Steroid-dependent modification of Hox function drives myocyte reprogramming in the *Drosophila* heart

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Summary

In the *Drosophila* larval cardiac tube, aorta and heart differentiation are controlled by the Hox genes *Ultrabithorax* (*Ubx*) and *abdominal A* (*abdA*), respectively. There is evidence that the cardiac tube undergoes extensive morphological and functional changes during metamorphosis to form the adult organ, but both the origin of adult cardiac tube myocytes and the underlying genetic control have not been established. Using in vivo time-lapse analysis, we show that the adult fruit fly cardiac tube is formed during metamorphosis by the reprogramming of differentiated and already functional larval cardiomyocytes, without cell proliferation. We characterise the genetic control of the process, which is cell autonomously ensured by the modulation of *Ubx* expression and AbdA activity. Larval aorta myocytes are remodelled to differentiate into the functional adult heart, in a process that requires the regulation of *Ubx* expression. Conversely, the shape, polarity, function and molecular characteristics of the surviving larval contractile heart myocytes are profoundly transformed as these cells are reprogrammed to form the adult terminal chamber. This process is mediated by the regulation of AbdA protein function, which is successively required within these persisting myocytes for the acquisition of both larval and adult differentiated states. Importantly, AbdA specificity is switched at metamorphosis to induce a novel genetic program that leads to differentiation of the terminal chamber. Finally, the steroid hormone ecdysone controls cardiac tube remodelling by impinging on both the regulation of *Ubx* expression and the modification of AbdA function. Our results shed light on the genetic control of one in vivo occurring remodelling process, which involves a steroid-dependent modification of Hox expression and function.

Key words: Reprogramming, Hox, Steroid, Heart

Introduction

Until recently, the differentiated state of a cell was thought to be maintained in a stable way. However, recent advances in mammalian stem cell research challenge this idea. Indeed, upon appropriate treatment in culture, stem cells can give rise to unrelated tissues by a process known as metaplasia (reviewed by Tosh and Slack, 2002). Transdifferentiation, which is the conversion of one differentiated cell type to another, is a further example of cell-type conversion. Given their potential interest for our understanding of normal or pathological development and for cellular therapy strategies (Burke and Tosh, 2005), both processes have received much attention in recent years. However, the majority of studies have involved in vitro systems, and the underlying molecular mechanisms remain largely unknown (Slack and Tosh, 2001; Tosh and Slack, 2002). Understanding how a cell committed to one phenotypic fate can acquire a different fate has important implications, ranging from regeneration to cancer, making important the analysis of naturally occurring reprogramming processes in genetically amenable organisms.

Drosophila melanogaster provides an invaluable model to analyse complex biological mechanisms in the context of an intact developing organism. In particular, metamorphosis offers the opportunity to study the genetic control of an in vivo occurring remodelling process in which the adult organism is, in part, reconstructed from already differentiated, functional organs and tissues.

Drosophila is a holometabolous insect whose metamorphosis is triggered by three peaks of the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) (Riddiford, 1993). Ecdysone exerts its effects through a heterodimer of two members of the nuclear receptor superfamily, the Ecdysone Receptor (EcR) and the fly RXR orthologue Ulstraspiracle (Usp). Ecdysone binding directly impinges on the transcription-regulatory activity of the heterodimer (reviewed by Riddiford et al., 2000; Thummel, 1996). During metamorphosis, most *Drosophila* larval tissues are eliminated by programmed cell death, and adult structures develop from imaginal cells that remain quiescent and have no functional requirement during larval life. However, some larval cells are not eliminated and are subjected to ecdysone-dependent changes in their shape and physiology. Such remodelling occurs mainly in the nervous system (Truman, 1990). For example, in mushroom bodies, the olfactory

learning and memory centre in insects, remodelling involves the pruning of larval projection and the subsequent acquisition of adult specific projections (Lee et al., 1999).

The cardiac tube of *D. melanogaster* is a simple linear tube that pumps and delivers haemolymph through the organism in an open circulatory system, as invertebrates have no vessels (Rizki, 1978; Rugendorff et al., 1994). Despite its simplicity, it is formed by highly conserved molecular mechanisms (Zaffran and Frasch, 2002). In the embryo and the larva, the cardiac tube extends from thoracic segment T1 to abdominal segment A7. Intra-segmental myocyte diversification is revealed in the abdominal segments by two transcription factors, Seven Up (Svp, the CoupTFII orphan nuclear receptor orthologue) and Tinman (Tin, the Nkx2.5 orthologue), which are respectively expressed in the two anterior and four posterior myocytes (Fig. 1C). Cooperating with this segmental information, the Hox genes *Ubx* and *abdA* trigger cardiac tube differentiation along the anteroposterior axis: *Ubx* expression is restricted to segments A1 to A4, which form the posterior aorta, while *abdA* is expressed in segments A5 to A7 and is required for heart differentiation (Lo et al., 2002; Lovato et al., 2002; Perrin et al., 2004; Ponzielli et al., 2002).

It has been reported that the *D. melanogaster* cardiac tube undergoes extensive changes during metamorphosis (Curtis et al., 1999; Molina and Cripps, 2001; Rizki, 1978), but both the precise origin of adult cardiac tube myocytes and the underlying genetic control had not been established until now. Using both time-lapse analysis of in vivo developing adult heart and cell tracing experiments, we demonstrate that myocytes in the larval cardiac tube are remodelled without proliferation to form the adult organ. In addition, we show that the organ remodelling involves a transcriptional reprogramming of each cell type. Finally, we investigate the genetic control of this process and show that it is cell autonomously controlled by the Ecdysone Receptors (EcRs), in a large part through the regulation of Hox gene expression and of the function of their protein products. In particular, EcR activation switches AbdA towards a new activity that leads *abdA*-expressing myocytes to be reprogrammed, and to acquire functions, morphology and transcriptional activity specific to the adult. This analysis provides several unexpected molecular insights into a naturally occurring cellular reprogramming process.

Materials and methods

Drosophila strains

The following UAS lines were obtained from the Bloomington *Drosophila* Stock Centre. Df(3R)Ubx-109, UAS-nls-lacZ, UAS-nls-GFP, UAS-mCD8-GFP, UAS-*Ubx Ia*, UAS-*abdA*, UAS-EcR-B1-ΔC655.F645A (EcR dominant negative). UAS-dsRNA>EcRcore was a generous gift from C. Antoniewski (Roignant et al., 2003). UAS-dsRNA>*abdA* and UAS-dsRNA>*Ubx* are described below. GAL4 drivers were: 24B-Gal4 (Brand and Perrimon, 1993), tinCΔ4-Gal4 (Lo and Frasch, 2001), and NP1029-Gal4 and NP5169-Gal4 [obtained from the Gal4 Enhancer Trap Insertion Database (GETDB), see http://flymap.lab.nig.ac.jp/~dclust/getdb.html]. P(tub-GAL80[ts]) was obtained from the Bloomington *Drosophila* Stock Centre. The *svp-lacZ* reporter line was from Kerber et al. (Kerber et al., 1998). *abd-A*^{M1} (Karch et al., 1990) mutants were obtained from Bloomington.

dsRNA-*abdA* and dsRNA-*Ubx* constructs

Inverted-repeat constructs were generated in order to produce genomic cDNA fusions predicted to form hairpin dsRNA molecules following splicing (Kalidas and Smith, 2002). Genomic and cDNA fragments were PCR amplified with primers containing unique restriction sites. The cDNA fragment was selected to avoid splice donor sequences (/GTNNGT) within the inverted repeat sequence. The dsRNA constructs were cloned into the pUAST transformation vector (Brand and Perrimon, 1993). Primer sequences are available upon request. Transgenic flies for UAS-IR constructs were generated as previously described using a w^{1118} strain as a recipient stock (Rubin and Spradling, 1982).

Control of Gal4 induction

UAS and P(tub-GAL80[ts]) (McGuire et al., 2003) transgenes were combined in the same lines and crossed with appropriate Gal4 lines. Development was allowed to proceed at 18°C until the late third instar larval stage and individuals were then shifted to the restrictive temperature (29°C).

Timing of pupal development

The onset of pupal development corresponds to white pupae that were selected on the basis of spiracle eversion, absence of reaction following forceps contact and absence of tanning. Individuals were kept for further development in an air incubator at 25°C.

Antibody staining

Dissections were done as described by Molina and Cripps (Molina and Cripps, 2001). For antibody staining, individuals were fixed for 1 hour in 1×PBS, incubated for 1 hour with 1×PBS, 1% Triton X-100, washed three times for 10 minutes each with BBT (1×PBS, 0.1% Tween, 0.1% BSA), incubated for 30 minutes with saturation medium (1×PBS, 0.1% Tween, 10% BSA) and overnight at 4°C with primary antibodies in BBT. After four 15-minute washes with BBT, samples were incubated for 30 minutes with saturation medium, incubated for 1 hour at room temperature with secondary antibodies in BBT and washed four times for 15 minutes each in BBT. Primary antibodies used: rabbit anti-β-galactosidase (Cappel), 1:500; mouse anti-GFP (Molecular Probes), 1:500; rabbit anti-Tin (Azpiazu and Frasch, 1993), 1:800; rabbit anti-D-Mef2 (Nguyen et al., 1994), 1:1000; mouse anti-Ubx (FP3.38) (White and Wilcox, 1985), 1:100; rat anti-Abd-A (Macias et al., 1990), 1:1000; rabbit anti-Synaptotagmin (Littleton et al., 1993), 1:1000; mouse anti-Wg, 4D4 (1:50) and mouse 22C10 (1:100), obtained from the Developmental Studies Hybridoma Bank. Affinity-purified secondary antibodies were coupled to alkaline phosphatase or biotin (Jackson ImmunoResearch Laboratories), or were Alexa-488 or Alexa-546 conjugated (Molecular Probes). All secondary antibodies were used at a dilution of 1:500. In the adult, better signals were obtained for Ubx with the aid of a Tyramide Signal Amplification kit (NEN Life Sciences).

Observations and photographs were carried out using a Leica MZ12 fluorescent binocular microscope, an Axiophot Zeiss microscope or a LSM 510 Zeiss confocal microscope.

In situ hybridisation

In situ hybridisation was performed as described previously (Ponzielli et al., 2002). For *Ih* and *Ndae1* detection, it was necessary to amplify the signal with the aid of the Tyramide Signal Amplification kit (NEN Life Sciences). In situ hybridisation on adults was performed as follows: individuals were dissected in PBS, fixed for 15 minutes in 4% paraformaldehyde in 1×PBS, rinsed three times for 10 minutes each in 1×PBS and rinsed three times in ethanol. Subsequent steps were identical to hybridisations on whole-mount embryos. Digoxigenin (Dig)-labelled antisense RNA probes specific for *Ih* were produced by transcription of an *Eco*RI-digested full-length cDNA (clone number GH 23838, BDGP release 1).

Phalloidin staining of F-actin

Dissected individuals were fixed in 1×PBS, 3.7% formaldehyde for 15 minutes at room temperature, washed three times for 10 minutes each in BBT, incubated for 20 minutes in saturation medium, incubated either with TRITC-phalloidin (Sigma; 1:60 in BBT, 10 minutes) or Alexa350-phalloidin (Molecular Probes; 1:20 in BBT, overnight) and rinsed three times for 10 minutes each in 1×PBS.

Results

Adult cardiac tube originates from the remodelling of larval cardiac tube myocytes

Our first aim was to supplement previous descriptions of the morphological and functional transformations of the cardiac tube during metamorphosis (Curtis et al., 1999; Molina and Cripps, 2001; Rizki, 1978). The morphological transformations of the cardiac tube during metamorphosis were visualised by polymerised actin (F-actin) staining with phalloidin, which reveals myofibril organisation and enables visualisation of the general morphology of the cardiac tube (see Fig. 1A,B). Major morphological and functional changes accompany the remodelling of the cardiac tube (see Fig. S2 and Movies 3-7 in the supplementary material).

In order to unambiguously determine the origin of the adult cardiac myocytes, we analysed cardiac tube-specific expression of GFP throughout metamorphosis. This approach indicated that the adult organ arises by the remodelling of the larval cardiac tube by a continuous and progressive process (see Movie 1 and Fig. S1 in the supplementary material for a detailed timetable description of cardiac tube remodelling). Time-lapse analysis of cardiac tube remodelling, performed from 27 to 64 hours after puparium formation (APF), demonstrates that adult myocytes directly derive from larval myocytes without cell proliferation (see Movie 2 in the supplementary material). In addition, cell tracing experiments (see Fig. S2A-C in the supplementary material) show that the adult heart is formed by the myocytes that constituted the larval posterior aorta (segments A1 to A4), and by segment A5 myocytes, which are a part of the larval heart and which constitute the posterior tip of the adult heart (the terminal chamber). Both approaches thus indicate that the adult heart is formed by persisting larval myocytes that are remodelled without proliferation or the addition of new cells. One identity for each adult cardiac myocyte can therefore be inferred from its larval origin. Fig. 1C summarises the fate of the larval myocytes during the remodelling process.

Remodelling is accompanied by transcriptional reprogramming of the cardiac tube myocytes

We next investigated whether the morphological and functional transformation of the cardiac tube myocytes is linked to changes in transcriptional activity during metamorphosis. During embryogenesis, *wg* is specifically expressed in the developing ostiae of the heart, precisely in the *svp*-expressing cells of segments A5 to A7 (Fig. 2A) (Lo et al., 2002). *wg* expression in the embryonic/larval cardiac tube is transient, and we failed to detect it in the third instar larval cardiac tube (data not shown). In a screen for genes whose expression pattern could account for the differences in physiology between aorta and heart myocytes, we identified *Ih*, which, like *Ndae1* (Perrin et al., 2004), is expressed in the Tin-expressing myocytes of the embryonic heart (Fig. 2D), but not in *svp*-expressing cells. *Ih* encodes the *Drosophila* homologue of the vertebrate HCN (hyperpolarisation-activated cyclic nucleotide-gated) channels.

Cardiac tube metamorphosis is accompanied by an anterior shift of *wg*, *Ih* and *Ndae1* expression. *wg* is transiently expressed at 30-36 hours APF in *svp*-expressing cells in

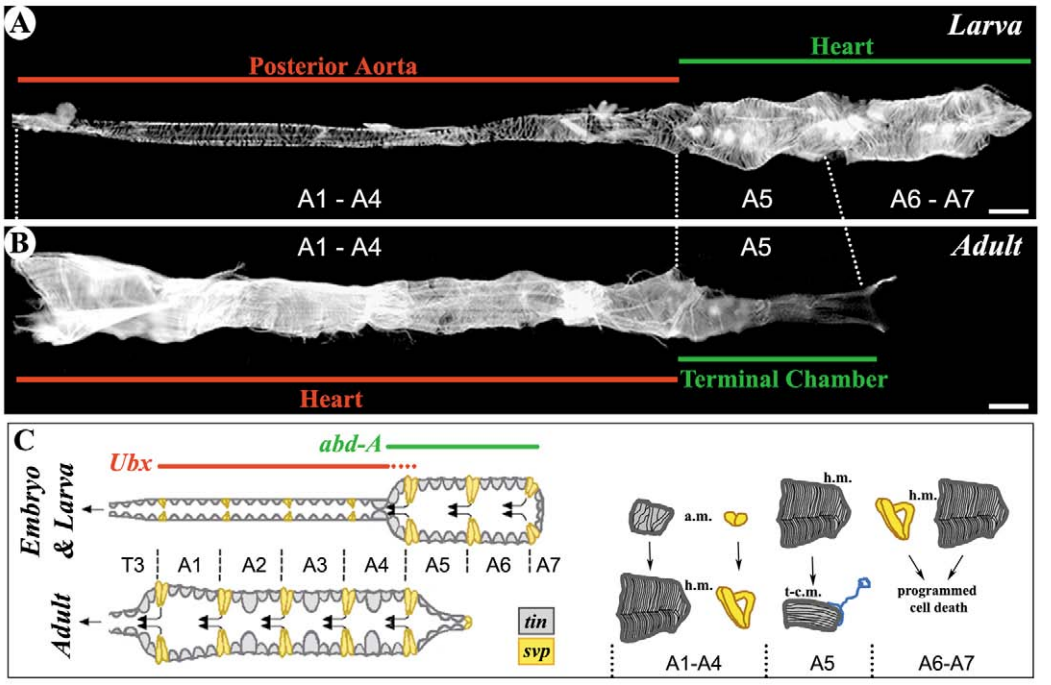

Fig. 1. *Drosophila melanogaster* cardiac tube remodelling. (A,B) Larval (A) and adult (B) cardiac tubes, stained with phalloidin for polymerised actin (F-actin), are shown at the same magnification. Scale bars: 50 μm. (C) Scheme of larval and adult cardiac tube. (Top) Larval cardiac tube. The *svp*, *tin*, *Ubx* and *abdA* expression patterns are indicated. (Bottom) Adult cardiac tube. *svp* and *tin* expression are indicated according to Molina and Cripps (Molina and Cripps, 2001) and to our own observations. The adult organ is formed by larval myocytes from T1 to A5 segments. Cardiac myocyte morphological modifications (including size, myofibril content and orientation, and innervations) are schematized (see Fig. S2 in the supplementary material). a.m., aorta myocytes; h.m., heart myocytes; t-c.m., terminal chamber myocyte.

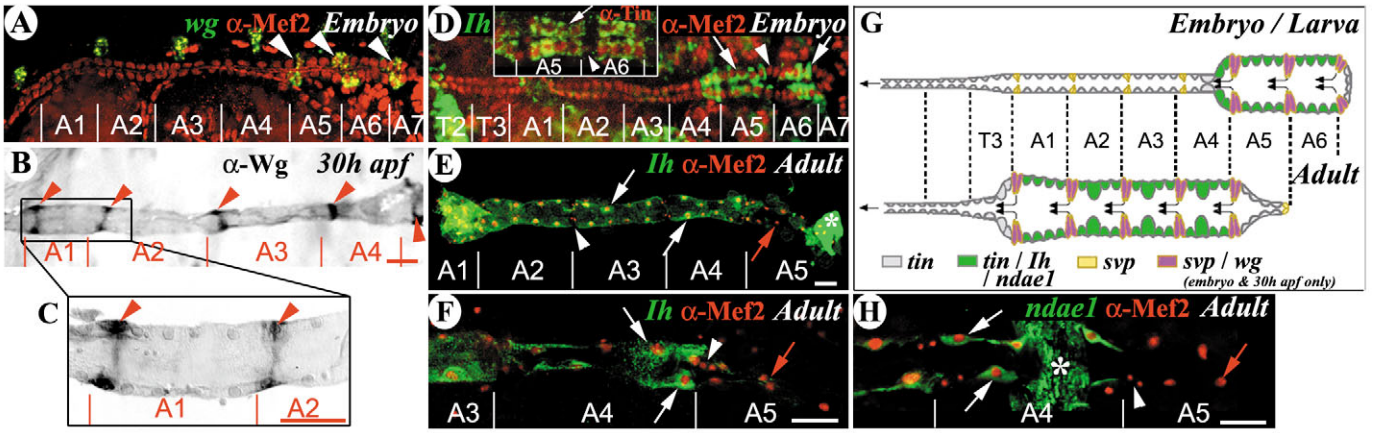

Fig. 2. Cardiomyocyte transcriptional reprogramming. (A,D) Embryonic expression of *wg* (A) and *Ih* (D) in stage 16 embryos. In situ hybridization (green), and anti-dMef2 staining (A,D, red) or anti-Tin staining (D, inset, red). (A) At stage 16, *wg* is expressed in *svp*-expressing cells in segments A5 to A7 (arrowheads) (Lo et al., 2002). (D) In the embryonic cardiac tube, *Ih* is expressed in the two posterior-most pairs of Tin-myocytes in segment A4, and in the four pairs of Tin-myocytes in segments A5 and A6 (arrows). *Ih* is not expressed in *svp*-expressing cells (which also do not express Tin, arrowheads), or in segment A7, or in the anterior and posterior aorta (segments T1 to A3 and anterior part of segment A4). (B,C) Anti-Wg staining of a 30-hour-APF pupal cardiac tube. Five pairs of cells express Wg. (C) At higher magnification and based on their morphology, Wg-expressing cells are identified as the *svp*-expressing myocytes of segments A1 to A5 (arrowhead). (E,F) *Ih* expression in the adult cardiac tube. *Ih* is expressed in all Tin-expressing myocytes of segments A1 to A4 (white arrows), but not in *svp*-expressing myocytes (arrowhead). *Ih* is no longer detected in segment A5 Tin-expressing myocytes (red arrow). (F) Detail of the posterior part of an adult cardiac tube. *Ih* is not expressed in *svp*-expressing myocytes (arrowhead), nor in segment A5 Tin-expressing myocytes (red arrow). (H) *Ndae1* expression in the adult heart. Detail of the posterior part of an adult cardiac tube. *Ndae1* is not expressed in *svp*-expressing myocytes (arrowhead), nor in segment A5 Tin-myocytes (red arrow), but is expressed in Tin-expressing myocytes of segments A1 to A4 (only A4 is shown, white arrows). Asterisk in E and H indicates fat body non-specific staining. (G) Schematic representation of *svp*, *Ih*, *Ndae1*, Tin and Wg expression in the embryonic and adult cardiac tubes. (A,D,E,F,H) Confocal images. Scale bars: 50 μm.

segments A1-A5 (Fig. 2B,C). Likewise, *Ih* is activated in Tin-expressing myocytes in segments A1-A4, which constitute the adult heart contractile myocytes (Fig. 2E,F), but, here again, is not activated in *svp*-expressing cells (Fig. 2E,F). Strikingly, *Ih* expression is turned off in segment A5 during remodelling (Fig. 2E,F). The same switch of expression pattern is observed for *Ndae1*, which becomes expressed in Tin-expressing myocytes in segments A1-A4 at adulthood, but which is repressed in segment A5 (Fig. 2H).

In conclusion, morphological and functional transformation of the cardiac tube myocytes is accompanied by transcriptional reprogramming during metamorphosis.

Adult heart differentiation requires the modulation of *Ubx* expression

In the embryonic cardiac tube, posterior aorta and heart identity are determined by the Hox genes *Ubx* and *abd-A*, respectively (Lo et al., 2002; Lovato et al., 2002; Ponzielli et al., 2002). We therefore investigated *Ubx* and *abd-A* function during cardiac tube remodelling. Ubx expression in the adult cardiac tube is restricted to *svp*-expressing myocytes (Fig. 3A,B), indicating that, compared with in embryos, its expression is repressed in Tin-expressing myocytes. The time course of Ubx expression during heart remodelling (Fig. 3C-H) indicates that Ubx remains expressed in Tin- and *svp*-expressing myocytes in segments A1-A4 up to 24 hours APF (Fig. 3C,D), but starts to be repressed in Tin-expressing cells at 30 hours APF (Fig. 3E,F) and is not detectable in these myocytes from 48 hours APF onwards (Fig. 3G,H). The dynamics of Ubx expression in the cardiac tube thus parallel remodelling (see Fig. S1 in the supplementary material), suggesting that *Ubx* repression in Tin-expressing myocytes is required for adult heart formation. To investigate this hypothesis, we induced *Ubx* expression in all muscle cells at the onset of metamorphosis using the 24B-Gal4 driver, which is expressed in all myogenic lineages, combined with the TARGET system (McGuire et al., 2003), in which the activity of the transcriptional activator Gal4 is under the control of a temperature-sensitive Gal80 inhibitor. As shown in Fig. 3J, artificial maintenance of *Ubx* expression during metamorphosis drastically alters adult heart formation. Tin-expressing myocytes in segments A1-A4 adopt characteristics of adult segment A5 Tin-expressing myocytes, including longitudinal orientation of the muscle fibres (Fig. 3L, compare with Fig. S2I in the supplementary material), and a lack of *Ih* expression (Fig. 3M). The same results were obtained when *Ubx* was driven by NP5169-Gal4, which is expressed in cardiac myocytes only (and not in ventral heart imaginal muscles; B.M. and L.P., unpublished). This strongly suggests that *Ubx* expression has to be repressed in the Tin-expressing myocytes for their proper development during metamorphosis.

In addition, the ventral heart imaginal muscles that develop beneath A1-A4 cardiac myocytes (see Fig. S2J-L in the supplementary material) are absent when Ubx overexpression is driven by NP5169-Gal4 in cardiac myocytes (not shown), indicating that their development depends on correct cardiac myocyte reprogramming.

Ubx overexpression does not, however, affect the differentiation of adult ostiae that develop from A1-A5 *svp*-expressing cells (arrowhead in Fig. 3L), suggesting that Ubx activity is either positive or neutral with regard to ostiae differentiation. To distinguish between these two possibilities,

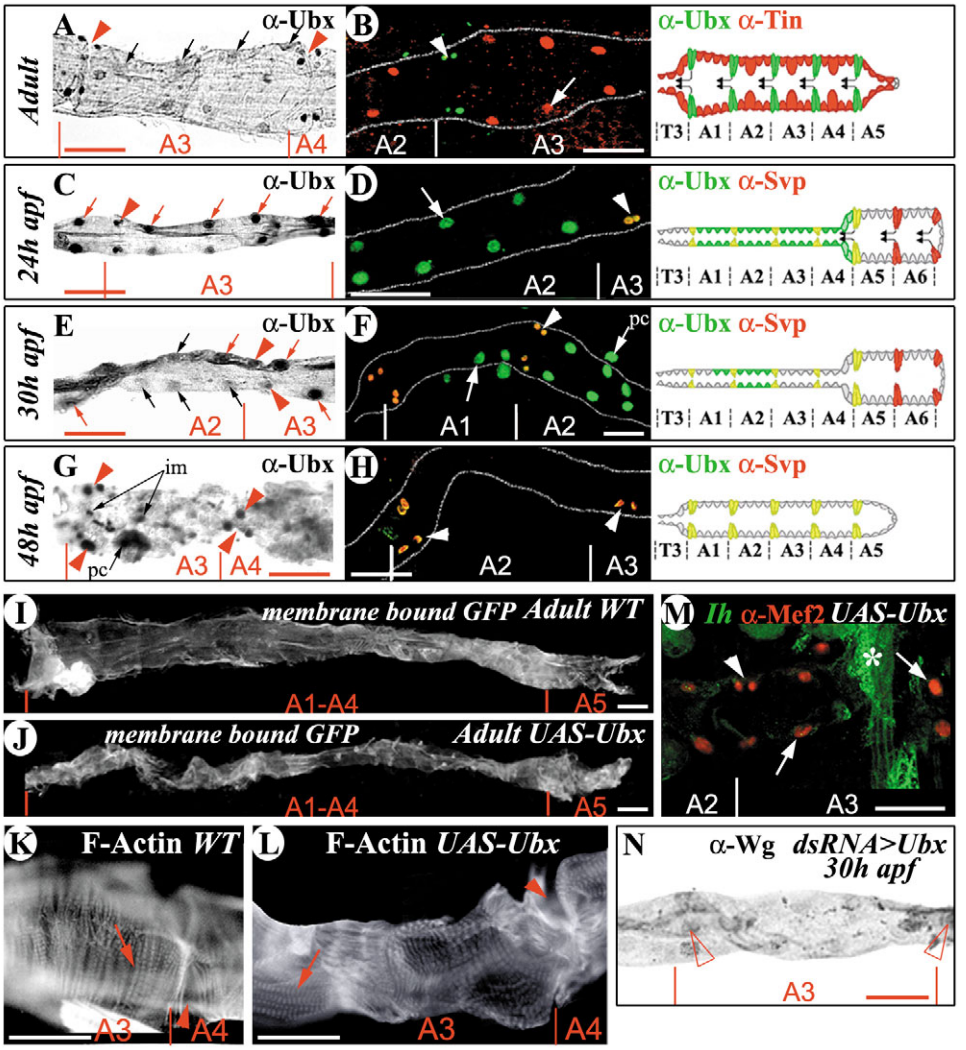

Fig. 3. Ubx expression and function during cardiac tube remodelling. (A-H) Ubx expression pattern during cardiac tube remodelling. A magnified view of the cardiac tube at the indicated stages is provided. (A,C,E,G) Anti-Ubx staining, revealed with Alkaline Phosphatase secondary antibody. (B,D,F,G; left) Fluorescent immunostaining against Ubx (green) and either β-Gal (red, D,F,G) in the *svp-lacZ* reporter strain or Tin (red, B). Cardiac tube limits are highlighted. Arrowheads indicate *svp*-expressing cells; arrows indicate Tin-expressing cells. (Right) Schematic drawing of the Ubx, Tin or Svp expression pattern at indicated stages. (A,B) Ubx expression pattern in the adult cardiac tube. Adult Ubx expression is restricted to the A1-A5 *svp*-expressing myocytes (arrowheads) that correspond to the ostium-forming cells. Arrows indicate unstained Tin-expressing myocytes, recognised by the size of the nucleus in A. (C,D) At 24 hours APF, Ubx is expressed in all Tin- (arrows) and *svp*-expressing (arrowheads) myocytes of segments A1-A4. (E,F) At 30 hours APF, Ubx expression becomes progressively repressed in some Tin-expressing myocytes (black arrows, E) but remains expressed in others (red arrows in E). pc, pericardiac cell unspecifically stained. (G,H) At 48 hours APF, Ubx expression is maintained in *svp*-expressing myocytes (arrowheads) but is not detected in Tin-expressing myocytes. pc, pericardiac cell; im, Ubx-expressing imaginal muscle cell nuclei. (I-M) Effects of Ubx overexpression during cardiac tube remodelling on myocyte differentiation (I-L) and *Ih* expression (M). In all cases, individuals were shifted to 29°C at the onset of metamorphosis. (I-L) Cardiac tubes from Gal80^{ts}, 24B>Gal4; UAS>mcd8-GFP alone (I,K) or Gal80^{ts}, 24B>Gal4; UAS>mcd8-GFP, UAS>*Ubx* (J,L) pharate adults. (I,J) Detection of membrane-bound GFP demonstrates that Ubx overexpression profoundly perturbs cardiac tube remodelling. (K,L) Phalloidin staining. Ubx overexpression has no visible effect on ostium differentiation (arrowhead in L), but perturbs the Tin-expressing myocyte remodelling that differentiates longitudinal myofibrils (arrow). (M) Cardiac tube from a Gal80^{ts}, 24B>Gal4; UAS>*Ubx* pharate adult stained for *Ih* transcripts (green) and dMef2 protein (red). Ubx overexpression prevents *Ih* expression in adult heart Tin-expressing myocytes (arrows). Asterisk indicates non-specific staining of the fat body; arrowhead indicates *svp*-expressing myocytes. (N) *Ubx* is required for *wg* expression in the *svp*-expressing myoctes at metamorphosis. Df(3R)Ubx-109; 24B>Gal4; UAS> dsRNA>*Ubx* cardiac tube at 30 hours APF, stained for Wg and detected with anti-mouse alkaline phosphatase-conjugated secondary antibody. Individuals were shifted to 29°C at the onset of metamorphosis. dsRNA>UAS-Ubx prevents Wg activation in *svp*-expressing cells (arrowheads). (B,D,F,H,M) Confocal images. Scale bars: 50 μm.

Ubx expression was inhibited by targeted, specific dsRNA in the cardiac tube during metamorphosis, and Wg expression was monitored as a landmark of ostiae differentiation. Targeted *Ubx*-dsRNA driven in the cardiac tube during metamorphosis consistently reduces Ubx expression (not shown). Under these conditions, Wg expression in *svp*-expressing cells is abolished (Fig. 3N), suggesting that Ubx function is required for adult ostiae development. In conclusion, the regulation of *Ubx* expression appears to be required to drive the remodelling of the posterior aorta into adult heart.

AbdA function is required for A5 remodelling

In the adult heart, AbdA is expressed in the terminal chamber, which is constituted by the Tin-expressing myocytes of segment A5 plus one *svp*-expressing myocyte pair in segment A6 (Fig. 4A-C). AbdA therefore remains expressed at a high level in those cardiac myocytes from the larval heart that persist in the adult. As described in Fig. S2 (in the supplementary material), these myocytes are directly targeted by specific nerve terminations during metamorphosis. In addition, myofibrils are re-oriented during the remodelling of A5 myocytes, becoming longitudinal in the adult, whereas they are transversal during larval stages, indicating a change of cell polarity (see Fig. S2H-I). To ascertain whether Abd-A is required for A5 segment remodelling, we inhibited its expression using dsRNA.

The targeted dsRNA method, when driven in the cardiac tube during metamorphosis by either the pan myogenic driver 24B-Gal4 or the heart-specific driver NP5169-Gal4, proved to be very efficient at inhibiting *abd-A* expression (not shown). *abdA* loss of function completely prevents terminal chamber differentiation. In particular, the organisation of the myofibrils in segment A5 is unmodified at metamorphosis. The fibrils remain oriented transversally, as in the larval heart, instead of being remodelled and longitudinally oriented, as observed in the wild-type adult organ [compare phalloidin staining of wild-type (Fig. 4E) and mutant (Fig. 4F) posterior cardiac tubes]. In addition, *abdA* loss of function prevents the formation of nerve terminations, without affecting axon growth (Fig. 4D). This indicates that *abdA* function is cell autonomously required in the myocytes of segment A5 for their innervations. By contrast, dsRNA>*abd-A* expression in the cardiac tube does not affect the histolysis of segments A6 and A7, which are eliminated upon *abdA* loss of function (Fig. 4D,F), suggesting that the

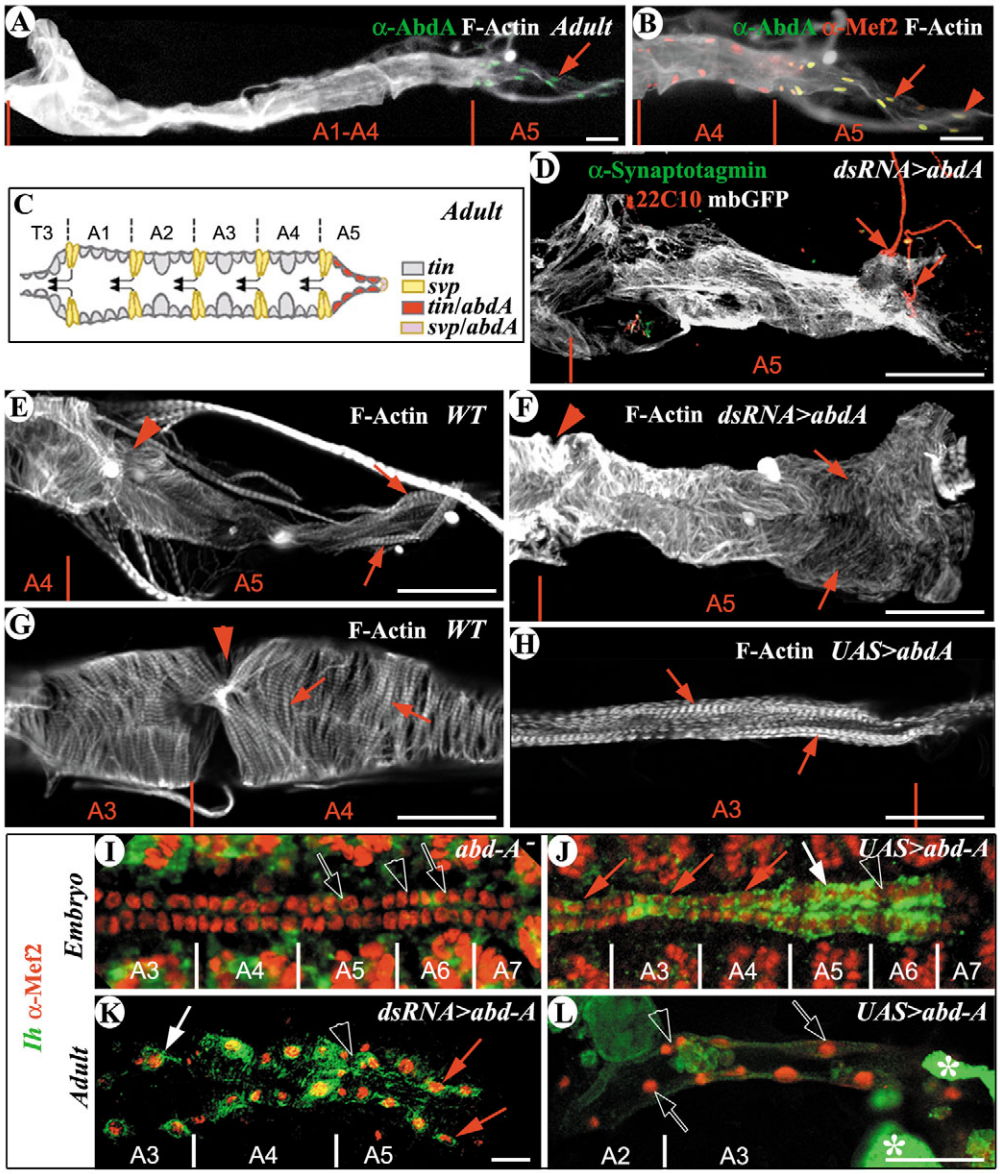

Fig. 4. AbdA expression and function during cardiac tube remodelling. (A,B) Adult heart stained for AbdA (green), polymerised actin (phalloidin, white) and dMef2 (B, red). AbdA is expressed in segment A5 Tin-expressing myocytes (arrow) and in the posterior-most pair of myocytes that correspond to the anterior-most segment A6 *svp*-expressing myocytes (arrowhead). (C) Schematic drawing of AbdA expression in the adult cardiac tube. (D-L) In all cases, individuals were shifted to 29°C at the onset of metamorphosis. (D) Posterior tip of a Gal80^{ts}, 24B>Gal4; dsRNA>*abdA; UAS>mcd8-GFP* adult cardiac tube, stained for Synaptotagmin (green), GFP (mb GFP, white) and 22C10 (red). 22C10 staining shows that neurones accurately extend to the posterior cardiac tube (red arrows), but *abdA* loss of function during metamorphosis prevents nerve termination to form synaptic contacts on A5 myocytes, as judged by the absence of sinaptotagmin staining on A5 myocytes (compare with Fig. S2M in the supplementary material). (E,F) Phalloidin staining of the posterior tip of wild-type (E) and Gal80^{ts}, 24B>Gal4; dsRNA>*abdA* (F) adult cardiac tubes. Arrows point to Tin-expressing myocytes; arrowheads indicate *svp*-expressing cells. Loss of *abdA* function during metamorphosis prevents segment A5 remodelling. Myofibrils are oriented transversally instead of longitudinally and the A5 segment is thicker than in wild type. In F, note that loss of *abdA* function does not affect histolysis in segments A6-A7. (G,H) Phalloidin staining of a wild-type (G) or Gal80^{ts}, 24B>Gal4; UAS>*abdA* (H) adult cardiac tube. In G, magnification of A3-A4 segments illustrates the transversal orientation of myofibrils. (H) The cardiac tube is thinner following AbdA overexpression during metamorphosis and myofibrils are longitudinal, instead of transversal as in the wild type. (I-L) Effects of *abdA* dosage on *Ih* expression in the cardiac tube during embryogenesis (I,J) and metamorphosis (K,L). In all cases, anterior is left, *Ih* transcripts are in green and dMef2 protein in red. Arrows indicate Tin-expressing myocytes, arrowheads *svp*-expressing myocytes. (I,J) In the embryo, loss of *abdA* function (I, *abd-A^{M1}*) prevents *Ih* expression in the cardiac tube, whereas *abdA* misexpression (J, 24B>Gal4; UAS>*abdA*) induces ectopic expression in Tin-expressing myocytes (red arrows, compare with Fig. 2D). (K) Gal80^{ts}, 24B>Gal4; dsRNA>*abdA* adult cardiac tube. Loss of *abdA* function during metamorphosis induces *Ih* expression in segment A5 Tin-expressing myocytes (red arrows). (L) Gal80^{ts}, 24B>Gal4; UAS>*abdA* adult. *abdA* overexpression represses *Ih* transcription in A1-A4 Tin-expressing myocytes (open arrow). Asterisk indicates non-specific fat body staining. Scale bars: 50 μm.

histolysis segment A6-A7 myocytes is independent of *abd-A* function.

Conversely, ectopic *abdA* expression during metamorphosis under the control of either the 24B-GAL4 or NP5169-Gal4 drivers combined with the TARGET system, led to an almost complete transformation of segments A1-A4 into structures characteristic of the terminal chamber. This transformation was characterized by the thinning of the cardiac tube and the longitudinal orientation of the myocyte muscle fibres (Fig. 4G,H). AbdA overexpression also prevented ventral imaginal muscle formation (not shown), further supporting the requirement of normal cardiac myocyte remodelling for proper ventral imaginal muscle development.

AbdA cellular function is modified at metamorphosis

Our results demonstrate that, during metamorphosis, *abdA* drives the remodelling of segment A5 and thus confers a terminal chamber identity onto A5 myocytes. This activity is clearly distinct from that previously described in the same cells during embryogenesis, as *abdA* is required there to confer a heart identity to the cardiac myocytes, and is therefore responsible for the differentiation of ostiae and contractile myocytes (Lo et al., 2002; Lovato et al., 2002; Perrin et al., 2004; Ponzielli et al., 2002).

Accordingly, *abdA* dosage manipulation has distinct consequences for *Ih* expression when analysed during embryogenesis and during metamorphosis. When *abdA* function is inhibited in the cardiac tube during remodelling, *Ih* expression is maintained in segment A5 (Fig. 4K). Reciprocally, targeted *abdA* overexpression within the whole cardiac tube during metamorphosis represses *Ih* expression in segments A1 to A4 (Fig. 4L). By contrast, during embryogenesis, *abd-A* is required for *Ih* expression (Fig. 4I) and *abdA* misexpression in the whole cardiac tube ectopically activates *Ih* transcription (Fig. 4J). These differential outputs of *abdA* activity during embryogenesis and metamorphosis, observed at the level of both cellular differentiation and *Ih* transcriptional control, highlight that the cellular function of AbdA is modified at metamorphosis.

Ecdysone signalling is required to cell autonomously trigger cardiac tube remodelling

What could be the temporal input that leads to the regulation of *Ubx* expression and the switch in AbdA activity? Because the reprogramming occurs at metamorphosis, we investigated whether ecdysone signalling has a role in the process. Indeed, the remodelling starts at 30 hours APF, which corresponds to the latest and major peak of ecdysone titre observed during *Drosophila* metamorphosis (Riddiford, 1993).

Ecdysone signalling is mediated by a heterodimer of nuclear receptors, consisting of one Ecdysone Receptor (EcR) isoform (either EcR-A, -B1 or -B2) and Ultraspiracle (Usp), which is activated upon ecdysone binding (Riddiford et al., 2000; Thummel, 1996). In order to evaluate the involvement of ecdysone-signalling in remodelling, we interfered with the reception of ecdysone signalling in two ways, either with the targeted dsRNA technique [with an UAS>dsRNA-EcR construct directed against the core common to all EcR isoforms (Roignant et al., 2003)], or with a dominant-negative form of EcR [UAS>EcR^{DN} (Cherbas et al., 2003)]. When driven in the cardiac tube during metamorphosis with either the 24B-Gal4 (Fig. 5A,B,D,E) or the NP5169-Gal4 (Fig. 5C) driver, both constructs dramatically inhibit heart remodelling. The cardiac tube retains its larval morphology, with the characteristic division into aorta in segments A1 to A4 and heart in segments A5 to A7 (Fig. 5A). In addition, inhibition of ecdysone signalling prevents Wg activation in *svp*-expressing cells (Fig. 5B), and A5 myofibrils retain their larval circular orientation (Fig. 5D,E). Furthermore, segment A5 innervations are prevented following EcR inhibition (Fig. 5D), segments A6 and A7 are not eliminated (Fig. 5A) and ventral imaginal muscles do not form. These results demonstrate that ecdysone signalling is cell autonomously required for all aspects of cardiac tube remodelling.

Ecdysone signalling impinges on *Ubx* regulation and on *abdA* activity

We next investigated whether ecdysone signalling acts upstream of *Ubx* expression and AbdA activity. Inhibition of ecdysone signalling through dsRNA>EcRcore expression in the Tin-expressing myocytes with the 5169-Gal4 driver results in the maintenance of *Ubx* expression during metamorphosis (Fig. 5C), indicating that *Ubx* repression, which is required for the correct remodelling of the larval posterior aorta into adult heart, is regulated by the ecdysone pathway. Interestingly, despite maintenance of *Ubx* expression, A1-A4 Tin-expressing myocytes are not reprogrammed. This contrasts with the above described effect of *Ubx* ectopic expression during metamorphosis, which affects Tin-expressing myocyte remodelling and induces the formation of longitudinal myofibrils (Fig. 3J,L). This result might indicate that the action of Ubx expression in Tin-expressing myocytes during metamorphosis requires the ecdysone signalling pathway. In agreement with this hypothesis, we failed to detect any formation of longitudinal myofibrils in cardiac myocytes in which Ubx and EcR^{DN} were co-expressed during metamorphosis (data not shown).

AbdA expression is maintained in A5 myocytes after EcR inhibition (Fig. 5E). Its expression is therefore not dependent on ecdysone signalling. However, A5 myocytes that do express *abdA* are unable to remodel when EcR activity is depressed, suggesting that ecdysone signalling is instrumental in driving the switch of AbdA activity at metamorphosis. Indeed, the consequences of inhibiting EcR function during remodelling are very similar to those observed upon loss of *abdA* function, including a lack of innervations and of myofibril orientation remodelling (Fig. 5D,E). Consistent with this, ecdysone signalling downregulation inhibits the effects of AbdA overexpression. Indeed, although it efficiently induces terminal chamber-like structures in segments A1 to A4 when expressed alone, targeted AbdA overexpression is unable to induce terminal chamber-like structures in the cardiac tube when EcR function is inhibited, not only in A1-A4 segments (Fig. 5F), but also in A5 (Fig. 5G), where myofibrils remain transversal. Together, these results indicate that the reprogramming of segment A5 myocytes requires the modulation of AbdA activity (but not of its expression) by the ecdysone-mediated metamorphosis program.

Discussion

Transdifferentiation is the process by which a cell committed to one phenotypic fate acquires a different fate, implying that

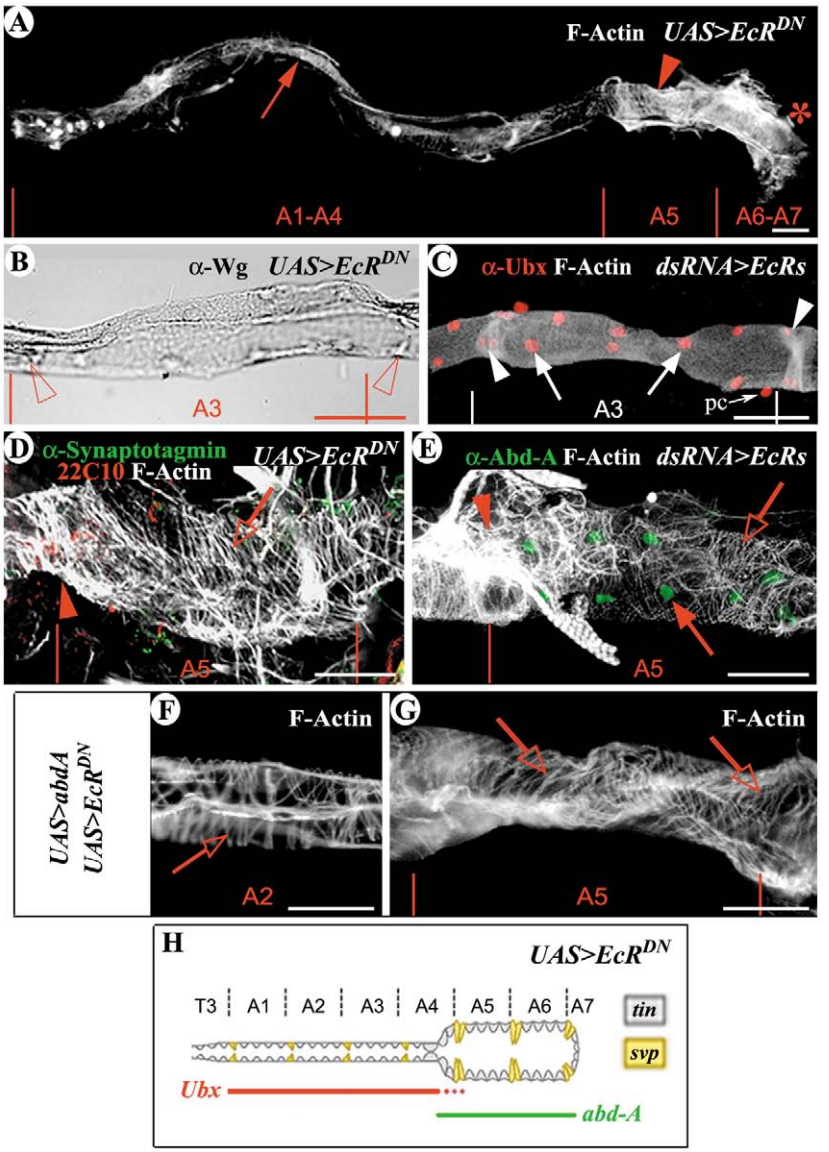

Fig. 5. Ecdysone signalling cell autonomously controls all aspects of cardiac tube remodelling. In all cases, individuals were shifted to 29°C at the onset of metamorphosis. (A) Phalloidin staining of a Gal80^{ts}, 24B>Gal4; UAS>EcR^{DN} pharate adult cardiac tube. Inhibition of EcR function prevents heart remodelling, including A6/A7 histolysis (red asterisk), A5 transformation (arrowhead) and posterior aorta remodelling, which remains thin anterior to A5 (arrow). (B) Gal80^{ts}, 24B>Gal4; UAS>dsRNA-*EcR* cardiac tube at 30 hours APF, stained for Wg, detected with anti-mouse alkaline phosphatase-conjugated secondary antibody. *wg* is not expressed in the *svp*-expressing myocytes (arrowheads). (C) Phalloidin (white) and anti-Ubx (red) staining of NP5169-Gal4; UAS>dsRNA-EcR pharate adult cardiac tubes. EcR inactivation in Tin-expressing myocytes prevents *Ubx* repression in segment A1-A4 Tin-expressing myocytes (arrows). pc, pericardial cell. (D) Anti-synaptotagmin (green), 22C10 (red) and phaloidin staining of Gal80^{ts}, 24B>Gal4; UAS>EcrDN pharate adult cardiac tubes. (E) Anti-AbdA (green) and phalloidin staining on Gal80^{ts}, 24B>Gal4; UAS>EcR^{DN} pharate adult cardiac tubes. In both cases, myofibrils are transversal (open arrow), indicating that EcR loss of function prevents terminal chamber differentiation. (D) Nerve terminations do not make synaptic contact with the myocytes, as judged by the absence of synaptotagmin staining. (E) EcR inactivation does not affect AbdA expression. (F,G) Phaloidin staining of Gal80^{ts}, 24B>Gal4; UAS>AbdA, UAS>EcR^{DN} pharate adult cardiac tubes. Inhibition of ecdysone signalling prevents AbdA from inducing terminal chamber structure both in A1-A4 segments (F) and in A5 (G). (H) Schematic representation of a cardiac tube in which EcR function has been inhibited during metamorphosis. Ubx and *abdA* expression are represented (see C and E). Scale bars: 50 μm.

some characteristics are lost while novel ones are acquired (Eguchi and Kodama, 1993; Slack and Tosh, 2001). Our study suggests that myocytes in segment A5 undergo a transdifferentiation process during cardiac tube remodelling: their shape and polarity are changed and they undergo transcriptional reprogramming. In addition, their physiological function is modified: they loose the rhythmic contractile activity they display during larval life, but gain specific innervations, implying that they may become involved in the regulation of cardiac activity. Indeed, adult A5 cardiac myocytes are directly innervated, whereas anterior innervations are targeted to ventral imaginal muscles but not to A1-A4 cardiac myocytes. Neurons innervating the terminal chamber were previously described as being positive for Crustacean Cardioactive Peptide [Ccap (Dulcis and Levine, 2003); Cardioacceleratory peptide – FlyBase] and might be involved in the regulation of the anterograde heart beat (Dulcis and Levine, 2005). A recent paper from the same group further supports this hypothesis (Dulcis et al., 2005).

Much attention has been given to transdifferentiation in recent years; however, the majority of studies have used artificial systems (Tosh and Slack, 2002) and the genetic control of the process is still largely unknown. Here, we have investigated the genetic and molecular control of a naturally occurring transdifferentiation process: the metamorphosis of the *Drosophila* larval cardiac tube. This situation offers the opportunity to analyse cell plasticity in an intact system, in which cells can be individually identified.

Like myocytes in segment A5, those in segments A1-A4 also gain a number of novel properties during remodelling. For example, Tin-expressing myocytes increase their myofibrillar content, activate *Ih* transcription and acquire contractile activity; *svp*-expressing cells transiently express *wg* and differentiate into functional ostiae. However, this may not represent transdifferentiation per se, as these cells do not appear to lose any of the differentiated characteristics they would have acquired during embryonic or larval life. Rather, we consider that larval A1-A4 myocytes are committed to differentiate into adult heart myocytes, but do not represent, on their own, a fully differentiated state. Interestingly, their final

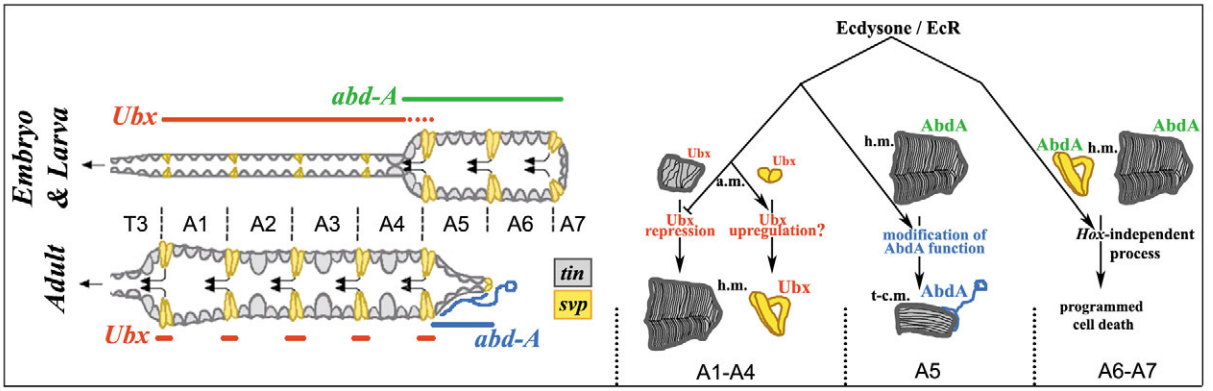

Fig. 6. Model of the ecdysone-dependent control of cardiac tube remodelling during metamorphosis. Ecdysone signalling triggers adult heart formation throughout the transcriptional regulation of *Ubx*. By contrast, ecdysone modifies AbdA function, whose activity is switched during remodelling towards a new genetic program that leads to differentiated cells with function, cell behaviour and transcriptional activity characteristic of the adult. Segment A6-A7 programmed cell death does not depends on *abdA* function. a.m., aorta myocytes; h.m., heart myocytes; t-c.m., terminal chamber myocyte.

differentiation relies on an ecdysone-dependent modulation of *Ubx* expression: *Ubx* expression has to be downregulated in Tin-expressing myocytes, while its maintenance, and probably its overexpression, is required in *svp*-expressing cells. *Ubx* function, however, appears to be unchanged in these cells. Indeed, when *Ubx* is overexpressed in the embryonic cardiac tube, it is able to activate *wg* expression in A1-A4 *svp*-expressing myocytes (Perrin et al., 2004), and to repress *Ih* transcription in Tin-expressing myocytes (B.M., unpublished). This clearly indicates that, at least at the *wg* and *Ih* transcriptional level, Ubx activity is unmodified during remodelling.

By contrast, the reprogramming of A5 myocytes depends on the modification of AbdA activity. Although *abdA* is required, during embryogenesis, for *Ih* transcription and for the acquisition of rhythmic contractile activity by the A5 myocytes that constitute part of the beating larval heart, its cellular function is modified at metamorphosis. During cardiac tube remodelling, *abdA* is indeed required for *Ih* repression, and for changes in the shape and polarity of myocytes, as well as for the acquisition of specific innervations. In these myocytes, the functional outcome of AbdA activity is thus dramatically different during embryogenesis and metamorphosis, indicating that *abdA* is instrumental in myocyte reprogramming.

The developmental switch triggering cardiac tube remodelling is mediated by ecdysone, which impinges on *Ubx* expression and on AbdA activity (see Fig. 6 for a summary model). Interestingly, like retinoic acid (RA) in the developing vertebrate hindbrain (Gavalas, 2002; Gavalas and Krumlauf, 2000), the outcome of ecdysone activity during the remodelling of segments A1-A4 is a 'posterior transformation': adult A1-A4 myocytes acquire properties characteristic of larval A5-A7 posterior myocytes. This is the first reported example of a steroid function in axial patterning in arthropods. In addition, we show that, like RA, which modulates Hox expression in vertebrates (Conlon and Rossant, 1992; Conlon and Rossant, 1995; Dupe et al., 1997; Kessel and Gruss, 1991), the ecdysone signalling pathway modulates *Ubx* expression. These observations strongly suggest that steroid activity on axial patterning is conserved from flies to vertebrates.

In contrast to its effects on *Ubx*, ecdysone does not affect *abdA* expression but impinges on its activity. This is the first observation of such an activity for a steroid hormone and it certainly warrants further study in other animal models. Is this also a property of steroids (such as RA) in vertebrates? To date, the only known activity of RA on Hox gene function is a modulation of their transcriptional expression. The data presented here recommend a re-examination of RA/Hox functional interactions in vertebrates.

Cellular identities along the rostrocaudal axis mainly depend on the Hox code, i.e. on the combinatorial expression of Hox genes (Hunt and Krumlauf, 1992; Kessel and Gruss, 1991; Kmita and Duboule, 2003). The fact that, as we show here, a steroid hormone can additionally control Hox activity greatly increases the number of cellular identities that can potentially be determined by the same Hox code. In addition, the situation described here concerning AbdA activity indicates the importance of the developmental context upon Hox specificity (Brodu et al., 2002; Lohmann and McGinnis, 2002; Rozowski and Akam, 2002). An important unanticipated result from the present study is that the change of AbdA activity can occur in the same cells, leading to a reprogramming between two differentiated states that are both controlled by the same Hox gene.

A future challenge will be to understand the molecular basis of the steroid-dependent Hox activity switch. Ecdysone signalling might change AbdA transcriptional activity, by specifying new transcriptional targets or by switching its activity from an activator to a repressor (and vice versa) on the same transcriptional targets. What could be the molecular mechanism by which EcR activation impinges on AbdA activity? Given the pivotal role played by transcriptional cofactors in Hox function (Mann and Affolter, 1998; Mahaffey, 2005), one hypothesis is that EcR activates or represses such a Hox cofactor. Exd/Hth are obvious candidates (Mann and Affolter, 1998) but do not seem to be involved; they are not expressed in the cardiac myocytes, neither during embryogenesis (Perrin et al., 2004) nor during the remodelling process (B.M., unpublished). Conversely, there is evidence that Hox transcriptional activity can be modified by

phosphorylation (Jaffe et al., 1997). An alternate possibility would therefore be that EcR activates or represses the expression of some kinases or phosophatases, which would in turn modify AbdA activity. In any case, elucidation of the molecular mechanisms will rely on the identification of direct *abdA* targets in the cardiac tube. *wg* and *Ih* are potential candidates, and are currently under investigation.

In conclusion, our study may shed light on Hox gene function and regulation in other cell reprogramming processes, such as pathological remodelling of the human heart, in which steroids appear to play a key role (Sun, 2002). In addition, cellular therapies for inherited myopathies are based on the cellular plasticity of the donor cells (either satellite cells or muscle-derived stem cells). Cell transplantations for the treatment of muscular dystrophies appear promising (Huard et al., 2003), and understanding the molecular basis of one example of myocyte reprogramming should help to unravel the underlying mechanisms.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/23/5283/DC1

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