Fig. 4 shows that tethered Twist homodimers, expressed under similar conditions, rescued early mesodermal defects associated with loss of endogenous *twist*. Genes that fail to be expressed in *twist* mutant embryos, including *Htl* (Fig. 4E,J,O) and *Mef2* (data not shown) were induced in these embryos. Mesoderm migration occurred, yet subsequent mesodermal differentiation

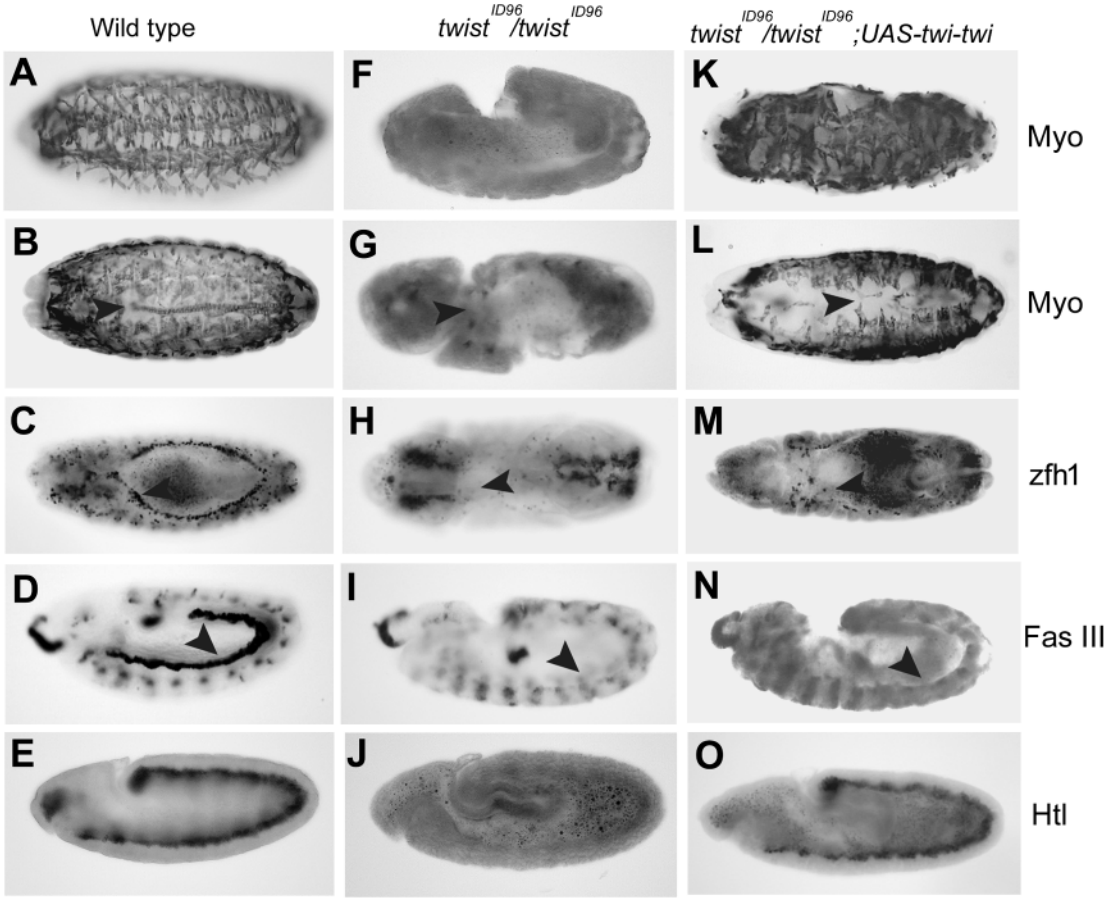

Fig. 4. Tethered Twist homodimers rescue some mesodermal defects associated with loss of Twist. (A-E) Wild-type embryos; (F-J) *twi^{ID96}* null mutant embryos; (K-O) *twi^{ID96}* embryos expressing UAS-Twist-Twist. *twist* null mutant embryos completely lack somatic musculature as shown by Mhc expression (F,G, arrowhead in G) as well as Zfh1-positive pericardial and cardial cells (H; arrowhead), Fas III-positive visceral muscle progenitors (I; arrowhead), and Htl-positive migrating mesoderm (J). Twist-Twist expression in mutant embryos rescued Htl expression during mesoderm induction (O), and somatic muscle formation, although muscle patterning is disrupted (K). Twist-Twist, however, did not rescue either the visceral mesoderm (N; arrowhead) or the heart (M; arrowhead). Instead, multinucleated, Mhc-positive cells appeared dorsally where the heart usually develops (L; arrowhead). Rescue with the Twist monomer or the Twist-Twist homodimer was 100% or 96% respectively.

was abnormal. Somatic muscles formed but with an aberrant pattern (Fig. 4A,F,K). Visceral muscle progenitors were missing (Fig. 4D,I,N), as well as the majority of both pericardial and cardial heart cells (Fig. 4C,H,M). Instead multinucleated somatic muscles were found dorsally where the heart normally develops (Fig. 4B,G,L) and around the gut (not shown). Thus, the two early functions of Twist, specification of mesoderm and allocation of somatic mesoderm, were rescued by the tethered dimer. However, the somatic muscle mispatterning and the severe reduction of visceral and heart muscle suggested that tethered homodimer expression failed to completely rescue all Twist functions during mesoderm development. This failure was not due simply to low levels of tethered dimer expression since somatic muscle allocation, which requires high levels of Twist (cf. Baylies and Bate, 1996), was rescued by this construct. Moreover, different UAS insertions give similar results. We conclude that Twist homodimers can execute two roles of Twist, the induction of mesoderm-specific genes and specification of the somatic muscle lineage.

Twist forms heterodimers with Daughterless, a ubiquitously expressed bHLH protein required for mesoderm development

As described above, the tethered Twist homodimers cannot completely rescue the *twist* null phenotype, raising the possibility that other Twist dimer forms are required for proper development. In this regard, vertebrate tissue culture experiments showed that *Drosophila* Twist can also heterodimerize with E proteins inhibiting muscle-specific gene activation (Spicer et al., 1996).

The *Drosophila* E protein homologue, Daughterless, like its vertebrate counterparts, is expressed ubiquitously and participates in a number of developmental processes (Cronmiller and Cummings, 1993; Cline, 1989; Caudy, 1988a; Caudy, 1988b; Cronmiller et al., 1988). We first investigated whether loss of Da had any effect on myogenesis. Reduction of both maternal and zygotic levels of Da had drastic consequences for mesodermal development. Twist expression was reduced during mesoderm induction and was nearly absent during mesodermal subdivision (Fig. 5E,J and D,I). Genes such as *Htl*, *Mef2*, and *zfh1* were detected but at lower levels (data not shown). Despite these alterations, the mesoderm migrated properly; however, further mesodermal development is impaired. For example, somatic muscle development was severely suppressed, with embryos showing reduced numbers of aberrantly placed, Mhc-positive syncytial muscles (Fig. 5A,F). Heart development was also repressed (Fig. 5B,G), although not as dramatically as visceral mesoderm (Fig. 5C,H). Since the maternal and zygotic loss of Da function gave such a drastic mesodermal phenotype, we decided to analyze the mesodermal phenotype of embryos lacking only the zygotic Da.

Mesodermal differentiation was less dramatically affected in zygotic null *da* mutant embryos. For example, visceral muscle developed normally (Fig. 6F,L), whereas losses in both pericardial and cardial cells were detected in a low percentage of embryos (Fig. 6E,K). In contrast, somatic muscles showed greater defects. These alterations, although detectable in every embryo, varied from segment to segment. We found both duplications of somatic muscles (Fig. 6A,G) and losses (Fig. 6B,H), suggesting a possible role for Da in the patterning of somatic muscles. These changes in the final muscle pattern could be correlated with increases and losses in founder cell gene expression. For example, we detected more Kr cells (data not shown) as well as losses in S59 (Slouch) expression in some clusters (Fig. 6D,J). In addition, we found ectopic Even-skipped (Eve) expression (Fig. 6C,I), suggesting that in these *da* mutant embryos, more somatic mesoderm is being allocated. Considering both sets of Da loss-of-function data,

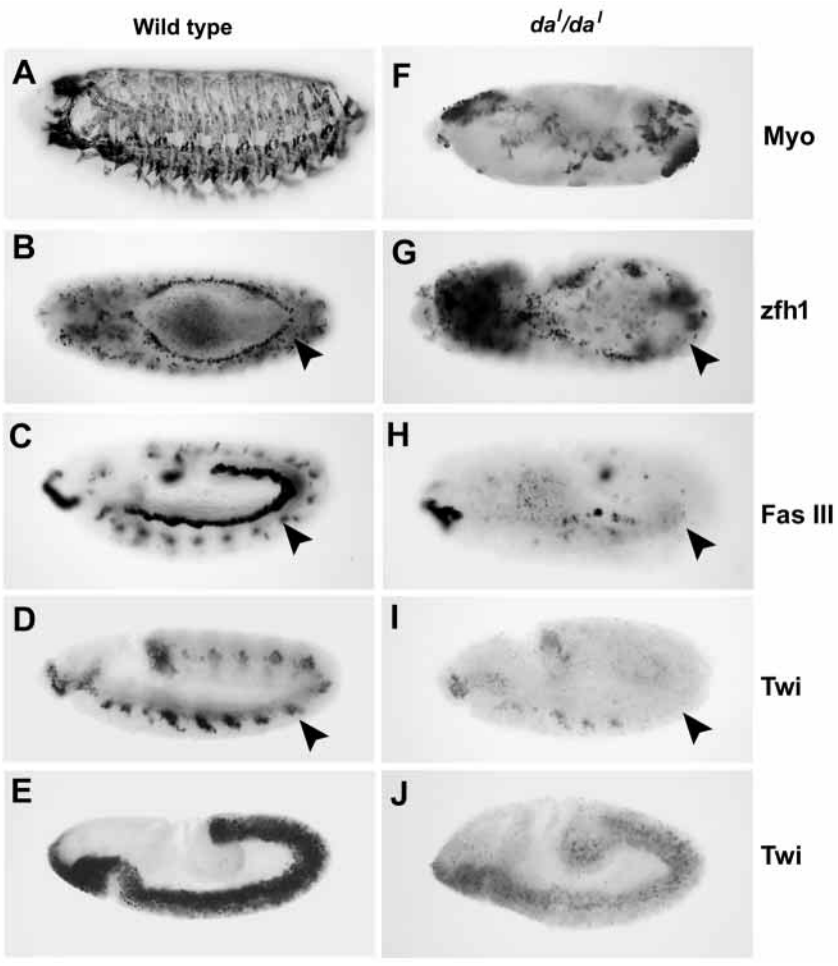

Fig. 5. Absence of both maternal and zygotic Da function leads to loss of mesodermal tissues. (A-E) Wild-type embryos; (F-J) *da* maternal and zygotic mutant embryos. The somatic musculature failed to form in *da* holonull embryos. Most somatic muscles were missing, although oddly patterned multinucleated fibres were detected (F). Absence of Fas III positive visceral muscle progenitors and Zfh1 positive pericardial and cardial cells was observed in mutant embryos (G,H; arrowheads). Although the mesoderm does migrate, Twist levels were reduced or absent, shown here in stage 8 embryos (J) and in stage 10/11 embryos when subdivision of the mesoderm occurs (I; arrowhead).

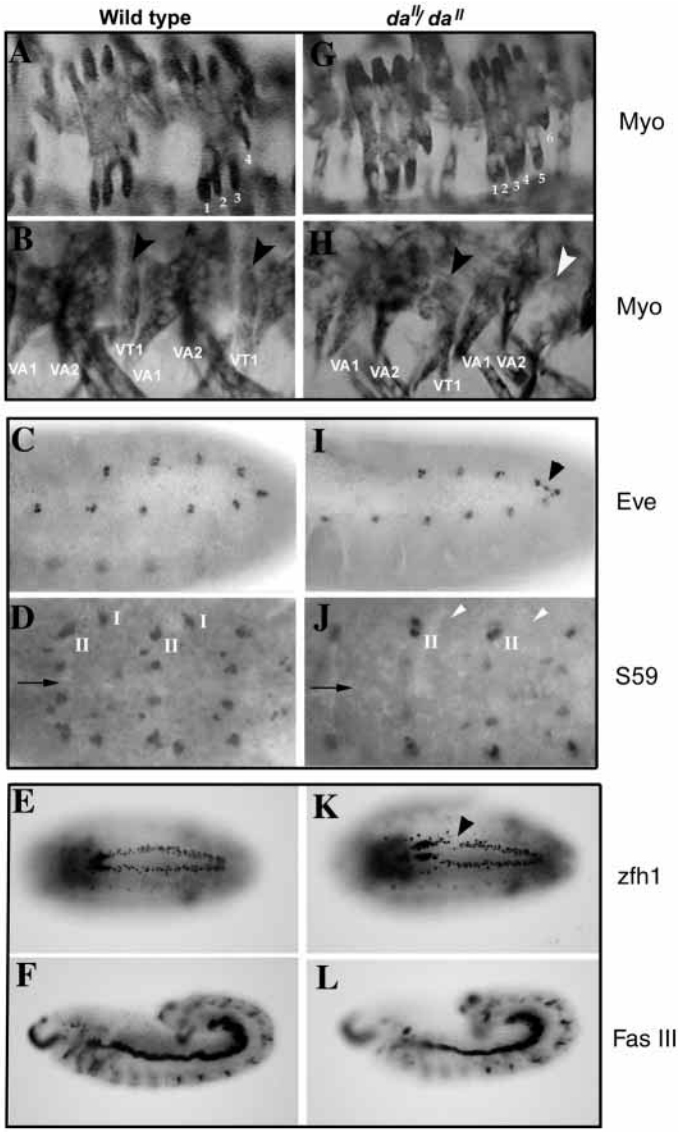

Fig. 6. Loss of zygotic Da leads to defects in allocation and patterning of heart and somatic muscle. (A-F) Wild-type embryos; (G-L) *da* zygotic mutant embryos. (A,B,G and H) show somatic muscles in embryos stained for Mhc. (G) An example of more muscle in *da* zygotic mutant embryos: Lateral muscles were duplicated as compared to wild-type. (H) Example of muscle loss. Muscle VT1 is absent is some abdominal segments (white arrowhead), but is present in others (black arrowhead). VT1 loss can be tied to loss of founder gene expression. *da* embryos lack S59 (*slouch*) expression in the cluster, which gives rise to this muscle (cluster I). Despite losses in S59 cluster I staining in many segments, not all VT1 muscles are absent. We attribute this effect to low levels of S59 expression and/or expression of other factors that allow formation of this muscle (J; arrowhead). (I) Ectopic Eve expression in the anterior region of the mesodermal segment (arrowhead) suggests a role for *da* in the allocation of somatic mesoderm. Zfh1-positive pericardial and cardial cells and Fas III-expressing visceral muscle progenitors were present in normal positions in the majority of analyzed embryos (K,L), however absence of some Zfh1-positive cells can be observed in some mutant embryos (arrowhead, K).

we conclude that Da plays a critical role at all stages in myogenesis: in mesoderm specification (through induction of high Twist levels), during mesoderm subdivision (as witnessed by founder gene expression in ectopic locations), and in the patterning of the somatic muscles (as shown by duplications and losses of somatic muscles).

Since Da could partner any number of bHLH proteins that are present at these different stages of mesodermal development (i.e. L'scute, Nautilus, etc.), we next asked whether Twist could physically interact with Da and what the outcome of this interaction would be.

Mobility shift experiments performed by mixing Twist and Da proteins produced DNA-protein complexes with intermediate mobility, indicative of heterodimer formation (Fig. 7A). DNA binding assays performed with two different E boxes, one a canonical Da binding site (CA**CC**TG) (Ohsako et al., 1994) and the other the Twist binding site from *rho* (CA**TA**TG) (Ip et al., 1992) indicated different binding affinities for Twist and Da dimers. Whereas the Twist/Da heterodimers had approximately equal affinity for either site, the apparent K_d for Twist homodimer's favored site (CA**TA**TG) was an order of magnitude greater than for the Da site (CA**CC**TG) (4.2×10^{-7} M versus 1.3×10^{-6} M respectively), even though the flanking sequences for both sites are the same (Fig. 7A).

Expression of Da alone or in combination with Twist in SL2 cells led to little activation of the *Mef2* reporter construct (Fig. 7B). Competition experiments in which Da levels were increased while Twist levels were held constant showed a decrease in reporter gene activation (not shown). These data indicate that Da did not activate the *Mef2* mesoderm-specific reporter and Da reduced Twist's ability to activate the reporter. Repression by Da could be achieved either by competing for E box binding as Da homodimers or by forming heterodimers with Twist. These Twist/Da heterodimers can lead to the reduction in reporter activation by competition for the target E box as well as titration of Twist monomers.

Since the in vitro experiments indicated that Twist and Da were able to form dimers and that these dimers repressed the activity of a gene required for myogenesis, we next sought in vivo evidence for Twist and Da interactions. Flies heterozygous for *twist* or *da* showed no mutant phenotype. However, when the zygotic dose of *da* was reduced by half while one copy of Twist was misexpressed in the mesoderm, we find greater losses in gut and heart forming mesoderm (Fig. 8K,L) as compared to misexpressing one copy of Twist in a wild-type background (Fig. 8E,F). This effect was enhanced further using two copies of Twist (Fig. 8H,I and N,O). In addition, we found more ectopic somatic muscle in these *da* heterozygous embryos (Fig. 8A,D,G,J,M). Conversely, overexpression of Da in the mesoderm of wild-type embryos had little to no effect on mesodermal development (data not shown). However, if we reduced the dose of Twist by half, while overexpressing Da in the mesoderm, we saw a dramatic suppression of somatic muscle fate (Fig. 9F). No increase in other mesodermal tissues was found (Fig. 9H,I). Taken together, both the in vitro data and the dosage experiments indicate that Twist and Da do physically interact and that the function of this heterodimer is to repress somatic muscle development.

Linked Twist-Da heterodimers repress myogenesis in vivo

To test directly whether the Twist/Da heterodimer repress

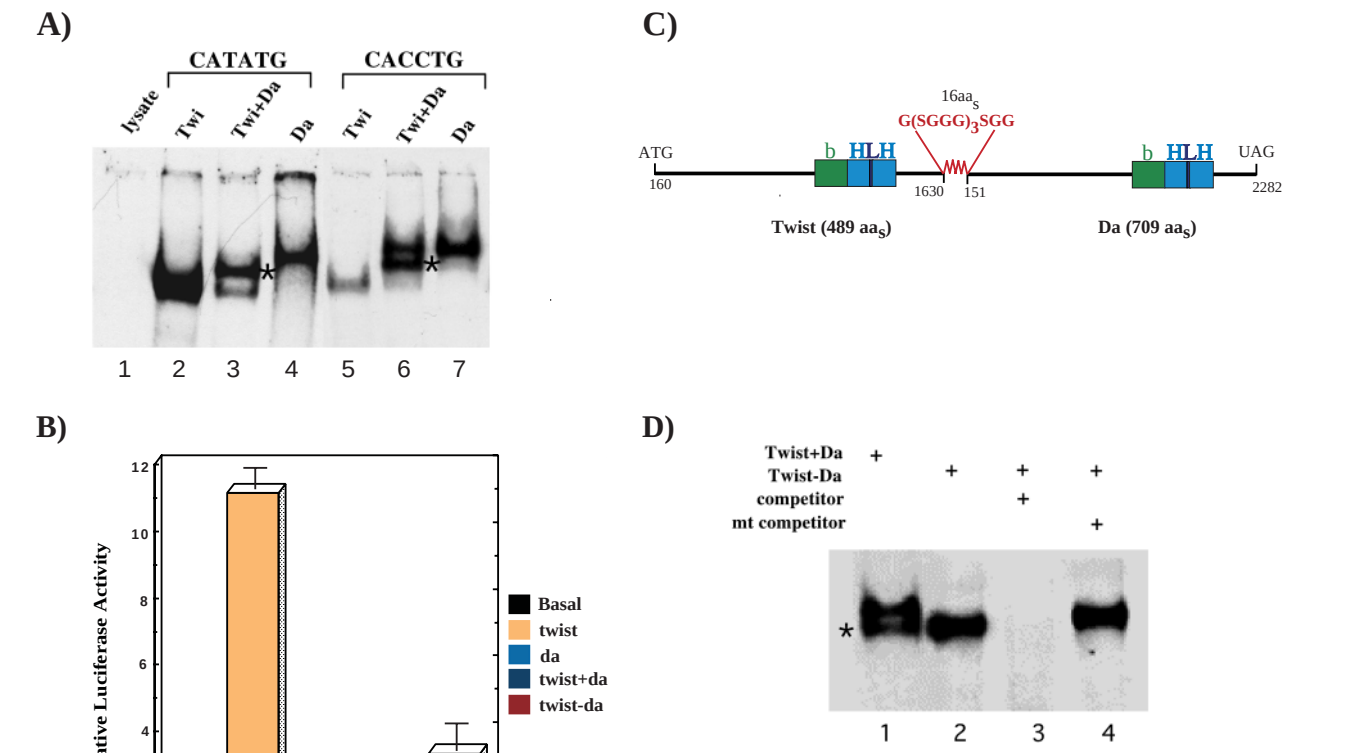

Fig. 7. Twist dimerizes with Da and represses muscle transcription in vitro. (A) Mobility shift analysis using *rho* CA**TA**TG (Ip et al., 1992) (lanes 2, 3, 4) and CA**CC**TG E boxes (lanes 5, 6, 7) performed with Twist (lanes 2, 5), Da (lanes 4, 7), and Twist + Da (lanes 3, 6). Equal amounts of protein are used in each lane. Heterodimer shifts are marked with an asterisk. The Da homodimer shows binding to class A sites (CA**CC**TG), whereas the Twist homodimer shows a marked affinity for a class B site (CA**TA**TG) (Ohsako et al., 1994). Cotranslation of both proteins and incubation with class A or B DNA sites results in formation of an intermediate shift between the Da homodimer and the Twist homodimer, which corresponds to the Twist/Da heterodimer (*). The apparent K_ds for Twi/Da and Da/Da on the *rho* E box are 1.6×10^{-6} and 3.2×10^{-6}, respectively. (B) Da represses Twist activation of the 175 bp *Mef2* enhancer in SL2 cells. SL2 cells were transfected with indicated amounts of *actin-lacZ* plasmid, the 175 bp *Mef2* enhancer-luciferase reporter plasmid, equimolar amounts of either Twist, Da, Twi+Da, or Twist-Da linked dimer expression vectors and carrier DNA. The values are expressed relative to controls (see Methods). Error bars represent the standard error of means of triplicated experiments. Transfection of Twist alone results in reporter gene activation. The same molar amount of transfected Da results in little activation. Cotransfection of equimolar amounts of Twist and Da results in reduction of reporter gene activity. Transfection of Twist-Da linked dimers led to little reporter gene activity. (C) Schematic representation of the tethered Twist-Da protein. DNA segments encoding Twist and Da are joined in frame via a flexible linker. The boxes denote the basic helix-loop-helix domain. Linker sequence is given in the single letter amino acid code. Numbers below the line represent the nucleotide sequence of *twist* and *da* cDNAs (Caudy et al., 1988; Cronmiller and Cline, 1988). (D) Mobility shift analysis with the tethered Twi-Da heterodimer. In vitro translated products are assayed for binding to class A E box. Twist-Daughterless linked dimers (lane 2 and 4) have the same DNA binding specificity as Twist/Da unlinked heterodimers (*, lane 1).

myogenesis in vivo, we made a tethered Twist-Da dimer. This construct, which linked the two proteins in a head to tail fashion, is shown in Fig. 7C. Mobility shift analysis indicated that the tethered dimers bind E boxes similarly to heterodimers formed by Twist and Da monomers (Fig. 7D). Consistent with previous results with the *Mef2*-enhancer reporter construct, transfection of the tethered dimer failed to activate this enhancer in SL2 cells (Fig. 7B). Competition experiments in which Twist or tethered Twist-Twist levels were held constant with increasing amounts of Twist-Da showed decreased activation, suggesting that Twist-Da heterodimers compete with Twist homodimers for E box binding (not shown). These in vitro experiments showed that tethered Twist-Da heterodimers function similarly to untethered Twist/Da heterodimers.

Transgenic flies in which the tethered Twist-Da heterodimers were over-expressed in the mesoderm using *twist-GAL4* revealed an extreme muscle phenotype. The somatic musculature is greatly reduced, with the remaining muscles showing defects in patterning and size (compare Fig. 9A,F,K). The tethered Twist-Da dimer produced defects that are generally more severe than overexpression of Da alone in the *twist* heterozygous background, with fewer intact Mhc-positive syncytial cells. Other mesodermal tissues, such as visceral muscle, are initially unaffected by over-expression of Da or tethered Twist-Da heterodimers, as measured by Bap expression

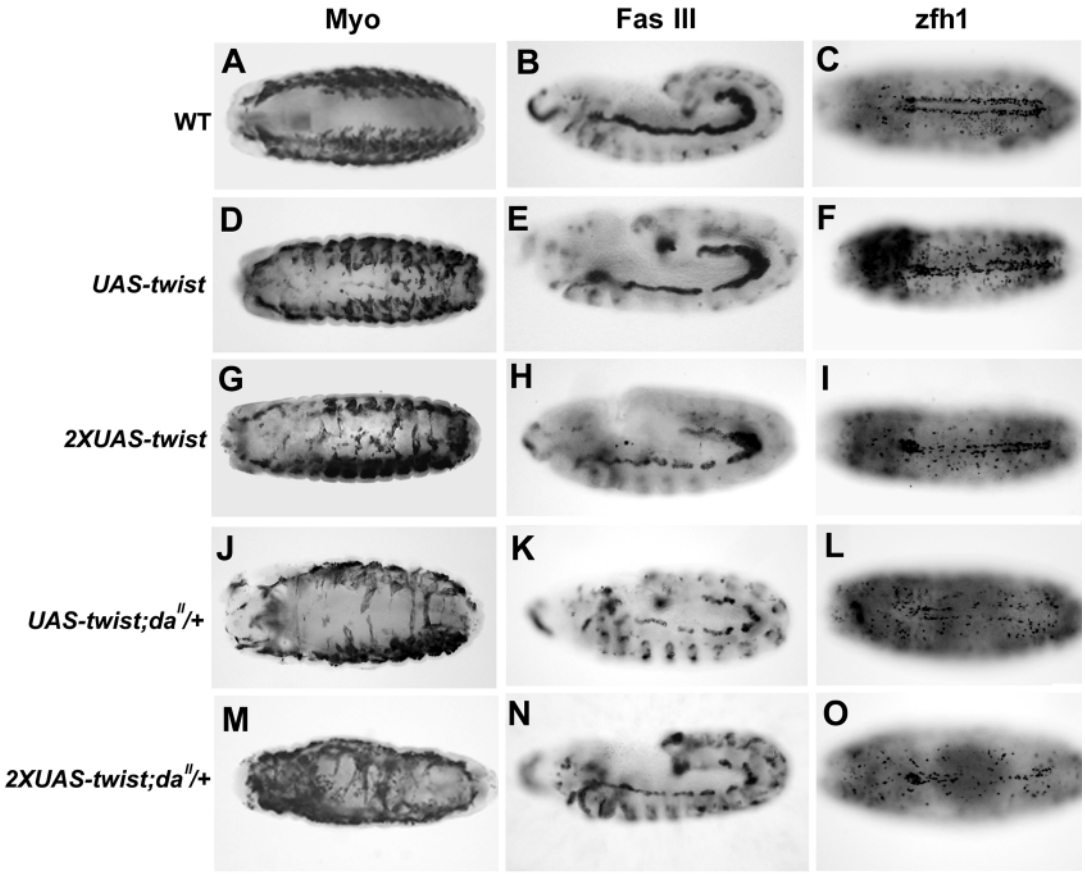

Fig. 8. Genetic interactions between *da* and *twist*. (A-C) Wild-type embryos; (D-F) Embryos expressing one copy of *UAS-twist* (G-I) or embryos expressing two copies of *UAS-twist* (2X UAS-twist) in a wild-type background; (J-L) Embryos expressing one copy of *UAS-twist* or (M-O) embryos expressing two copies of *UAS-twist* in a *da* heterozygous background. Increasing Twist levels in the mesoderm while decreasing Da levels by 50% led to an increase of ectopic somatic muscle throughout the embryo measured by Mhc staining, shown here in ventral view (A,D,G,J,M), as well as a decrease in Fas III0-positive visceral muscle progenitors (B,E,H,K,N) and Zfh1-positive pericardial and cardial cells (C,F,I,L,O).

(Fig. 9D,I,N). However, mild defects in the later differentiation of these tissues were found, presumably due to prolonged expression of the protein in our experiments (Fig. 9C,H,M).

Since Mhc expression gives a late readout of myogenesis, we determined when somatic muscle development first went awry in these embryos. Fig. 9 shows that the defect caused by over-expression of Da or tethered Twist-Da occurred just after the allocation of mesodermal cells to the somatic muscle fate. The number of founder cells, marked by Kr expression is decreased in the Twist-Da or Da over-expression embryos (Fig. 9B,G,L). In addition, the earliest marker of individual muscle formation, lethal of scute (L'sc), was significantly reduced or absent in these embryos (data not shown). Consistent with the transient transfection assays, we also saw reduction in *Mef2*-reporter activation in vivo (Fig. 9E,J,O), indicating that steps which initiate programs common to all somatic muscles such as fusion, regulation of the contractile apparatus, etc, were also suppressed in these embryos. Although cells were accurately allocated to the somatic mesodermal pathway as measured, for example, by Twist expression in the Da over-expression experiments, the subsequent differentiation of these cells was blocked. Earlier mesodermal events (prior to subdivision) such as migration are unaffected under these conditions (data not shown). We conclude that the earliest somatic muscle defect associated with tethered Twist-Da heterodimer or Da over-expression occurred just after mesodermal cells were allocated to somatic myogenesis. We find a repression of somatic muscle differentiation at a time when Twist homodimers activate this process.

DISCUSSION

Twist, a bHLH transcription regulator, plays multiple roles during *Drosophila* mesoderm development. The question of how Twist activity is modified such that it progressively determines more specific cell fates is the subject of our investigation. Using a combination of in vitro and in vivo approaches, we show two distinct Twist functions that depend on choice of dimerization partner. We note that the use of tethered dimers to dissect function in vivo is a powerful approach, yet the conclusions from these experiments alone could be subject to alternative explanations without data from our genetic experiments. Higher order complexes can be found in vitro (Fig. 1C) and we cannot rule out the existence of these in vivo. However, considering the genetic and biochemical data together, we find that homodimers of Twist are capable of inducing genes required for mesoderm and somatic muscle development. Heterodimers of Twist and Da repress these genes. Our observations support the model that dimer partners provide diversity in response and possibilities for regulation (Jones, 1990; Kadesch, 1993).

Different partner, different function

Unlike previous reports where functions of an HLH protein are attributed exclusively to a heterodimer (i.e. Achaete (Ac)/Da; Cabrera and Alonso, 1991) or homodimer forms (i.e. Hairy/Hairy; Ohsako et al., 1994), we find distinct activities associated both with Twist homodimers and Twist/Da heterodimers. The only other bHLH protein to which distinct

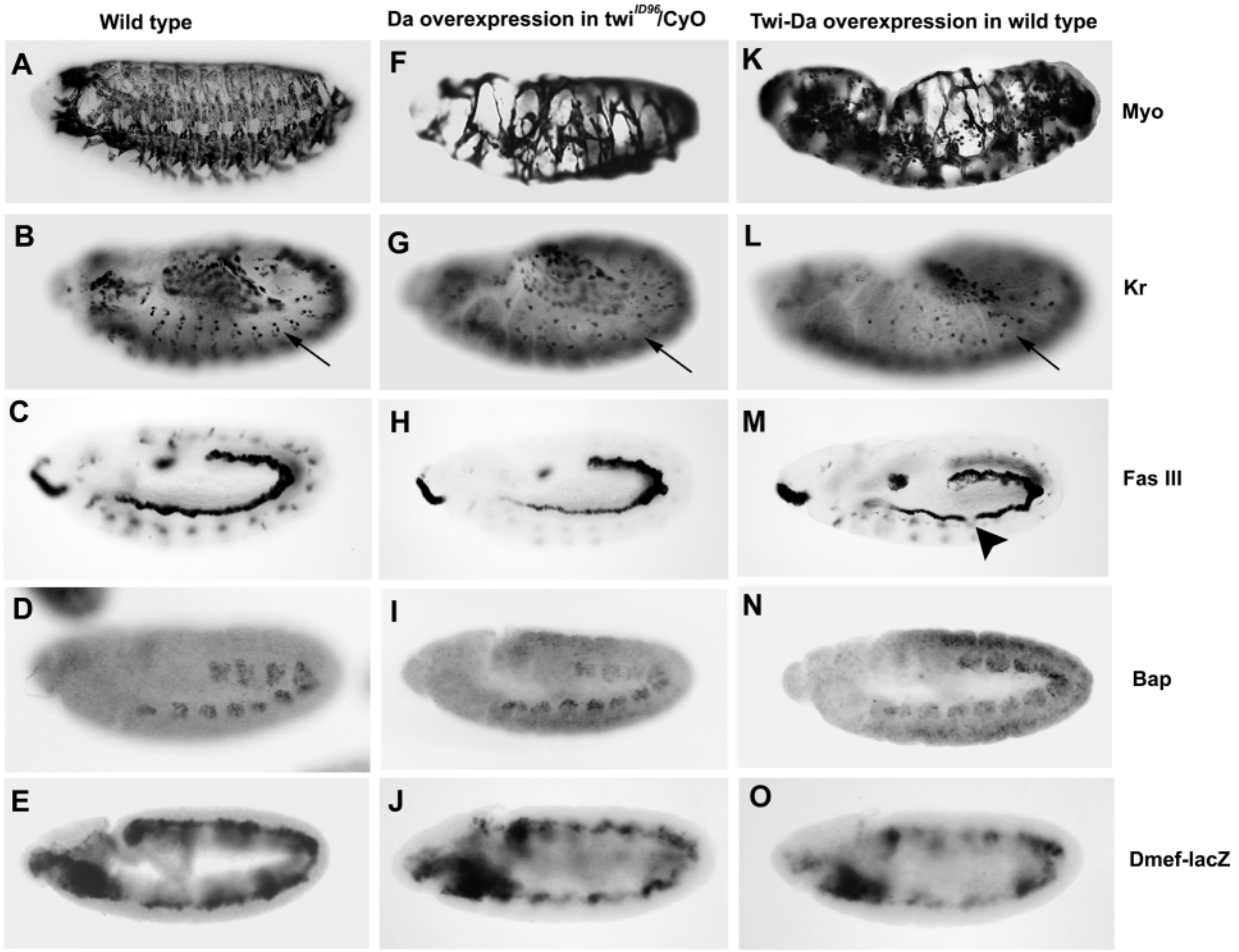

Fig. 9. Twist/Da heterodimers repress somatic muscle formation in vivo. (A-E) Wild-type embryos; (F-J) embryos expressing Da in a heterozygous *twist* background; (K-O) embryos expressing Twist-Da linked dimer in a wild-type background. Overexpression is achieved using *twist-GAL4*. (A,F,K) Mhc expression in stage 16 embryos. Embryos expressing Da or Twist-Da in mesoderm show severely reduced numbers of muscle cells. (B,G,L) Kr protein in stage 12 embryos. Kr expression in muscle precursors is highly reduced in embryos overexpressing Da (arrow). This phenotype is more severe in embryos expressing Twist-Da (L, arrow). (C,H,M) Fas III expression in stage 12 embryos. Fas III mesodermal expression is normal in embryos expressing Da protein (H), however some subtle defects can be observed in embryos expressing Twi-Da linked dimer (M; arrowhead). (D,I,N) Bap expression in stage 11 embryos. Progenitor cells of the visceral mesoderm and fat body show normal levels of Bap. (E,J,O) β-galactosidase expression in stage 10 embryos. Transgenic flies carrying a 175 bp *Mef2-lacZ* enhancer (the same enhancer used in transient transfection assays) were stained for β-galactosidase in the absence of ectopically expressed protein (E), in the presence of Da expression in a *twist* heterozygous background (J), or in the presence of Twist-Da expression in a wild-type background (O). The 175 bp enhancer was active in wild-type embryos, however, expression of Da or Twist-Da caused reduction of enhancer activity as seen in tissue culture cells. Using other GAL4s (i.e., 24BGAL4; Brand and Perrimon, 1993; Baylies et al., 1995) to drive expression of the tethered Twist-Da heterodimer in a wild-type background or Da in the *twist* heterozygous background resulted in similar defects.

functions have been linked to different dimer forms is the ubiquitously expressed vertebrate E proteins. E homodimers act uniquely in B cells to promote transcriptional activation from the IgH enhancer in the heavy chain locus, whereas heterodimers of E with other tissue-specific bHLHs such as MyoD activate transcription of target genes (Kadesch, 1992; Sigvardsson et al., 1997). Our results suggest that alternate functions for dimer pairs maybe a more general phenomenon, extending to tissue-specific bHLH proteins as well.

Previous data suggest that Twist functions to induce mesoderm and the somatic myogenic program (Leptin, 1991; Baylies and Bate, 1996). We interpret our data as showing that Twist homodimers are responsible for activation of early mesodermal and somatic myogenic programs. Domain mapping suggests that an amino-terminal region of Twist is required for transcriptional activation (I. C. and M. K. B., unpublished). Experiments are in progress to determine how this domain functions in transcriptional activation and to assess why one domain is not sufficient for activation when Twist is dimerized with Da. One possibility is that pairing of this region recruits co-activators essential for Twist activation and this region is masked upon dimerization with another HLH protein.

Like the vertebrate E proteins, Da functions in many different processes during fly development (Cline, 1989; Jan and Jan, 1993). We show here that Da also plays a significant role during mesodermal development. In particular, Da is responsible primarily for inducing high levels of Twist (also shown by Gonzalez-Crespo and Levine, 1993). Since levels of

Twist are so critical to mesodermal development, many effects seen in the *da* maternal and zygotic loss-of-function could be explained by this reduction in Twist levels. Similar reductions in heart, visceral muscle and somatic muscle tissues can be detected in embryos carrying hypomorphic Twist alleles either over a deficiency or over themselves as well as in embryos carrying the temperature sensitive allelic combination of *twist* grown at the nonpermissive temperature prior to gastrulation (Thisse et al., 1987; Leptin et al., 1992; Baylies unpub.). However, the data also suggest that Da performs additional roles in the mesoderm, both in the allocation and patterning of cells of the heart and somatic muscle. In this regard, it is interesting that other bHLH proteins including dHand, L'scute, and Nautilus are expressed in the heart and/or somatic muscles and may partner Da to execute these functions (Carmena et al., 1995; Moore et al., 2000; Michelson et al., 1990; Paterson et al., 1991).

Dimers of Da with tissue-specific proteins critical for neurogenesis, such as the *achaete-scute* family members, have been detected both in vitro and in embryo extracts. Genetic analyses suggest that Da/Ac and Da/Sc dimers are required for activation of genes essential for neural fate (Dambly-Chaudiere et al., 1988; Cabrera and Alonso, 1991). Two activation domains have been predicted in Da (Quong et al., 1993) and confirmed in SL2 cells (K. G. and M. K. B., unpublished), supporting Da's role as a transcriptional activator. Dimers of Da and Emc, an HLH with no basic domain (Ellis et al., 1990; Garrell et al., 1990), have also been detected. These Da/Emc dimers fail to bind DNA and lead to the effective decrease in available Da monomers (Martinez et al., 1993; Cabrera et al., 1994; Van Doren et al., 1991). In this report, we present another function for Da as a partner for Twist: Twist/Da heterodimers result in repression of somatic myogenesis genes. This repressive role for Da is unlike any previously described Da roles. Although Da, like Twist, is an activating protein in other contexts, dimerization of Twist/Da in the mesoderm leads to repression.

Repression by Da can be mediated by several mechanisms. First, Da homodimers compete for binding to mesoderm/somatic muscle E boxes. Second, Da monomers compete with Twist monomers to form heterodimers. Third, both our in vitro and in vivo experiments indicate that Twist/Da heterodimers also compete with Twist homodimers for DNA binding to E boxes. These modes of competition effectively reduce activator Twist homodimer levels, consistent with reduction in the *Mef2*-enhancer reporter activity that we detect both in tissue culture and in vivo upon tethered Twist/Da heterodimer expression. Our data also indicate that Twist homodimers have a greater affinity for the Twist E box compared to Twist/Da heterodimers or Da homodimers. Whether this difference is significant in vivo awaits further tests, but the results do highlight how different dimer partners alter binding affinity, and as a result, the eventual fate of the cell.

Opposing functions for Twist in a developmental context: Twist activity during the Subdivision of the mesoderm

We suggest that these data clarify how Twist acts during subdivision of the mesoderm. At stage 10, in response to transcriptional regulators such as Sloppy paired and Even skipped as well as signals from the overlying ectoderm such as Wingless, the uniform expression of Twist modulates into regions of high and low expression within each segment (Azpiazu et al., 1996; Riechmann et al., 1997; Bate and Rushton, 1993). Da is expressed uniformly in the mesoderm at this time. The region that maintains high Twist levels subsequently give rise to somatic muscles whereas the region that has lower Twist levels gives rise to tissues such as visceral muscle, fat body, gonadal mesoderm and some glia cells (Dunin-Borokowski et al., 1995; Baylies and Bate, 1996) The heart is derived from the region that initially expresses high levels of Twist; however these cells lose Twist expression, an event necessary for the execution of heart fate (this work, Baylies and Bate 1996). Expressing high Twist levels in cells destined to become visceral muscle, for example, blocks visceral muscle differentiation and promotes somatic muscle. Reduction of Twist levels in cells normally expressing high Twist levels blocks somatic myogenesis (Baylies and Bate, 1996).

We now provide several possible mechanisms to explain these observations and illustrate the in vivo roles for the two opposing activities of Twist homodimers and Twist/Da heterodimers (Fig. 10). Regions that normally express lower Twist levels do not form somatic muscles owing to higher concentrations of Twist/Da heterodimers as compared to Twist homodimers. These heterodimers repress transcription of pro-muscle genes, such as *l'sc* as well as founder cell genes such as *Kr*, thereby prohibiting somatic muscle development. Other differentiation programs for visceral muscle or fat body development can proceed unaffected. We find no evidence that Twist/Da heterodimers promote visceral mesoderm or fat body fate through the direct activation of targets such as Fas III. Regions that normally express higher Twist levels do form somatic muscle owing to higher concentrations of Twist homodimers as compared to Twist/Da heterodimers. Dimer competition, then, restricts the developmental potential of mesodermal cells, by not allowing Twist homodimers to convert all mesodermal cells into somatic muscle.

These conclusions are consistent with the observations that increasing Twist/Da levels, either by overexpression of Da or the tethered Twist-Da heterodimer, repress the earliest steps in somatic myogenesis. These are the same steps that are activated by Twist homodimers. For example, L'sc expression, which marks clusters of equipotential cells that segregate the muscle founder cells (Carmena et al., 1995), is drastically reduced or absent upon an increase of Twist/Da heterodimers. This indicates an early failure in the somatic muscle program. Likewise we see failure in subsequent steps; for example, few founder cells as well as few identifiable muscles are detected. We interpret these failures in muscle development as an outcome of the initial block in the differentiation pathway. We have not, however, eliminated the possibility that overexpression of Da or of Twist-Da could directly repress these subsequent steps. Gal4 lines that drive expression at later stages of muscle development or in particular subsets of muscle cells (i.e., the S59-expressing founder cells) could provide insight into this alternative.

We also have not ruled out the possibility that Twist forms dimers with HLH proteins in addition to Da. Although our in vitro results indicate that Twist does not dimerize with mesodermal HLH proteins such as Emc or L'sc (unpublished), Twist may dimerize with new HLH proteins predicted from the

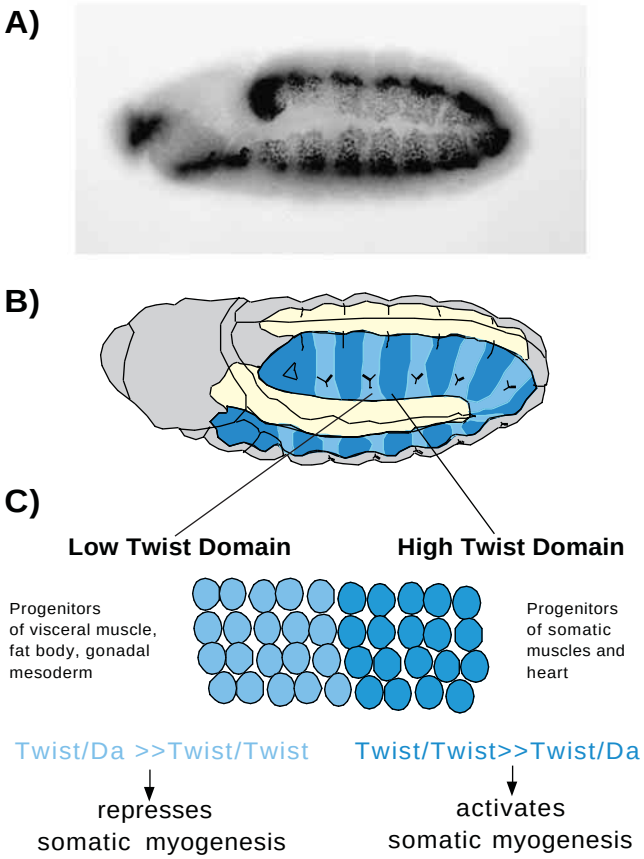

Fig. 10. The role of Twist homodimers and Twist/Da heterodimers during the subdivision of the mesoderm. (A) Lateral view of a stage 10 embryo showing modulated expression of Twist. Each segment has an anterior domain that expresses lower Twist levels and a posterior domain that expresses higher levels of Twist. (B) Cartoon of similar staged embryo. High Twist levels are indicated by dark blue, lower levels by light blue. Da is expressed uniformly in the mesoderm at this stage. (C) Close up of a mesodermal segment at stage 10. Cells that express high Twist levels favor formation of Twist homodimers relative to that of Twist/Da heterodimers. Twist homodimers in these cells promote the somatic muscle program. By increasing Twist/Da heterodimers levels either by reducing Twist levels or by overexpressing the linked Twist-Da dimer, the somatic muscle program is inhibited. Formation of Twist/Da heterodimers are favored relative to Twist/Twist homodimers in domains that express low Twist levels. Higher Twist/Da heterodimers levels lead to repression of somatic myogenesis in these cells, which are destined form tissues such as visceral muscle, fat body and gonadal mesoderm.

genome sequence (Moore et al., 2000). These dimers may be required for the accurate patterning of the somatic muscles.

Twist and the HLH network in vertebrates – multiple functions through dimerization?

Tissue culture experiments reveal that vertebrate Twist and in particular, mouse Twist, repress myogenesis induced by members of the MyoD family. Spicer et al. (Spicer et al., 1996) concluded that the form of mouse Twist that mediates this repression is a heterodimer between Twist and the Da homologue, E protein. This mouse Twist/E heterodimer mediates repression by blocking E box binding by MyoD, by titrating E protein and by inhibiting MEF2 transactivation. Thus, evolution has conserved one activity for Twist that spans both fly, mouse and perhaps human – a repressive activity in the form of Twist/Da (E).

Data from Twist knockout mice (Chen and Behringer, 1995) or from patients with Saethre-Chozten syndrome, which is caused by mutations in human Twist (Howard et al., 1997; el Ghouzzi et al., 1997), fail to provide any evidence of an activator role for vertebrate Twist during skeletal myogenesis. However, an activator role for vertebrate Twist, be it in an homodimeric form or with an unknown partner, cannot be ruled out for Twist's activity in neural crest, limb or for its newly described role as an apoptosis inhibitor. Our data suggest that vertebrate Twist homodimers can readily be detected in mobility shift assays using E boxes that are favored by *Drosophila* Twist homodimers (manuscript in prep). The sequence of this particular subset of E boxes may predict targets of Twist homodimers both in fly and in vertebrates.

In *Drosophila*, there is only one Twist; in mice, Twist belongs to a family of closely related bHLH genes, including Twist, Scleraxis, Dermo 1 and Paraxis (Cserjesi et al., 1995; Li et al., 1995; Burgess et al., 1995). These additional family members have overlapping patterns of expression. For example, Twist and Scleraxis are both expressed in the early mesoderm as well as the sclerotome (Fuchtbauer, 1995; Stoetzel et al., 1995; Gitelman, 1997; Brown et al., 1999). Hence in vertebrates, there is added complexity for dimerization between Twist and E protein families. These various homodimer and heterodimer forms provide more potential for regulating "Twist" activities. Our work with *Drosophila* Twist lends support to the notion that cell fate is the sum of the activities of a complex number of HLH proteins rather than the omniscient activity of just one protein.

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