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Tup/Islet1 integrates time and position to specify muscle identity in *Drosophila*

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SUMMARY

The LIM-homeodomain transcription factor Tailup/Islet1 (Tup) is a key component of cardiogenesis in *Drosophila* and vertebrates. We report here an additional major role for *Drosophila* Tup in specifying dorsal muscles. Tup is expressed in the four dorsal muscle progenitors (PCs) and *tup*-null embryos display a severely disorganized dorsal musculature, including a transformation of the dorsal DA2 into dorsolateral DA3 muscle. This transformation is reciprocal to the DA3 to DA2 transformation observed in *collier* (*col*) mutants. The DA2 PC, which gives rise to the DA2 muscle and to an adult muscle precursor, is selected from a cluster of myoblasts transiently expressing both Tinman (Tin) and Col. The activation of *tup* by Tin in the DA2 PC is required to repress *col* transcription and establish DA2 identity. The transient, partial overlap between Tin and Col expression provides a window of opportunity to distinguish between DA2 and DA3 muscle identities. The function of Tup in the DA2 PC illustrates how single cell precision can be reached in cell specification when temporal dynamics are combined with positional information. The contributions of Tin, Tup and Col to patterning *Drosophila* dorsal muscles bring novel parallels with chordate pharyngeal muscle development.

KEY WORDS: Tailup/Islet1, Collier/EBF, Tinman/Nkx2.5, Myogenesis, Temporal cascades, *Drosophila*

INTRODUCTION

Understanding how a cell acquires its identity is a fundamental problem in developmental biology. The somatic musculature of the *Drosophila* embryo is a classical model with which to study the regulatory processes that generate cellular diversity in response to positional information provided by embryonic body axes. Each hemi-segment shows a stereotypical arrangement of 30 somatic muscles connected to the exoskeleton, one alary muscle that attaches to the dorsal vessel, which is the equivalent of the heart, and six muscle stem-like cells [known as adult muscle precursors (AMPs)] (Bate and Rushton, 1993; Bate et al., 1991; Figeac et al., 2010; LaBeau et al., 2009). Each skeletal muscle is a syncytium seeded by a founder cell (FC), which undergoes multiple rounds of fusion with fusion-competent myoblasts. FCs are born from the asymmetric division of progenitor cells (PCs), themselves selected by Notch (N)-mediated lateral inhibition from equivalence groups of myoblasts, termed promuscular clusters at distinct positions within the somatic mesoderm (Carmena et al., 1995; Ruiz-Gómez and Bate, 1997). Characterization of selected muscle lineages has shown that muscle shape, size and orientation reflect the expression of specific 'identity' transcription factors (iTFs) in each PC and FC (Bate and Rushton, 1993; Baylies et al., 1998; Frasch, 1999; Tixier et al., 2010). Maintaining the expression of a subset of iTFs in one FC, but not its sibling (Ruiz-Gómez et al., 1997; Rushton et al., 1995), and propagation of this iTF code into all nuclei to the growing myofibre (Crozatier and Vincent, 1999; Dubois et al., 2007; Knirr et al., 1999) determine the final identity of each muscle. There are four dorsal muscles (DA1, DA2, DO1, DO2) and six dorsolateral (DL) muscles (DA3, DO3, DO4, DO5, DT1 and LL1) (Bate and Rushton, 1993; Nose et al., 1998). Detailed studies of the iTF Even-skipped (Eve) expression in the DA1 and DO2 lineages showed that *eve* cis-regulation integrates positional information provided by ectodermal Decapentaplegic (Dpp) and Wingless (Wg) signalling, tissue-specific information provided by the mesodermal transcription factors Twist (Twi) and Tinman (Tin), and Receptor Tyrosine Kinase (RTK) signals (Carmena et al., 1998; Duan et al., 2007; Knirr et al., 1999). Although these pioneering studies have provided a useful framework, there remain many gaps in our understanding of how each PC acquires a specific identity according to its position.

From a genetic screen designed to identifying new muscle identity genes (L.D., unpublished), we found that removal of the LIM homeodomain TF Tailup/Islet1 (Tup) causes a DA3 duplication. Islet1 was first characterized in vertebrates, as a regulator of insulin expression in the pancreas (Karlsson et al., 1990) and in motoneuron specification (Pfaff et al., 1996). Recently, it has been shown to be necessary for second heart field (SHF)-derived cells to populate the heart (Laugwitz et al., 2005; Moretti et al., 2006). *Drosophila tup* was initially characterized for its role in neuronal identity and axon pathfinding (Thor and Thomas, 1997), and was later shown to be required for development of the lymph gland and the specification of cardiac cells (Mann et al., 2009; Tao et al., 2007).

We report here that Tup is expressed in the four dorsal muscles and the alary muscles, and that the dorsal musculature is severely disorganized in *tup*-null embryos. We then focus our studies on the DA2 to DA3 muscle transformation. The DA2 PC is selected from myoblasts transiently expressing both Col and Tin. Tup activation in this PC during this time window is required to repress *col* transcription and impose the DA2 identity. Tup is then required for maintaining its own expression in dorsal muscles beyond the PC stage, suggesting a handover relay mechanism between two, early and late, cis-regulatory modules. Our findings provide a new dynamic view of transcriptional regulation that patterns the

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Drosophila dorsal musculature and novel parallels with specification of pharyngeal muscles in chordates.

MATERIALS AND METHODS

Genetics

Drosophila mutant and transgenic strains were: tup^{ex4} (de Navascués and Modolell, 2010), col^{1} (Crozatier et al., 1999), *rp298-lacZ* (Nose et al., 1998), *Mhc-GFP* (Eric Olson and Elisabeth Chen, Dallas, USA), twist-Gal4 (Baylies and Bate, 1996), IsletH-GFP, *UAS-tup* (Thor and Thomas, 1997), N^{55e11} (Salzberg et al., 1994), tin^{EC40} (Bodmer, 1993) and *Vg-lacZ* (Richard Cripps, Albuquerque, USA). Mutant strains were balanced over marked chromosomes: *Cyo wg-lacZ*; *Cyo dfd-EYFP*; *TM3 twist-lacZ*. Df(2L)Exel7072 and Df(2L)Exel7073 were obtained from the Bloomington Stock Center.

Constructions of transgenic reporter lines

The *4_0.9col-moeGFP* construct fuses a *col* genomic DNA fragment that contains 4 kb of upstream sequence and 560 bp of transcribed sequence extending into the Col protein region, in frame with a D-moesin GFP fusion (Polesello et al., 2002). It was inserted into the AttP-pS3AG transgenesis vector (Williams et al., 2008). *tup DME-lacZ* was constructed by placing a PCR-amplified *tup* genomic fragment, FlyBase positions 2L: 18897397..18900688 (release: r5.42), in the *attB-inslacZ* vector (Enriquez et al., 2010). *4_0.9col.moeGFP* and *DME-lacZ* were inserted at position 68A4 on the third chromosome by injection into nosC31NLS;attP2 embryos (Bischof et al., 2007; Markstein et al., 2008).

Immunohistochemistry and in situ hybridization

Antibody staining and in situ hybridization with intronic probes were as described previously (Dubois et al., 2007). Primary antibodies were: guinea pig anti-Col (1:5000; Adrian Moore, Saitama-Ken, Japan), anti-L'sc (Stephen Crews, Chapel Hill, USA), anti-SrB (Talila Volk, Reohovot, Israel), mouse anti-Col (1:100) (Dubois et al., 2007), anti-Tup (1:100; Developmental Studies Hybridoma Bank), anti-GFP (1:100; Torrey Pines Biolabs), anti-β-galactosidase (1:1000; Promega), rabbit anti-Tin (1:750; Manfred Frasch, Erlangen, Germany), anti-Nau (1:100; Bruce Paterson, Bethesda, USA), anti-Kr (Ralf Pflanz, Goettingen, Germany), anti-Eve (1:1000) (Kosman et al., 1998), anti-b3-tubulin (1:5000; Renate Renkawitz-Pohl, Marburg, Germany), anti-Zfh1 (1:5000; Alain Garces, Montpellier, France), anti-Vg (Andrew Simmonds, Edmonton, Canada) and rat anti-Runt (1:300) (Kosman et al., 1998). Secondary antibodies were: Alexa Fluor 488- and 555-conjugated antibodies (1:300; Molecular Probes), and biotinylated goat anti-mouse (1:1000; Vector Laboratories). The *tup* intronic probe spans 2 kb of the first intron. Confocal sections were acquired on a Leica SP5 microscope at 40× magnification and 1024/1024 pixel resolution. Images were assembled using Photoshop software.

tup CRM predictions

tup CRM predictions were based on the positions of Tin, Twi and Mef2 in vivo binding sites (Sandmann et al., 2007; Zinzen et al., 2009). These data were accessed via the Eileen Furlong's lab webpage (http://furlonglab.embl.de/data/browse_chip_mod/).

RESULTS

tup: a new muscle identity transcription factor

Our knowledge of the regulatory program that controls the exquisite pattern of *Drosophila* larval muscles is still fragmentary. To identify new players, we undertook a genetic screen, starting from a collection of small overlapping deficiencies covering the second chromosome and using Col expression as a read-out. Two overlapping deficiencies, Df(2L)Exel7072 and Df(2L)Exel7073, revealed a duplication of the Col-expressing DA3 muscle in most segments (81%, *n*=249; 82%, *n*=230, respectively; Fig. 1A-D). The genomic region deleted in both deficiencies includes two genes, *short spindle 3* (*ssp3*) and *tup* (Fig. 1A). tup^{ex4} null embryos (de Navascués and Modolell, 2010) show a DA3 duplication in 78% of segments (*n*=102) (Fig. 1E-E′) and a similar phenotype is observed in hemizygous tup^{ex4}/Df(2L)Exel7072 embryos (not shown). Lack of *tup* is therefore responsible for the DA3 duplication phenotype

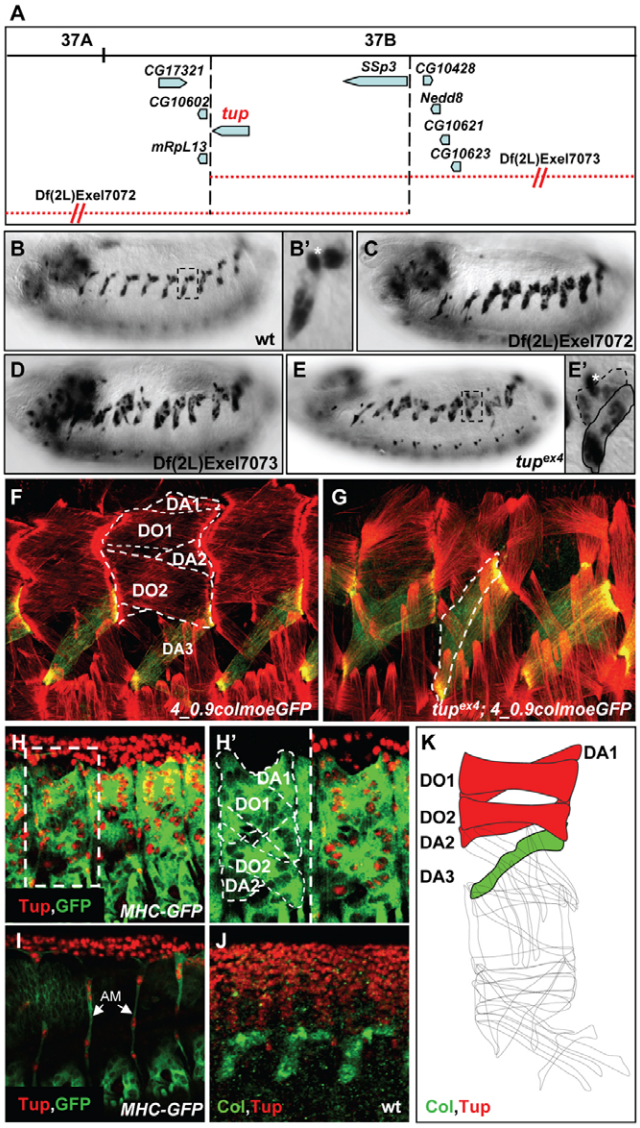

Fig. 1. *tup*: a muscle identity gene. (**A**) The 37A-37B chromosomal interval and overlapping Df(2L)Exel7072 and Df(2L)Exel7073 deficiencies (dotted red lines). The genes surrounding and within the deleted genomic region are indicated by blue arrows. (**B-E'**) Lateral views of stage 15 embryos stained for Col. (B,B′) Wild type (wt), (C) Df(2L)Exel7072, (D) Df(2L)Exel7073 and (E,E′) tup^{ex4} homozygous embryo. (B′,E′) Enlarged views of one segment. A duplication of the DA3 muscle is observed in absence of *tup*. Asterisks indicate dorsal md neurons that express Col. (F,G) Dorsolateral views of stage 16 4_0.9colmoeGFP embryos stained with phalloidin to visualize muscle fibres. Three segments are shown. (**F**) Wild-type embryo; the contours of the DA1, DA2, DO1 and DO2 dorsal muscles can be unambiguously identified (dotted white lines); GFP marks the DA3 muscle. (**G**) tup^{ex4} embryo; the dorsal muscles are ill-formed and GFP is expressed in a second, DA3-like muscle (framed). (**H-I**) Dorsolateral views of two segments of stage 15 Myosin Heavy Chain (MHC)-GFP embryos stained for Tup (red) and GFP (green), showing Tup expression in the four dorsal muscles (H) and the alary muscles (white arrows in I). (H′) High-magnification view of the region framed in H; left, GFP staining outlining the dorsal muscles (dotted white circled). (**J**,**K**) Tup (red) and Col (green) expression at stage 15; schematic in K.

observed in Df(2L)-Exel7072 and Df(2L)Exel7073 embryos. To better visualize the DA3 muscle, we expressed a moe-GFP fusion protein under control of a DA3-specific cis-regulatory module (CRM) (4_0.9*col*moeGFP). Double phalloidin and GFP staining confirmed the transformation of one dorsal muscle into a DA3-like muscle in tup^{ex4} embryos (Fig. 1F,G). Phalloidin staining also revealed that all dorsal muscles are affected to some extent in tup^{ex4} embryos, with individual muscles often not recognisable from segment to segment, showing that *tup* activity is crucial for patterning the dorsal musculature.

Tup contributes to the iTF code of dorsal muscle PCs

Each muscle can be uniquely identified by its position and shape at stage 15. Staining for Tup showed its expression in the four dorsal muscles (Fig. 1H,H′), complementary to DA3 Col expression (Fig. 1H,J,K), in addition to alary muscles (Fig. 1I) (Tao et al., 2007). As muscle identity is conferred by activation of specific combinations of iTFs in each PC, we looked at Tup expression at the PC stage. As general marker of PCs/FCs, we used rp298*lacZ* (Beckett and Baylies, 2007; Enriquez et al., 2012; Nose et al., 1998). Staining of late stage 11 rp298*lacZ* embryos for *lacZ* and either Tup or Col, showed that Tup expression is expressed in the four dorsal-most PCs (Fig. 2A). The dorsal FCs have been positioned relative to each other and identified, based on the expression of specific iTFs, except for DA2 (Beckett and Baylies, 2007). We tentatively identified the DA2 PC as a cell expressing Nautilus (Nau), but not Col, and located immediately dorsal to the DA3/DO5 PC (Enriquez et al., 2010; Enriquez et al., 2012). Double staining for Tup and either Eve or Kr confirmed that this cell expresses Tup, and is Eve and Kr negative (Fig. 2D,E). Thus, Tup is expressed in the four dorsal PCs and contributes to their specific iTF code. Kr expression is reduced in the DO1 muscle in *tup* hypomorphic mutants (Mann et al., 2009). Accordingly, Kr expression is completely lost in tup^{ex4} embryos, confirming that it crucially depends upon Tup (data not shown). On the contrary, Eve and Runt remain expressed in the DA1 and DO2 muscles, respectively, although an abnormal clustering of Runt-expressing nuclei correlates well with the abnormal morphology of the DO2 muscle in *tup* mutant embryos (supplementary material Fig. S1). Together, these data indicate that Tup acts both upstream of some ITFs and in combination with others in specifying dorsal muscle identities.

The DA2 muscle originates from a mixed muscle/AMP lineage

Only two dorsal muscle lineages, DA1 and DO2, were previously established, based on Eve expression. Both are mixed muscle/Eve pericardial cell (EPC) precursor lineages (Carmena et al., 1998; Speicher et al., 2008). To establish the DA2 lineage, we used a 7 kb *tup* cis-regulatory region, IsletH, which was initially characterized for its ability to drive GFP expression in a subset of post-mitotic motoneurons and interneurons (Thor and Thomas, 1997). We found that IsletH is active at stage 11 in the DA2 PC, as identified by its position, immediately dorsal to the DA3/DO5 PC (Fig. 2G). Following IsletH-GFP expression showed that the DA2 PC divides at stage 12. Double staining for GFP and Zfh1, a general AMP marker (Figeac et al., 2010; Sellin et al., 2009), established that this division is asymmetric, with one daughter cell becoming an AMP (Fig. 2H). The DA2 PC thus gives rise to a mixed somatic muscle/AMP lineage. Staining of stage 16 embryos for Zfh1 and GFP showed that the DA2 sibling AMP is the dorsal-most DLAMP (Fig. 2I). This AMP still expresses Zfh1 in *tup* mutant embryos, suggesting that *tup* is not required for its specification (not shown).

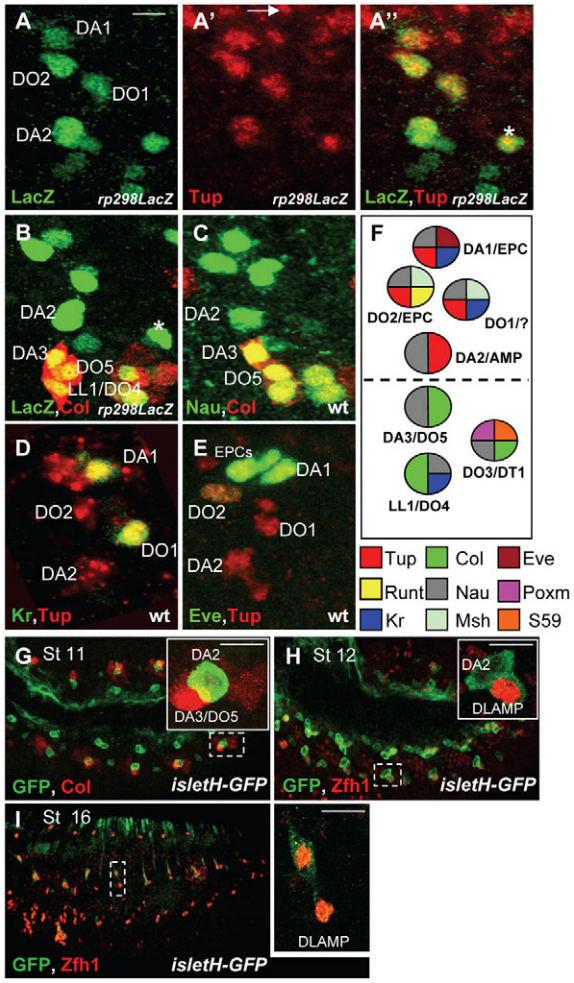

Fig. 2. Tup: the iTF code of dorsal PCs and the DA2/AMP lineage. (A-G) Staining of late stage 11 embryos for different iTFs. (**A-B**) rp298*lacZ* embryos stained for *lacZ* (green) and (red), either Tup (A-A″) or Col (B). Tup is expressed in cardioblasts (arrow in A′), the DA1, DO2, DO1 and DA2 PCs and a cell of unknown identity (asterisk in A″,B). Col is expressed in the DA3/DO5, DO3/DT1 and LL1/DO4 PCs. (**C-E**) Wild-type embryos stained for Nau (green) and Col (red) (C), Tup (red) and (green) Kr (D) or Eve (E), as indicated in each panel. The dorsal region of one abdominal segment is shown. The DA2 PC expresses Tup, but neither Eve nor Kr. (**F**) Schematic map of the dorsal and DL PCs, at late stage 11, with the iTF code colour-coded. (**G-I**) Staining of stages 11, 12 and 16 isletH-GFP embryos for GFP (green) and either Col (G) or Zfh1 (H,I) (red). (G) The DA2 PC expresses IsletH-GFP and divides into the DA2 FC and the dorsal-most DLAMP (H,I). Insets in G-I are enlarged views of framed areas. Scale bars: 10 μm.

Tup is required to specify the DA2 muscle identity, via Col repression

To determine which muscle ectopically activates Col expression and adopts a DA3 morphology upon loss of *tup*, we double stained wild-type and tup^{ex4} embryos for Col and Vestigial (Vg). Vg marks the DA1, DA2, DA3 and LL1 muscles (Bate et al., 1993). In wild-type embryos, only the DA3 muscle expresses Col (Fig. 3A,A′). In

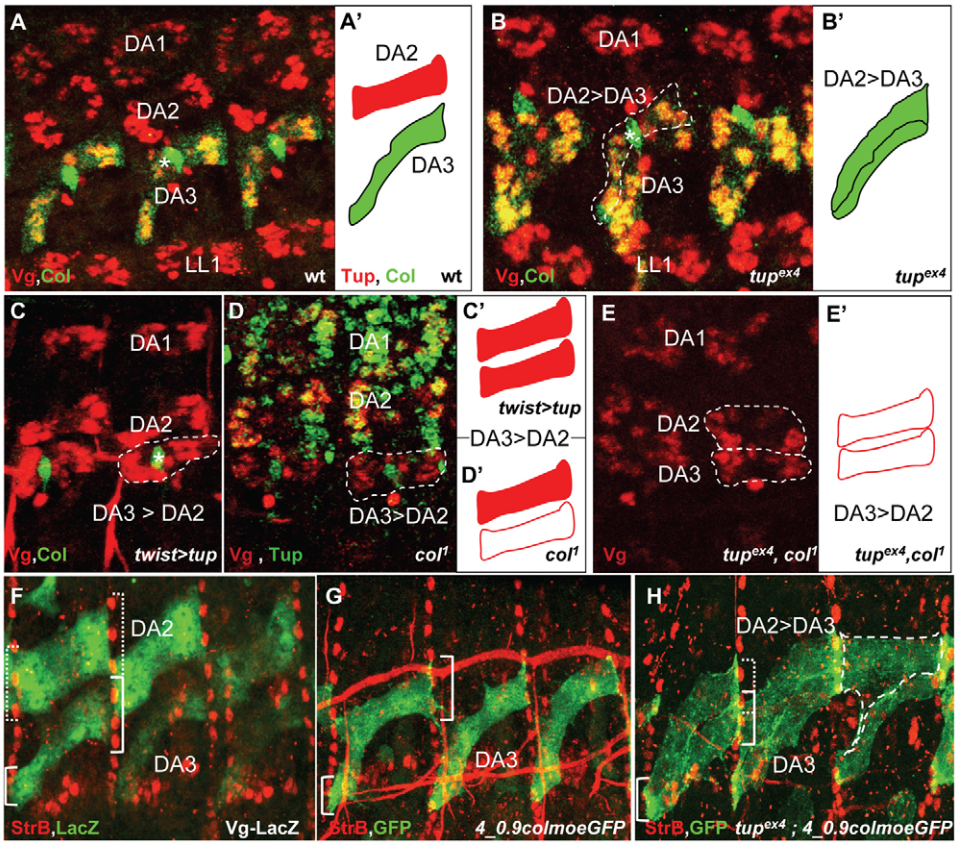

Fig. 3. Tup activity distinguishes between the DA2 and DA3 fates by repressing Col. Dorsolateral views of stage 15 embryos. (A) Wild-type, (B) *tup*^{ex4} (C), *twist>tup*, (D) *col*^{1} and (E) *tup*^{ex4}, *col*^{1} embryos stained for Vg (red) and either Col (A-C) or Tup (D) (green). (**A**) Vg labels the DA1, DA2, DA3 and LL1 muscles, and Col the DA3 muscle. (**B**) The DA2 muscle expresses Col and adopts DA3 (DA2>DA3) morphology. (**C**) The DA3 muscle expresses Tup and adopts DA2 (DA3>DA2) morphology. (**D**) The DA3 muscle (dashed circled) adopts DA2 morphology without expressing Tup. (**E**) The DA2 and DA3 muscles display DA2 morphology. (**A′**,**B′**,**C′**,**E′**) Schematic drawings of the DA2 and DA3 phenotypes with Tup and Col expression in red and green, respectively. (**F**) Vg-*lacZ* embryo stained for *lacZ* (green) and SrB (red), showing the anterior and posterior attachment sites of the DA2 (dotted white brackets) and DA3 (white brackets) muscles. (**G**,**H**) *4_0.9colmoeGFP* (G) and *tup*^{ex4}*; 4_0.9colmoeGFP* (H) embryos stained for GFP (green) and SrB (red). In *tup*^{ex4} embryos, the DA3 and DA2>DA3 attachment sites overlap; triangular-shaped muscles (white dotted circled) are observed. Three abdominal segments are shown in A,B,F-H; two abdominal segments are shown in C-E. The white asterisks in A-C indicate Col expression in multidendritic neurons.

tup^{ex4} embryos, the DA2 muscle also expresses Col, correlating with its abnormal morphology, showing that it is transformed into DA3 in absence of *tup* activity (Fig. 3B,B′).

The DA2>DA3 transformation observed in *tup* mutants is also observed upon Col ectopic expression in the entire mesoderm (Twi>Col) (Enriquez et al., 2012). This phenotype similarity suggested that Tup could antagonize Col activity. To test this possibility, we overexpressed Tup in the entire mesoderm (Twi>Tup embryos). Staining for Col and Vg showed a loss of Col expression in the DA3 muscle (Fig. 3C) and high frequency of DA3>DA2 transformations (88% of segments, *n*=77), similar to a *col* loss-of-function phenotype (Fig. 3D). We conclude that Tup is able to repress Col expression in the DA3 muscle, correlating with the DA3>DA2 identity shift (Fig. 3C′,D′). The opposite DA2>DA3 and DA3>DA2 transformations observed in *tup* mutants or *col* gain-of-function and *col* mutants or *tup* gain-of-function, respectively, show that Col and Tup distinguish between the DA2 and DA3 muscle identities. Of note, however, Col is activated in the DA2>DA3 muscle in *tup*^{ex4} embryos, whereas the DA3>DA2 transformation in *col* embryos occurs without Tup activation (Fig. 3B′,D′). This difference suggested that Tup is not instructive for the DA2 fate. To test this idea further, we looked at the muscle pattern of *col*^{1}*; tup*^{ex4} double mutant embryos. β3-Tubulin staining showed a cumulative phenotype; that is, dorsal muscle defects and a lack of DA3 muscle (supplementary material Fig. S2). Staining *col*^{1}*; tup*^{ex4} embryos for Vg confirmed that, in absence of *tup* and *col* activity, the DA2 and DA3 muscles display a DA2 morphology (Fig. 3E,E′). It indicates that DA2 is default morphology and suggests that DA2 iTFs other than Tup remain to be identified.

To describe more precisely the DA2>DA3 morphological transformation in *tup* mutants, we compared the epidermal attachment sites of the DA2 and DA3 muscles in wild-type and in *tup*^{ex4} embryos. Staining Vg-*lacZ* embryos for *lacZ* and Stripe B (StrB), a marker of tendon cells (Volk and VijayRaghavan, 1994; Volohonsky et al., 2007), indicated that the wild-type DA2 and DA3 attachment sites are not overlapping (Fig. 3F). StrB staining of wild-type and *tup*^{ex4}; *4_0.9colmoeGFP* embryos showed an overlap between the wild-type DA3 and *tup*^{ex4} DA2>DA3 insertion sites (Fig. 3G,H). Thus, in absence of *tup*, the DA2 muscle targets the DA3 tendon cells. Some partial DA2>DA3 transformations were also observed in *tup* mutant

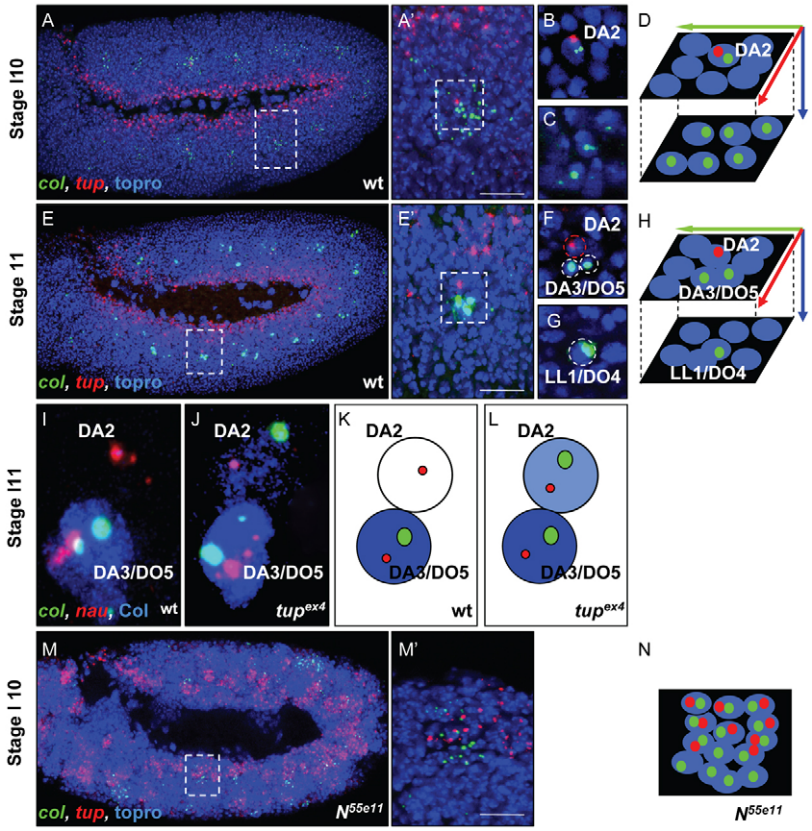

Fig. 4. Transcriptional repression of *col* by Tup in the DA2 PC. (A-H) *col* (green) and *tup* (red) primary transcripts. Nuclei are stained by Topro (blue). (**A**,**A′**) Late stage 10 embryo; *tup* is detected in a single cell among a *col*-positive cluster; outlined region is enlarged in A′. (**B-D**) High-magnification views of two *z*-sections of the squared region in A′. The DA2 PC singles out from the *col* cluster, shown schematically in D. (**E**,**E′**) Stage 11 embryo. The outlined region in E is enlarged in E′. (**F-H**) High-magnification views of different *z*-sections of the squared region in E′, shown schematically in H. The DA2 PC maintains *tup* transcription (red circled in F). *col* is transcribed in the DA3/DO5 and LL1/DO4 PCs (white circles in F and G, respectively). (D,H) The green, red and blue arrows indicate the anteroposterior, dorsoventral and mediolateral axes, respectively. (**I-L**) Late stage 11 embryos. *col* (green) and *nau* (red) primary transcripts in wild-type (I) and tup^{ex4} (J) embryos. In tup^{ex4} embryos, *col* transcription and Col protein (blue) are detected in the DA2PC, shown schematically in K,L. (**M-N**) *col* (green) and *tup* (red) primary transcripts in late stage 10 N^{55e11} embryos. Nuclei are stained by Topro (blue). M′ is an enlargement of the outlined region in M. Several myoblasts co-transcribe *col* and *tup,* shown schematically in N. Scale bar: 10 μm.

embryos, with triangular shaped muscles (Fig. 3H). This phenotype underlines the existence of two successive steps in the attachment of elongating muscles to specific tendon cells, a transient attachment, followed by a selection of the final attachment sites, with the second step being tightly regulated by the iTF code (Enriquez et al., 2012). Whether iTFs other than Col and Tup contribute to the robustness of the selection of DA2 versus DA3 attachment sites remains an unresolved issue.

Tup represses *col* transcription in the DA2 progenitor

Temporal aspects of Col expression in the DL PCs showed that they are specified sequentially. The DA3/DO5 PC is specified first, followed by the DO3/DT1 PC (only in the A1-A7 segments; not further considered here) and, finally, the LL1/DO4 PC (Enriquez et al., 2012). We previously noticed that the Nau-positive cell now identified as the DA2 PC (Fig. 2), singles out from the Col-positive cluster earlier than the DA3/DO5 PC and transiently expresses low amounts of Col (Enriquez et al., 2012). This observation suggested that the repression of Col by Tup was exerted in the DA2 PC, raising the question of the relative kinetics of *tup* and *col* transcription in this cell. To address this question, we used in situ hybridization with intronic probes to detect *tup* and *col* nascent transcripts. Double in situ hybridization revealed that *tup* is strongly transcribed in only one cell among the cluster of myoblasts that transcribe *col* at stage 10 (Fig. 4A,A′). Examination of single confocal sections showed that this unique *tup*- and *col*-positive cell has singled out and adopted a more external position than the rest of the cluster (Fig. 4B-D). By stage 11, however, whereas *tup* remains transcribed, *col* transcription is not detected anymore in this cell, unlike in the DA3/DO5 FCs and LL1/DO4 PC, which is singling out at this stage (Fig. 4E-H). We conclude that *col* and *tup* are co-transcribed in the DA2 PC, prior to *col* extinction. To determine whether *tup* maintenance and *col* extinction are linked, we compared *col* transcription in the DA2 PC in wild-type and tup^{ex4} embryos, using *nau* transcription as an internal reference. In wild-type embryos, *col* and *nau* transcripts are detected in the DA3/DO5 PC, identified by the accumulation of Col protein, while only *nau* transcripts are detected in the DA2 PC (Fig. 4I,L). In tup^{ex4} mutant embryos, *nau* and *col* transcripts, and Col protein are detected in the DA2 PC (71% of segments; n=60) in addition to the DA3/DO5 PC (Fig. 4J,L), showing that Tup represses *col* transcription in the DA2 PC. Promuscular *col* transcription at stage 10 is not affected in twi>tup embryos (supplementary material Fig. S3), confirming that Tup repression of *col* is exerted at the PC stage. The lower level of Col protein in the DA2, compared with DA3 PC, in *tup* mutant embryos (Fig. 4J), suggests, however, another level of *col* regulation.

Eve and S59 expression in selected lineages showed that PC-specific expression of some iTFs is established in two steps: first, activation in an equivalence group of competent cells, followed by restriction to PC(s), which involves N-mediated lateral inhibition (Buff et al., 1998; Carmena et al., 2002; Knirr and Frasch, 2001). *col* expression in the DL PCs follows this scheme (Fig. 4A-F) (Crozatier and Vincent, 1999; Enriquez et al., 2010). To determine whether *tup* expression in the DA2 lineage is first activated in an equivalence group, we looked at *tup* transcription in N mutant (N^{55e11}) embryos where lateral inhibition is defective. In absence of *N*, several myoblasts co-transcribe *col* and *tup* (Fig. 4M,M′), revealing the existence of a group of cells from which the DA2 PC is selected. Only the dorsal-most cells of the *col* cluster transcribe *tup* (Fig. 4M,N), providing an explanation of why *col* repression

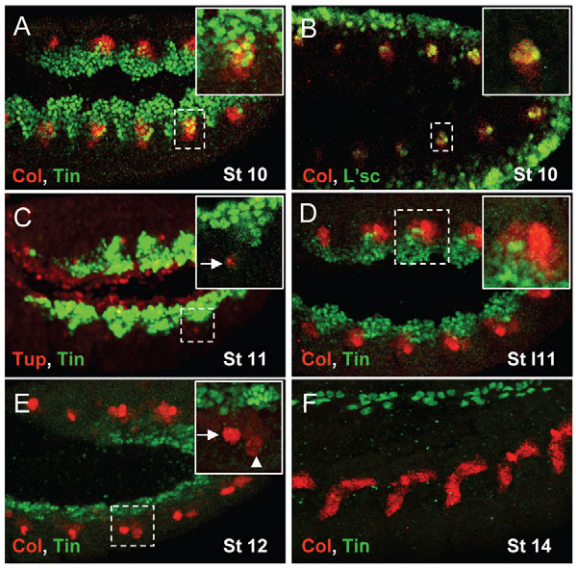

Fig. 5. The DA2 PC singles out from a promuscular cluster transiently co-expressing Col and Tin. (**A**,**B**) Col (red) and Tin (A) or L'sc (B) (green) expression in stage 10 embryos. The small region of Tin/Col expresses L'sc. (**C**) Tup (red) and Tin (green) expression in stage 11 embryos at the onset of dorsal restriction of Tin expression. Tup labels the DA2 PC (white arrow). Other Tup- and Tin-positive cells include dorsal muscle PCs and cardiac progenitors (Mann et al., 2009). (D-F) Tin (green) and Col (red) expression at stage 11 (D), 12 (E) and 14 (F). (**D**) Dorsal restriction of Tin expression and ventral expansion of Col expression is observed. (**E**) The DA3/DO5 (arrow) and DO3/DT1 (arrowhead) PCs have singled out; insets in A-D correspond to single confocal sections. (**F**) Tin expression in the heart.

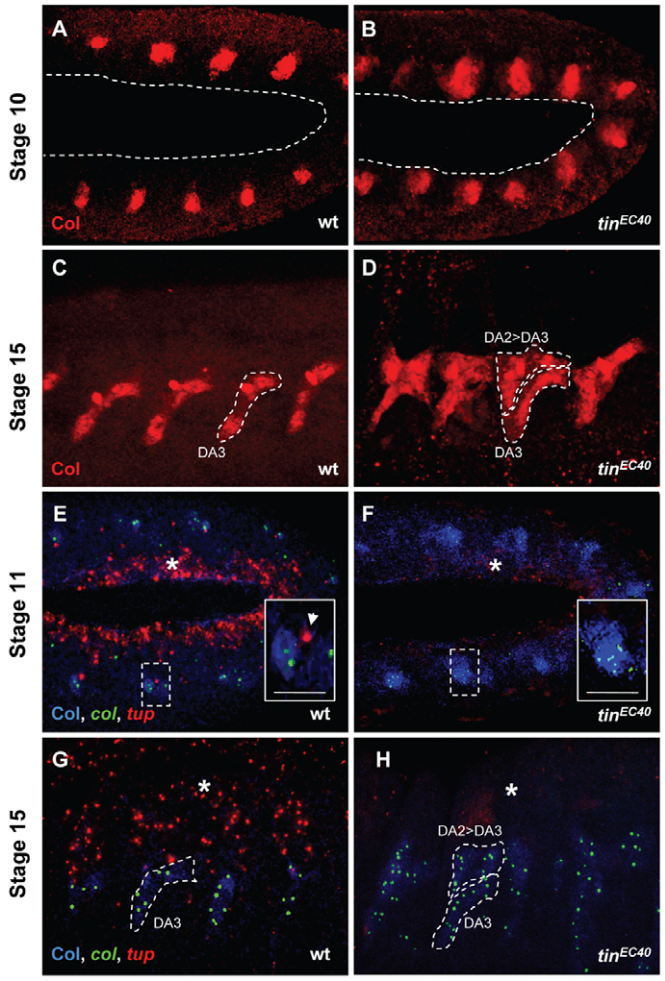

Fig. 6. Tin is required for *tup* transcription in the DA2 PC and DA2 identity. (**A-D**) Col expression in wild-type (A,C) and tin^{EC40} (B,D) embryos. In A,B, the dotted-white line delineates the amnioserosa. In *tin* mutants, Col expression extends dorsally (B); the DA3 muscle (dotted-white circled in one segment) is duplicated (D). (**E-H**) *col* (green) and *tup* (red) primary transcripts in wild-type (E,G) and tin^{EC40} (F,H) embryos. Col protein is in blue. Stage 11 *tup* transcription in the dorsal mesoderm (white asterisk) and DA2 PC (white arrow), is lost in tin^{EC40} embryos. (H) *tup* transcription in muscles (asterisk) is lost, whereas *col* is ectopically transcribed in the DA2>DA3 muscle. Scale bars: 10 µm.

by Tup only occurs in the DA2 PC. In turn, it raised the question of which mechanisms control the different registers of *tup* and *col* activation.

The DA2 progenitor is issued from a promuscular cluster transiently expressing Tin

Tin is a major regulator of cell fate in the dorsal mesoderm (Bodmer, 1993; Liu et al., 2009), which shows a dynamic expression pattern (Azpiazu and Frasch, 1993; Bodmer, 1993; Johnson et al., 2011; Xu et al., 1998; Yin et al., 1997). Tin is first expressed in the entire trunk mesoderm, followed by upregulation in the dorsal mesoderm, in response to Dpp signalling from the dorsal ectoderm (stage 10, Fig. 5A). It is during this second phase that Tin has been proposed to specify different dorsal mesoderm fates. Later, Tin expression is restricted to cardiac progenitors (late stage 11, Fig. 5D), before directing heart cell diversification (Fig. 5E,F). Of specific interest here, is the relationship between Tin, Col and Tup expression during the second phase of Tin expression. At stage 10, a small group of myoblasts express both Col, Tin and the proneural protein Lethal of scute (L'sc) (Fig. 5A,B), suggesting that it corresponds to the equivalence group of *tup*/*col* transcribing myoblasts revealed in *N* mutant embryos (Fig. 4N). Accordingly, residual Tin is detected in the DA2 PC (Fig. 5C). The overlap between Tin and Col expression is transient, however, because at late stage 11, while the Col-expressing cluster has expanded ventrally, Tin expression has retracted dorsally (Johnson et al., 2011; Reim and Frasch, 2005), such that Col and Tin are now expressed in complementary domains (Fig. 5D). As a consequence, the DA3/DO5 (and DT1/DO3) progenitors, which are born more ventrally, and later than the DA2PC, are selected from cells that do not anymore express Tin (Fig. 5E).

Tup acts downstream of Tin in the DA2 PC

Tin role in patterning the dorsal muscles has not been studied in detail, except for its requirement for Eve expression in the DO2 and DA1 progenitors (Knirr and Frasch, 2001). In silico search and chip-on-chip experiments suggested that Tin could directly regulate the expression of several iTFs, including *col* (Enriquez et al., 2010; Liu et al., 2009; Philippakis et al., 2006). Promuscular Col expression is not lost in tin^{EC40}-null embryos, however, but extends dorsally, to reach the dorsal edge of the embryo proper and abut the amnioserosa, which is expanded in these mutants (Bodmer, 1993) (Fig. 6A,B). Thus, during its first

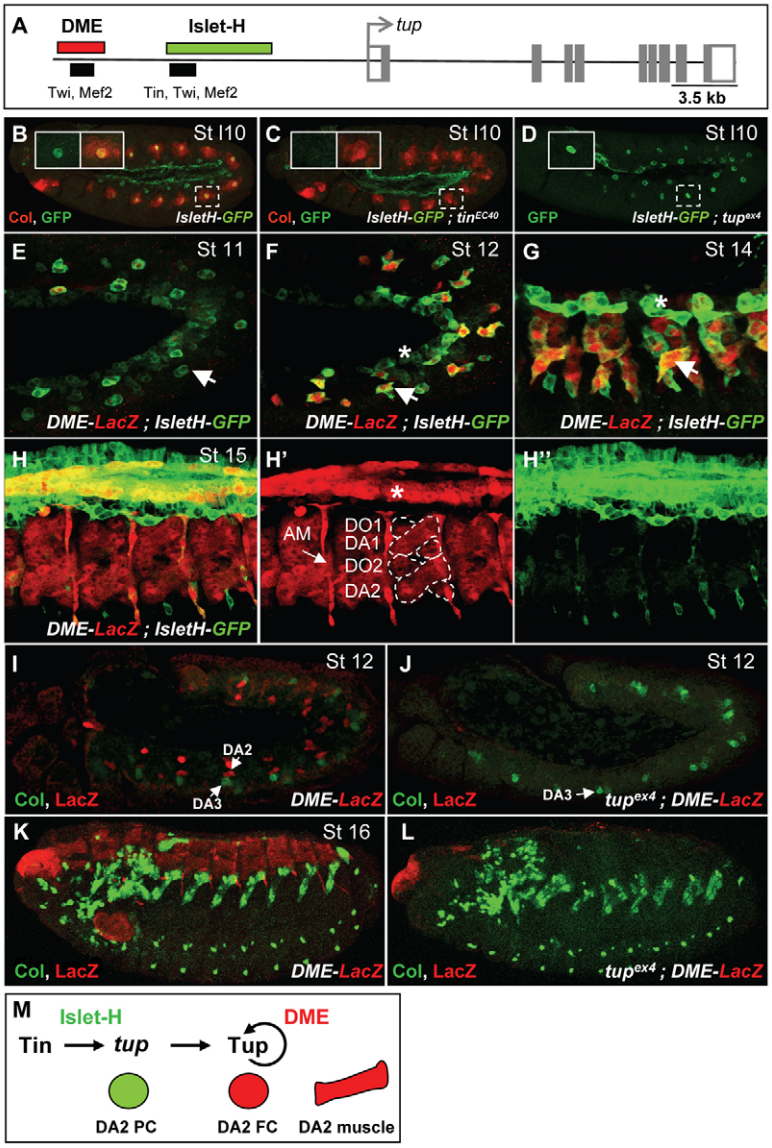

Fig. 7. Two separate cis-regulatory regions control *tup* transcription in the DA2 lineage. (**A**) Schematic view of the *tup* genomic region. The transcription start is indicated by an arrow; coding exons are in grey. The IsletH (Thor and Thomas, 1997) and DME cis-regulatory regions are represented by green and red boxes, respectively. Positions of meso-ChiP CRMs are indicated by black boxes. (**B-D**) IsletH-GFP expression in the DA2 PC at late stage 10 (B), is lost in tin^{EC40} (C) but not in tup^{ex4} (D) embryos. (**E-H″**) *lacZ* (red) and GFP (green) expression in DME-*lacZ*/IsletH-GFP embryos. (E) DME activity in the DA2 PC (white arrow) is first detected at stage 12. (G) IsletH and DME activity in elongating dorsal muscles, including the DA2 muscle (white arrowhead); IsletH is also expressed in heart cells (asterisk). (H-H″) Only DME activity is detected in dorsal muscles in stage 15, in addition to alary muscles (white arrow) and cardiomyocytes (white asterisk). (**I**,**L**) DME-*lacZ* (red) and Col (green) expression at stage 12 (I,J) and 15 (K,L). DME is inactive in the DA2 PC (J), and dorsal and alary muscles (L) in tup^{ex4} embryos. (**M**) Scheme of the *tup* CRM handover, allowing maintenance of *tup* expression in dorsal muscles, independently of Tin.

phase of expression, Tin sets the dorsal limit of Col expression, via a mechanism that remains unknown. At stage 15, Col is ectopically expressed in the DA2 muscle in 56% of tin^{EC40} segments (n=146), correlating with a DA3-like morphology (Fig. 6C,D). This mutant phenotype, reminiscent of *tup* mutants, prompted us to compare *tup* and *col* transcription between wild-type and tin^{EC40} embryos. At stage 11, while *col* is transcribed in several PCs in *tin* mutant embryos, *tup* transcription is strongly reduced in all dorsal cells and lost in the DA2 progenitor (Fig. 6F), indicating that Tin acts upstream of *tup* in specifying DA2 identity. Consistent with this loss of *tup* transcription, *col* remains transcribed in the DA2 muscle throughout differentiation (Fig. 6H). However, it is not expressed in more dorsal muscles where *tup* expression is also lost.

In summary, the staggered patterns of Tin and Col activation in stage 10 embryos define three distinct types of PCs along the DV axis: the DA1, DO1 and DO2 PCs, which express Tin and Tup; the DA2 PC, which expresses Tin, Tup and Col; the three DL PCs, which express Col but not Tin. Both spatial and temporal aspects of Tin, Tup and Col regulation are involved in distinguishing the DA2/AMP and DA3/D05 identities (Fig. 8).

Two separate cis-regulatory regions control Tup activation and maintenance in the DA2 lineage

The absence of *tup* transcription and IsletH expression in the DA2 PC in tin^{EC40} embryos (Fig. 7B,C) suggested that activation of *tup* transcription by Tin is mediated by IsletH, consistent with in vivo binding of Tin (Sandmann et al., 2007) (Fig. 7A). IsletH activity in the DA2 muscle is only transient, however (Fig. 7H), indicating the need for (an)other cis-regulatory region(s) responsible for maintaining *tup* transcription throughout muscle development. To identify other CRMs active in the mesoderm, we profited from data concerning the position of ChIP-validated binding sites of Tin, Twist and Mef2, which define so-called meso-ChiP CRMs (Liu et al., 2009; Sandmann et al., 2007; Zinzen et al., 2009). One such CRM maps to IsletH and overlaps tup-F4, a 1.5 kb IsletH subfragment previously shown to drive GFP in the heart, lymph gland and alary muscles (Tao et al., 2007); tup-F4 and IsletH give identical GFP patterns in dorsal muscles (data not shown). A second Meso-Chip CRM located upstream of IsletH binds Twi and Mef-2, but not Tin (Fig. 7A). An overlapping DNA region was retrieved from an in silico search for non-coding sequences selectively enriched for combinations of Twi-, Tin- and Ets-binding

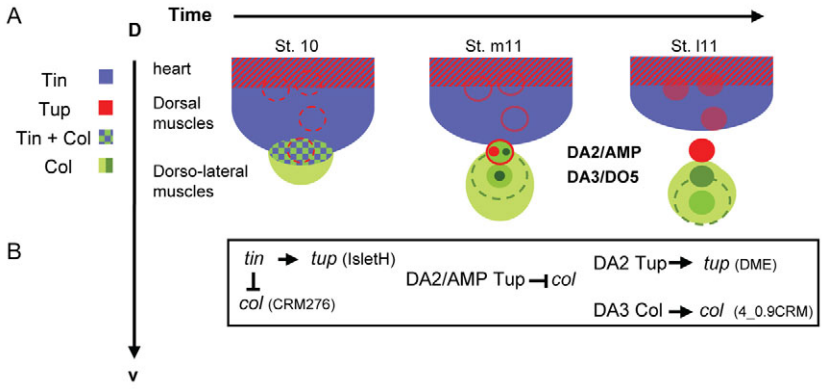

Fig. 8. Model for the sequential specification of the DA2/AMP and DA3/DO5 PCs. (**A**) Tin (blue) and Col promuscular expression (pale green) partly overlap at stage 10. The early born DA2 PC is specified from the Col- and Tin-expressing myoblasts. At mid stage 11, Tin and Col expression abut each other, owing to dorsal regression of Tin expression. Tin-mediated activation of *tup* transcription (red dot) in the DA2 PC, leads to repression of *col* transcription (dark green dot). The second-born DA3/DO5 PC transcribes *col* but not *tup*. At late stage 11, Tin expression becomes limited to cardiac progenitors. Tup and Col are expressed in a mutually exclusive manner in the DA2/AMP and DA3/DO5 PCs, respectively. Tup and Col are expressed in other dorsal and DL PCs, respectively. (**B**) *col* promuscular/PC expression is driven by an early CRM (CRM276); *tup* activation by Tin in the DA2 PC is via IsletH Tup and Col subsequently autoregulate their own expression in the DA2 and DA3 lineages, via the 4_0.9CRM (Enriquez et al., 2010) and DME, respectively.

sites (Philippakis et al., 2006; Warner et al., 2008) (A. Aboukhabil and M. Bulyk, personal communication). Taking into account ChiP-ChIP data, and sequence conservation during evolution, we identified a 3 kb fragment, designated below as DME (dorsal muscle enhancer), that drives *lacZ* expression in heart cells, the dorsal and the alary muscles (Fig. 7A,H; supplementary material Fig. S4). DME starts to be active in the DA2 FC at stage 12 (Fig. 7E,F) and remains active in the dorsal and alary muscles up to stage 16, at a time when IsletH-driven expression is no longer detected (Fig. 7G,H). The sequential activity of IsletH and DME in the DA2 lineage was reminiscent of the sequential activity of an early CRM (CRM276) and a late CRM (4_0.9*col* CRM) in the control of mesodermal *col* transcription (Enriquez et al., 2010). It raised the possibility of a similar relay process, whereby Tup protein expressed in the DA2 PC under control of IsletH, would subsequently auto-regulate its own expression via DME. To test this possibility, we examined IsletH and DME-*lacZ* expression in tup^{ex4} embryos and found that, while IsletH remains active (Fig. 7D), DME-*lacZ* expression is completely lost from the DA2 PC (Fig. 7I,J). Staining stage 16 embryos confirmed that DME activity in the dorsal muscles is dependent upon Tup (Fig. 7K,L). In situ hybridization confirmed that *tup* transcription in dorsal muscles is lost in the absence of *tup* (not shown). We conclude that Tup-positive autoregulation, mediated directly or indirectly by DME, is required for *tup* expression in dorsal muscles throughout differentiation (Fig. 7M).

DISCUSSION

Drosophila Tup/Islet plays key roles in specification of neurons (Thor and Thomas, 1997) and cardiac and lymph gland progenitor cells (Mann et al., 2009; Tao et al., 2007). We show here that it is required for somatic muscle development.

Intertwined cascades of iTF regulation in embryonic and adult muscle progenitors

The pattern of rp298*lacZ* expression and the three-dimensional arrangement of FCs distinguished four groups: dorsal, dorsolateral, lateral and ventral (Beckett and Baylies, 2007; Nose et al., 1998). Whether this topology reflects specific genetic programs has remained unclear. Tup and Col are expressed in the four dorsal and three DL PCs, respectively, supporting the notion of DV regionalization of the somatic mesoderm. This notion was evoked by regional Pox meso (Poxm) expression in most ventral and lateral FCs (Duan et al., 2007). As other known iTFs are only expressed in subsets of dorsal PCs/FCs (Tixier et al., 2010), it raised the possibility that Tup could reside at the top of dorsal iTF cascades. Our data show that this is not the case, as Tup, although required for Kr expression in the DO1 PC (Mann et al., 2009) (data not shown) and for Col repression in the DA2 PC, is not required for expression of Eve, Runt and Vg in the DA1, DO2 and DA2 and DA1 lineages, respectively (Fig. 2; supplementary material Fig. S1). Likewise, Col is required for expression of some iTFs but not others in DL PCs (Enriquez et al., 2012). Together, the patterns of Col, Eve, Kr, Poxm, Runt, Tup and Vg expression in wild-type and *tup* or *col* mutant conditions underline the intertwined, combinatorial nature of transcriptional regulatory networks specifying muscle identity. The DA2 PC gives rise to the DA2 muscle/DL AMP mixed lineage. Each abdominal hemisegment features six AMPs at stereotypical positions (Figeac et al., 2010). Other AMPs originate from mixed lineages, e.g. the ventral VA3/AMP and lateral SBM/AMP lineages. The VA3/AMP and SBM/AMP PCs express Poxm and S59, and Lb, respectively (Jagla et al., 1998; Ruiz-Gómez and Bate, 1997). Tup expression in the DA2/AMP lineage confirms that different AMPs express different iTFs at the time of specification. Whether, as for somatic muscles, the iTF code confers specific properties to each AMP remains an unresolved issue.

Temporal progression of Tin expression translates into unique muscle identities

How PCs born at similar positions in the somatic mesoderm come to express different combinations of iTFs and acquire distinct identities has remained elusive. For example, what distinguishes the fate of the two Eve-expressing PCs, which are sequentially born from the same dorsal cluster, is unknown (Carmena et al., 2002; Carmena et al., 1998). One other example is the expression of S59

and Lb, each in one of two abutting ventrolateral PCs: the LO1/VT1 and SBM PCs. Activation of both Lb and Slo expression in the two PCs is controlled by the same upstream regulator, Org-1 (Schaub et al., 2012). Subsequent reciprocal secondary cross-repression results in exclusive S59 or Lb expression (Junion et al., 2007; Keller et al., 1997), but the nature of the presumed positional bias responsible for the oriented resolution of this cross-repression has not been explored. In the case of the adjacent DA2 and DA3 PCs, we show here that Tup activation by Tin in the DA2 PC is instrumental in distinguishing between DA2 and DA3 identities. The DA2 PC is selected from a small group of cells at the intersection between Tin and Col expression domains. Thus, the relative registers of *tin* and *col* expression along the DV axis provide precise positional information. Another key is timing. The overlap between Tin and Col expression is only transient, such that only the earlier-born Col-expressing PC expresses Tin. This provides a unique temporal window for Tup activation and Col repression (Fig. 8). The transient overlap is due to the dorsal restriction of Tin expression to cardial cells during stage 11 (Yin et al., 1997). This dynamic process is controlled by JAK-STAT signalling activity in the mesoderm, which is itself modulated by Tin activity (Johnson et al., 2011; Liu et al., 2009). The key function of Tup in the DA2 PC, which is to distinguish between two muscle identities, illustrates how cell identity can be specified with single-cell precision when temporal dynamics are combined with positional information.

The CRM handover process: a general mode of muscle identity propagation?

Some iTFs are expressed during all steps of myogenesis, from promuscular stage to muscle attachment (Baylies et al., 1998; Frasch, 1999). Schematically, two major phases of expression can be distinguished: (1) PC specification when multiple iTFs are expressed in different PCs and extensive cross-regulation occurs, leading to FC-specific iTF patterns (Enriquez et al., 2012; Junion et al., 2007; Knirr et al., 1999; Ruiz-Gómez and Bate, 1997) (Fig. 2; supplementary material Fig. S1); and (2) muscle differentiation when the FC pattern is maintained and propagated into the syncytial fibre (Rushton et al., 1995) via transcriptional activation of the iTF code in newly fused FCMs (Crozatier and Vincent, 1999; Dubois et al., 2007; Knirr et al., 1999). Analysis of *col* regulation in the DA3 lineage showed that these two phases rely on two separate, early (CRM276) and late (4_0.9) CRMs, the activity of the late CRM requiring Col provided under the control of the early CRM. We termed this auto-regulatory loop a CRM handover mechanism (Dubois et al., 2007; Enriquez et al., 2010). Here, we have provided evidence that *tup* transcriptional regulation in the DA2 muscle follows the same rule. On the one hand, *tup* activation by Tin is mediated by an early CRM, IsletH; on the other, *tup* expression is maintained in differentiating muscles via a late CRM, DME, the activity of which depends upon Tup (Fig. 7). We propose that this handover relay mechanism could be a widespread mode of iTF regulation, as it efficiently links early steps of muscle specification in response to positional information with final muscle identity.

New parallels between the transcriptional regulatory networks that pattern *Drosophila* dorsal muscles and vertebrate jaw muscles

Tup and Tin are key components of the transcriptional regulatory cascade that controls early cardiogenesis, with Tin acting to activate Tup, the expression of which then persists after Tin has ceased to be expressed. We have now shown that a similar cascade operates in the somatic muscle mesoderm. Tup and Tin expression in both the heart and dorsal somatic muscles recalls Nkx2.5 (Tin ortholog) and Islet1 expression in the pharyngeal mesoderm, which contributes to some head muscles and part of the vertebrate heart (Kelly et al., 2001; Lescroart et al., 2010). Nkx2.5 is required for deployment of the SHF (Moretti et al., 2006; Prall et al., 2007) and Islet1 marks SHF progenitors that contribute both to the right ventricle and the arterial pole of the forming heart and a subset of skeletal pharyngeal muscles (Cai et al., 2003; Nathan et al., 2008; Tzahor and Evans, 2011). Similarly, in the simple chordate *Ciona intestinalis*, Nk4 (Tin/NKx2.5) marks the cardio-pharyngeal mesoderm at the origin of the heart and atrian siphon muscles (ASMs) that are evocative of vertebrate pharyngeal muscles. Islet-expressing cells also contribute to ASMs (Stolfi et al., 2010; Tolkin and Christiaen, 2012), suggesting an evolutionarily conserved link between cardiac and pharyngeal muscle development. Interestingly, the ascidian Col/EBF ortholog Ci-COE, is expressed in ASM precursors and is a crucial determinant of the ASM fate (Stolfi et al., 2010), reminiscent of *Xenopus* XCoe2 expression and requirement in pharyngeal arches for aspects of jaw muscle development (Dubois et al., 1998; Green and Vetter, 2011). It is now well established that distinct genetic networks govern skeletal myogenesis in the vertebrate head and trunk (Bothe and Dietrich, 2006; Sambasivan et al., 2011). The repertoire of TFs differentially deployed in the head mesoderm includes Tbx1, the *Drosophila* ortholog of which, Org-1, has recently been shown to act as a muscle iTF (Schaub et al., 2012). Tin/Nkx2.5, Tup/Isl1, Org-1/Tbx1 and Col/EBF may thus be part of a repertoire of transcription factors co-opted and diversified to regulate muscle patterning in *Drosophila* trunk and head muscle patterning in chordates.

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Competing interests statement

The authors declare no competing financial interests

Supplementary material

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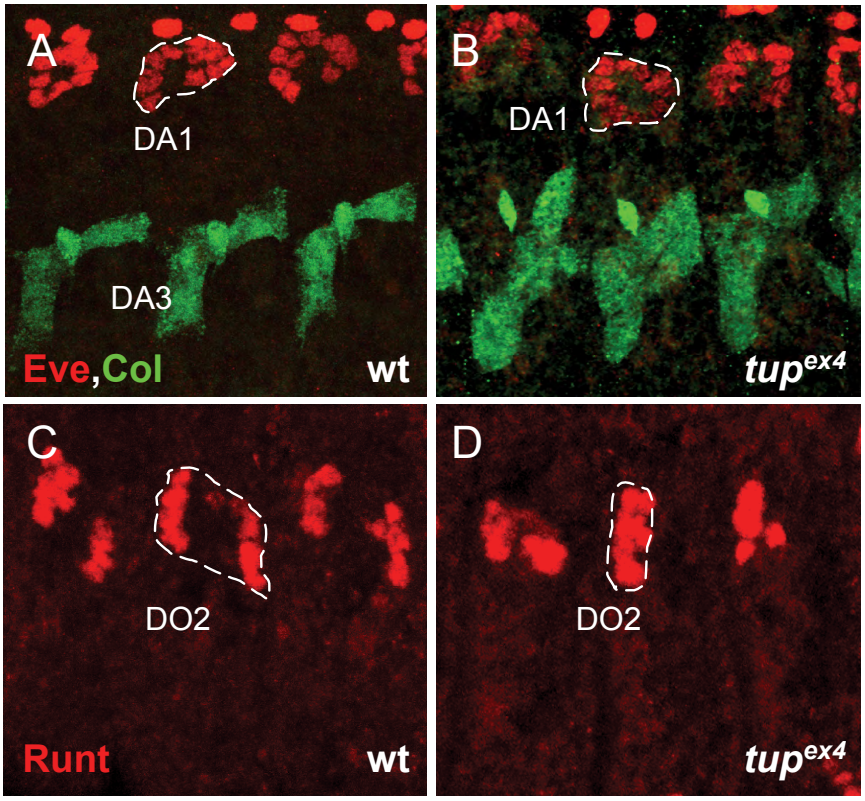

Fig. S1. Eve and Runt expression in dorsal muscles in *tup* mutant embryos. Dorsolateral views of three abdominal segments of stage 15 embryos are shown. (**A**) Wild-type embryo. Eve (red) and Col (green) are expressed in the DA1 (dotted circled in one segment) and DA3 muscles, respectively. (**B**) DA1 Eve expression is preserved in *tup*^{ex4} embryos. (**C**) Wild-type Runt expression in two rows of nuclei in the DO2 muscle (white-dotted circled in one segment). (**D**) Runt expression reveals an abnormal clustering of the DO2 nuclei.

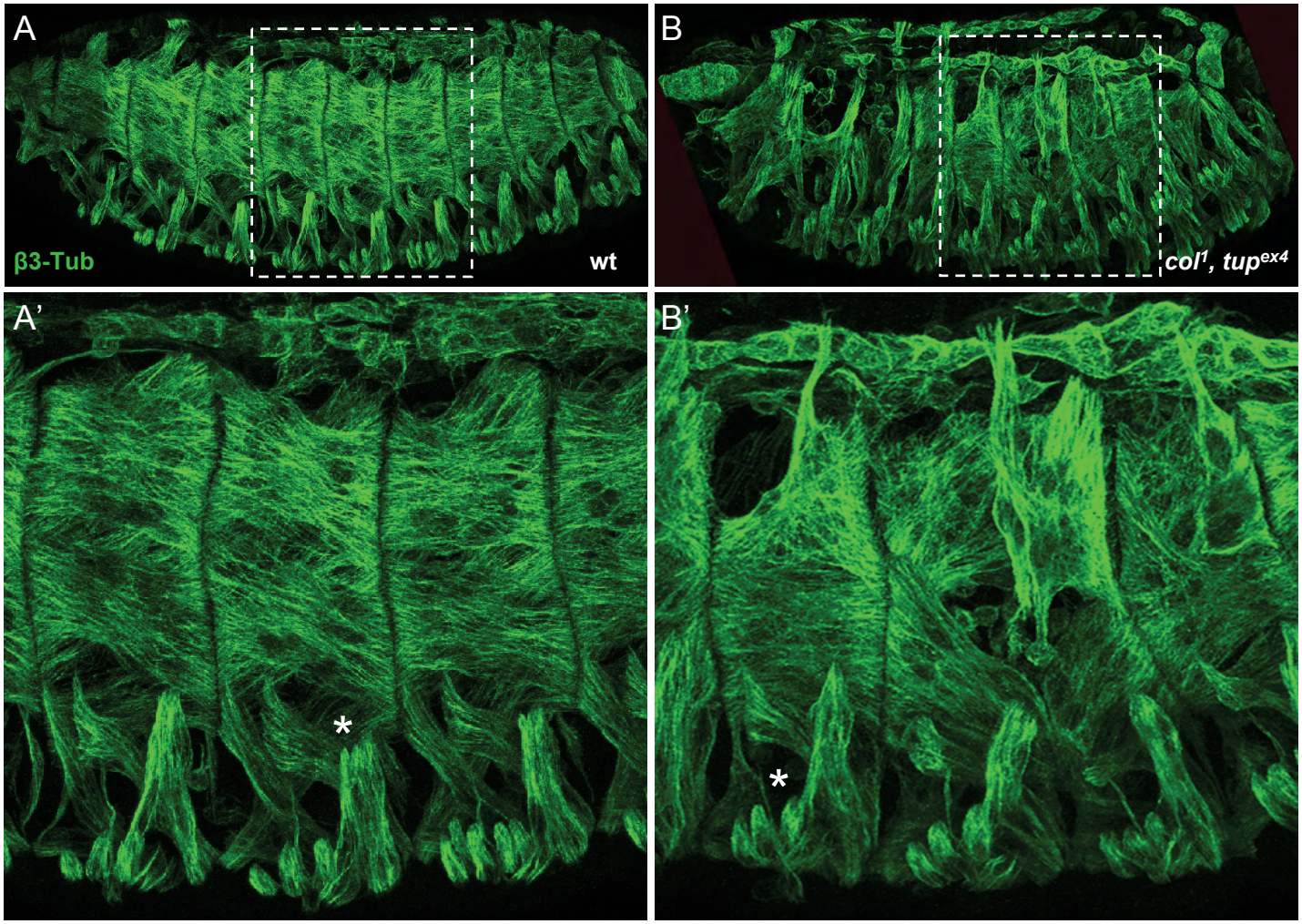

Fig. S2. Cumulative muscle defects in double *col*^{1}, *tup*^{ex4} mutant embryos. (**A**,**B**) β3-Tubulin staining of stage 16 wild-type (A) and *col*^{1}, *tup*^{ex4} (B) embryos. (A′,B′) High-magnification views of the dorsal and dorsolateral muscles in three segments (framed region in A,B). (B,B′) Major disorganization of the dorsal musculature and lack of muscle at the DA3 position (white asterisk) are observed.

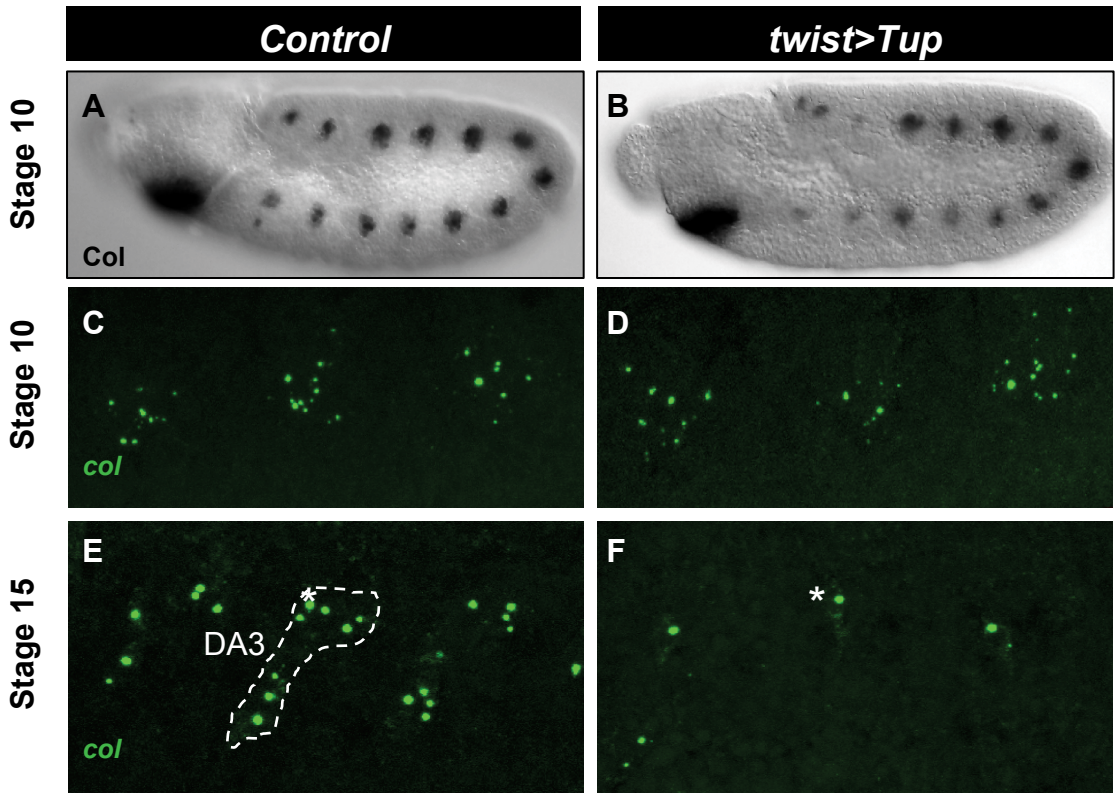

Fig. 3. Tup does not repress *col* transcription prior to the PC stage. (**A**,**B**) Col expression in wild-type (A) and *twi>tup* (B) embryos at stage 10. (**C-F**) *col* transcription in wild-type (C,E) and *twi>tup* (D,F) embryos. (A-D) Promuscular *col* expression is unaffected in *twi>tup* conditions; (E,F) DA3 *col* transcription is fully repressed at stage 15. The DA3 muscle is dotted circled in one wild-type segment. The white asterisk indicates *col* transcription in multidendritic neurons.

Fig. S4. Nucleotide sequence of *D. melanogaster* Tup-DME. FlyBase positions (release: r5.42) 2L: 18897397..18900688. Conserved sequences in *D. melanogaster*, *D. sechellia, D. simulans, D. yakuba, D. erecta, D. pseudoobscura, D. persimilis, D. ananassae* and *D.willistoni* are in black bold characters. Putative binding sites for Mef2 (purple) and Twi (yellow) were positioned using Jaspar, Consite and Genomatix (MatInspector) matrix models. Their position is indicated in the margin. Only matrix similarities greater than 0.9 were considered. In grey is region corresponding to the ChiP-ChIP data from E. E. Furlong laboratories (http://furlonglab.embl.de/data/browse_chip_mod/). This region includes late binding sites for twist (6-8 hours) and for Mef2 (6-8 hours, 8-10 hours and 10-12 hours).