Notch directly regulates cell morphogenesis genes, *Reck*, *talin* and *trio*, in adult muscle progenitors

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SUMMARY

There is growing evidence that Notch pathway activation can result in consequences on cell morphogenesis and behaviour, both during embryonic development and cancer progression. In general, Notch is proposed to co-ordinate these processes by regulating expression of key transcription factors. However, many Notch-regulated genes identified in genome-wide studies are involved in fundamental aspects of cell behaviour, suggesting a more direct influence on cellular properties. By testing the functions of 25 such genes we confirmed that 12 are required in developing adult muscles consistent with roles downstream of Notch. Focusing on three, *Reck*, *rhea/talin* and *trio*, we verify their expression in adult muscle progenitors and identify Notch-regulated enhancers in each. Full activity of these enhancers requires functional binding sites for Su(H), the DNA-binding transcription factor in the Notch pathway, validating their direct regulation. Thus, besides its well-known roles in regulating the expression of cell-fate determining transcription factors, Notch signalling also has the potential to directly affect cell morphology/behaviour by modulating expression of genes such as *Reck*, *rhea/talin* and *trio*. This sheds new light on functional outputs of Notch activation in morphogenetic processes.
INTRODUCTION

Notch signalling is a local cell communication mechanism highly conserved throughout the animal kingdom. It is implicated in a variety of developmental and physiological processes and aberrant Notch activity is linked to many different diseases, including cancers and neurodegenerative disorders (Bolos et al., 2007; Louvi and Artavanis-Tsakonas, 2012). The Notch family of receptors and the Notch ligands, Delta and Serrate (Jagged in vertebrates), are cell-surface type I transmembrane proteins. Upon ligand binding, Notch receptors undergo two proteolytic cleavages that lead to the release of the Notch intracellular domain (Niced). Niced is the active form of the receptor and acts in the nucleus as a transcriptional regulator, in cooperation with the DNA-binding protein CSL (also known as Su(H), CBF1 and LAG-1) and its co-activator Mastermind (Bray, 2006). Thus, transduction of Notch signalling is relatively simple and primarily results in the regulation of target genes. The identification and characterization of Notch target genes is therefore crucial to fully understand the function of Notch in developmental processes.

The best characterized target-genes of Notch encode transcription factors of the HES/E(spl) and Hey/Hesr/Herp gene families (Iso et al., 2003) but more recent, genome-wide studies have uncovered a broader spectrum of Notch regulated genes (Djiane et al., 2013; Hurlbut et al., 2009; Krejci et al., 2009; Mazzone et al., 2010; Terriente-Felix et al., 2013; Wang et al., 2011). Amongst these were genes directly involved in cell shape, cell organisation and cell behaviour, whose functional relevance downstream of Notch has not been explored despite the fact that Notch has been implicated in different morphogenetic processes independently of cell fate. These include the formation of boundaries between different cell populations, such as during somitogenesis and in the Drosophila wing imaginal disc (Becam and Milan, 2008; Major and Irvine, 2006), cell migration (Schober et al., 2005; Wang et al., 2007) and axon guidance (although the latter may involve a non canonical pathway (Le Gall et al., 2008)). In the majority of contexts, such regulation of morphogenesis involves a transcriptional hierarchy, where activation of Notch results in the expression of a key transcription factor which in turn co-ordinates the cell behaviours (Niessen et al., 2008; Saad et al., 2010; Schober et al., 2005; Wang et al., 2007). However, it remains plausible that, in some tissues, Notch activity may have a more direct role in co-ordinating the genes that implement cell shape changes, although there is as yet little evidence to support this.

One context where Notch is required for regulating the behaviour of a specified group of cells is in the Adult muscle progenitors (AMPs) in Drosophila. As in mammals, Notch activity is required to prevent premature differentiation of the AMPs, which are specified in the embryo and ultimately give rise to the adult muscles of the fly. (Anant et al., 1998; Delfini
et al., 2000; Hirsinger et al., 2001). During larval stages, these progenitors proliferate to expand the pool of myoblasts and remain associated with imaginal discs (Bate et al., 1991). Then, at the beginning of metamorphosis the AMPs detach from the epithelium of the imaginal discs and migrate as a ‘swarm’ of associated cells (Roy and VijayRaghavan, 1998). At the target sites, myoblasts fuse with templates formed either from founder myoblasts or, in a few cases, from persistent larval muscles. In doing so, the fusing cells contribute to the differentiating muscle so that it achieves the appropriate size and structure. (Fernandes et al., 1991). Thus adult myogenesis is a complex morphogenetic process, involving proliferation, migration, cell fusion and differentiation. As Notch signalling is active both in the proliferating AMPs, where it inhibits differentiation, and in the semi-differentiated migrating myoblasts (Bernard et al., 2006; Gildor et al., 2012) it could regulate many aspects of this morphogenetic process. Indeed, a significant fraction of the genes directly regulated by Notch in DmD8 cells encode cytoskeletal regulatory proteins. DmD8 cells are related to AMPs and in particular express the transcription factor Twist which has been shown to function as a cooperating transcriptional activator for many Notch target genes (Bernard et al., 2010; Krejci et al., 2009). This suggested that Notch could have a direct role in co-ordinating genes that control cell behaviours in this setting.

Amongst twenty-five putative Notch-regulated genes encoding cell morphogenesis related proteins that were identified through genome-wide studies, (Djiane et al., 2013; Krejci and Bray, 2007; Terriente-Felix et al., 2013), we found that twelve are essential for generating adult flies capable of flight, consistent with appropriate functions in muscle formation. Focusing on three, Reck, talin and trio, we demonstrate that they are expressed in adult muscle progenitors and exhibit Notch regulation. Thus, these data support the model that Notch activity has the potential to directly regulating genes that co-ordinate cell morphology, in addition to its more widely accepted role in regulating such characteristics through cell fate-determining transcription factors.

RESULTS

Identification of Notch target genes involved in adult myogenesis.

We first selected a set of genes with known or inferred function in the control of cell shape, organisation and behaviour from genome-wide datasets documenting genes associated with Su(H)-bound regions in response to increased Notch signalling in cultured cells and in wing imaginal discs (Djiane et al., 2013; Krejci and Bray, 2007; Terriente-Felix et al., 2013). Such genes encode different types of proteins including Rho/Rac GTPase Exchange Factors.
(e.g. trio), cytoskeleton binding proteins, a Netrin receptor implicated in cell migration and axon guidance (unc-5; (Keleman and Dickson, 2001)) and the Reck Matrix Metalloprotease inhibitor, an inhibitor of cell invasion in cancer (Takahashi et al., 1998)(Table 1). To test their function in adult myogenesis, a morphogenetic process where Notch signalling is implicated in both cell fate and cell migration (Anant et al., 1998; Gildor et al., 2012), expression of RNAi targeting the individual genes was directed to the AMPs (using 1151-Gal4 as a driver). Progeny from these crosses were assayed for their ability to fly, as an indication of muscle formation/function, and all genes were categorised according to the percentage of flight deficient individuals observed for each line (Fig. 1A, Tables 1 and S1). This approach had the advantage that gene expression was ablated at all post-embryonic stages of AMP development, from their proliferation in the larvae through to the forming muscles in pupae, but was not disrupted in the larval muscles nor in their embryonic progenitors (Anant et al., 1998). Finally, to avoid false positives from off-target effects or from side effects of RNAi insertions, where possible we used lines from different sources that contained different types of RNAi constructs.

In most cases (18 out of 25) the results were consistent between different RNAi lines targeting the same gene (Table S1). For example, lines targeting Reck and trio all induced a high percentage of flightless flies. However, four genes (ena, GEFmeso, singed and CG6891) were classified as “weak-wt” since the results were inconclusive due to variability between lines, although overall there was little indication of any requirement in myogenesis. Furthermore, for three genes (Klar, wb and cdep) the results were contradictory because one RNAi line induced a severe phenotype whereas other lines targeting the same gene had no or very little effect. Such differences could be due to differences in the depletion efficiencies or to the inhibition of off target genes, but we have not pursued those genes further.

In total, knock down of twelve genes gave consistent phenotypes suggesting that they are required for myogenesis (Fig. 1A, Tables 1 and S1). Three of these (chic, rhea/talin and sls) gave lethality at pharate stage when inhibited. This is consistent with a deficiency in the adult muscles and fits with previous data showing that rhealtalin and sls have roles in myogenesis (Brown et al., 2002; Burkart et al., 2007). Furthermore, in a few instances the adults were found half emerged from the pupal case and, very occasionally a viable flightless fly eclosed. This suggests that the observed lethality was, at least in part, due to the inability to emerge from the puparium rather than to a major developmental defect. In agreement, one of the chic lines gave weaker phenotypes, generating adult flies of which 50% were flightless.

Inhibition of nine other genes, including corn and unc-5, resulted in flight defects with variable penetrance. In all cases the degree of flightless-ness amongst the progeny varied,
ranging from completely flightless flies to weak fliers. These appeared to represent a continuum in the severity of the same phenotype, because flightless flies were only observed when the progeny had a high percentage of flight defects, whereas weak fliers were often observed along with normal fliers. Nevertheless these included good candidates to mediate effects from Notch. For example both corn and unc-5 were shown to be expressed in AMPs and unc-5 contains a Notch responsive enhancer (Krejci et al., 2009).

A previous genome-wide RNAi screen to identify genes with function in muscle formation (Schnorrer et al., 2010) differed from our assay by using mef2-Gal4 as the driver, which is expressed in the muscle lineage from embryonic stages onward. Despite this difference, the results are broadly consistent (Table 1). Only two (NijA and cher) of the ten genes with no or little effects in our assay were found to give a muscle phenotype with the more widespread depletion used previously. Likewise, among the six genes with a strong phenotype in our analysis, five were found to be required for correct muscle formation in the previous screen (Schnorrer et al., 2010). Notably, three genes (chic, rhea/talin and sls) whose depletion induced lethality at pharate stage in our assay gave embryonic / early larval lethality when inhibited from embryonic stage onwards so their role in later development was not considered.

**Reck, talin and trio are required in AMP lineages.**

Three genes with a penetrant phenotype obtained with all tested RNAi lines, Reck, rhea/talin (hereafter referred to as talin) and trio were selected for further analysis, since they had been shown to be associated with Su(H)-bound regions in response to increased Notch signalling in the AMP-related DmD8 cells (Table 1). In addition, they are well-conserved between Drosophila and vertebrates and have been previously linked to muscle formation: Reck, a metalloprotease inhibitor, is expressed in developing muscles in the mouse where its expression is regulated by myogenic factors (Echizenya et al., 2005); Talin, a key component of integrin mediated cell adhesion is involved at different stage of myogenesis in Drosophila (Brown et al., 2002); Trio, a GTPase exchange factor (GEF) has a role in muscle formation in mammals, where it activates Rac1 to promote myoblast fusion (Charrasse et al., 2007; O’Brien et al., 2000).

Because 1151-Gal4 expression is not specifically restricted to AMPs (for example expression occurs in the salivary gland and some neural cells (Anant et al., 1998)), it was possible that the observed phenotypes were due to Reck, talin and trio inhibition in tissues other than AMPs. We thus first confirmed the requirement of these genes during adult muscle formation by using two other drivers, E(spl)m6-Gal4 and mef2-Gal4, to direct RNAi
expression in AMPs (Fig. S1). While small differences were observed in the penetrance obtained with the three drivers (likely due to different expression levels and timing), overall the phenotypes observed for all three genes were very similar (Table 2). As expected, one line targeting talin induced lethality at embryonic / early larval stage when driven by mef2-Gal4. Since mef-2-Gal4 is also expressed in embryonic and larvae muscles, these results are consistent with the requirement for talin during embryogenesis (Brown et al., 2002; Schnorrer et al., 2010). Similar lethal phenotypes were obtained when Notch-RNAi was expressed under the same conditions. Thus, these results confirmed that Reck, talin and trio are required in the myogenic lineages.

Next we sought to determine at what stage the three genes are required. To do so, we combined UAS-RNAi lines targeting Reck, talin and trio with the 1151-Gal4 driver in the presence of a thermo-sensitive derivative of Gal80 (Gal80<sup>ts</sup>), the Gal4 inhibitor (McGuire et al., 2003). RNAi expression was then induced at different stages by incubating larvae at temperatures where Gal80<sup>ts</sup> was inactivated. As a control, the experiment was first performed with an RNAi line targeting the transcription factor twist, which is required in AMPs but is down-regulated when myoblasts differentiate (Anant et al., 1998). In agreement, we observed a severe phenotype when RNAi expression was initiated two days before pupation but not when it was initiated within 12 hours after pupation (Table 3). In contrast, inhibition of Notch after pupation resulted in a highly penetrant phenotype (albeit less penetrant/severe than when Notch was inhibited prior to pupation), suggesting a longer requirement for Notch compared to Twist. With Reck, talin and trio, the consequences were intermediate between the two. Severe phenotypes were observed when RNAi expression was induced before pupation but the phenotypes were considerably reduced when the animals were shifted within 12 hours after pupation. Indeed, with trio-RNAi, ameliorated phenotypes were already evident with larvae shifted 1 day prior to pupation and were almost non-existent when shifted after pupation. Although it is not possible to distinguish exactly when the gene function will be perturbed, as the precise moment depends on the RNA and protein stability, these results suggest that the three genes are required for myogenesis at the end of larval stages and the beginning of pupation corresponding to the time when the AMPs start to migrate and fuse to form muscle fibres (Fernandes et al., 1991).

In order to visualize AMPs shape, morphology and organisation we used beta-3-tubulin, which is highly expressed specifically in the AMPs (Fig. 1B). Consistent with the requirement for Trio during the larval stages, we found that the AMPs were disorganized and had altered morphology when trio expression was inhibited. Indeed, in ~30% of wing discs, AMP cells were abnormally dispersed, with evident gaps between cells (Fig. 1B). The same
phenotype was observed with two independent RNAi constructs (K18214R-1 and V40138, n=5/15 and 6/20 discs respectively), and suggests that Trio is important in sustaining the integrity of the AMP layer. No such robust changes were detected when Reck or talin expression was perturbed at this stage, although some cells appeared to have lost the expression of the myogenic protein beta-tubulin with one Reck RNAi (data not shown). Furthermore, although individual flies emerging after inhibition of Reck, talin and trio presented a wing posture defect (Fig. 1C and data not shown), using polarized light we did not detect any gross morphological abnormality of indirect flight muscles in these surviving flightless adults. Thus overall it seems likely that the flightless phenotype itself is due to subtler defects in muscle function and that there are also perturbations at earlier developmental stages that correlate to lethality before the flies eclose.

**Reck, talin and trio are expressed in AMP cells.**

Given the implication that Reck, talin and trio are required in AMPs for normal development, we assessed whether they are expressed in these cells, as predicted. Although their overall patterns differed, all three genes were expressed in AMPs. Expression of Reck was largely restricted to the AMPs (Fig. 2A-B and Fig. S2, for comparison, see expression of AMP markers, Cut and Mef2; Fig. 2D and H) where it was present at all stages examined, including young third instar (L3) larvae (Fig. 2A) and early pupae (Fig. 2B). Interestingly the expression levels of Reck seemed to correlate with their maturation, as higher expression levels were detected at the onset of pupation. In addition, Reck was also detected in the peripodial margin possibly reflecting a function in disc eversion (Srivastava et al., 2007). In contrast, Talin was broadly expressed throughout the wing-disc, with a fairly ubiquitous pattern as described previously (Brown et al., 2002). However there was also a clear accumulation in the AMPs, making it plausible that there could be specific regulation of talin in these cells (Fig. 2C-D, and Fig. S2). Similarly, Trio protein appeared to be expressed widely throughout the wing disc, including in the AMPs (Fig. 2E, and Fig. S2). Furthermore, *in situ* hybridizations suggested some differential regulation of trio, with higher expression in the notal region / AMPs (Fig. 2F). Similar enrichment in the AMPs was detected using a trio ‘enhancer trap’ line (corresponding to a P element insertion at the transcription start of the longest transcript of trio (Bateman et al., 2000)) and this high level of LacZ reporter expression was found to co-localize with the AMP marker Mef2 (Fig2. G-H, and Fig. S2). Thus, Reck, talin and trio are all expressed in AMPs, consistent with their proposed function in adult myogenesis.
Notch regulated enhancers from Reck, talin and trio.

Reck, talin and trio were identified as putative direct targets regulated by Notch signalling in the muscle lineage. To verify this, we first tested whether the regions occupied by Su(H) in these three loci can function as Notch responsive enhancers (NREs). DNA fragments corresponding to these Su(H)-bound regions (in brown in Fig. 3A-C) were cloned upstream of a minimal Luciferase reporter and their ability to respond to the activated form of Notch (Niced) analyzed in co-transfection assays using the DmD8, Twist-expressing, cell line. All three enhancers gave increased luciferase expression in the presence of Niced in DmD8 cells and this response was compromised when the Su(H) binding motifs were mutated (Fig. 3D; p<0.001 for trio and p<0.05 for talin and Reck, in each case the two best matches to the Su(H) position weight matrix were mutated). Of the three, the NRE from the trio locus gave the highest fold change in reporter expression in the presence of Niced (6.5 for trio NRE versus 4.3 and 2.6 for talin and Reck NRE respectively) and also gave the most dramatic reduction when the Su(H) motifs were mutated. In contrast, the consequences on Reck NRE from mutating two Su(H) motifs was modest, although significant. This suggests that additional motifs, with lower Patser scores, may also be important in this NRE. Nevertheless all of the three identified regions behave as NREs consistent with the Su(H) occupancy detected in ChIP.

To further refine the NREs, smaller regions, centred on the Su(H) motifs, were assayed in a similar manner (circa 800pb, in green in Fig. 3A-C). The fold of activation obtained with the short Reck and talin NREs showed a reduced ability to respond to Notch activation compared to their longer counterparts, suggesting that there are important regulatory elements in the surrounding region (Fig. 3D). Indeed, shortened Reck NRE was no longer responsive to Nicd, suggesting there are relevant Su(H) motifs in the larger fragment which would explain the residual activity of the mutated long Reck NRE. Furthermore, all three long NREs contain Twist (Twi) binding regions, which are absent from their shorter counterparts (see “Twi ChIP”, blue, Fig. 3A-C). Thus, the reduced expression in the short Reck and talin NREs is consistent with the model that, in DmD8, Twi functions as a cooperating transcriptional activator for many Notch target genes (Bernard et al., 2010). Surprisingly, the short trio NRE gave a fold change 50% higher than that of the longer trio NRE, even though it lacks the region previously found occupied by Twi. Thus this trio-NRE may bind Su(H) independently of Twi. In agreement, Su(H) was found bound to trio NRE not only in DmD8 cells but also Kc cells which do not express Twi (Terriente-Felix et al., 2013) whereas Reck and talin NRE were only bound to Su(H) in DmD8 cells.
**Reck and Talin NRE drive Notch dependent GFP expression in AMP cells in vivo.**

To further confirm the relevance of the NREs for *Reck*, *talin* and *trio* expression during adult myogenesis, we tested whether they could drive expression of a reporter in the AMPs. To do this, the identified NRE sequences were cloned upstream of a minimal GFP reporter (Housden et al., 2012) and the resulting expression patterns analysed in wing discs from transgenic flies. Both *Reck* and *talin* NREs clearly directed expression of GFP in the AMPs (Fig. 4 A-D’ and G) although in both cases only in a subset of the AMPs. Interestingly, the subsets differed according to the NRE, which could reflect different stages in the maturation of these cells or different pre-patterns amongst the progenitors. In contrast the *trio*-NRE was not functional in the AMPs, although it did direct some expression in the wing disc epithelium (not shown). Therefore despite its ability to function effectively in transient transfection assays, this fragment appears to lack features critical for activity in vivo. Finally, we tested whether the expression from the *Reck* and *talin* NREs were affected when Notch signalling was compromised. Inhibition of Notch in AMPs, using Notch-RNAi driven by 1151-Gal4, caused a clear reduction in the proportion of AMPs expressing the GFP (p<0.01 for both, Fig. 4 C-F’ and G). Thus the activity of the enhancers appears responsive to Notch signalling in vivo, supporting the hypothesis that *Reck* and *talin* are direct Notch targets during adult myogenesis. We also detected weak and variable reductions in expression from the endogenous genes under similar conditions of Notch RNAi (Fig S3).

**Notch can induce ectopic expression of Reck, talin and trio.**

As a further test of whether the three genes can be regulated by Notch activity, we monitored their response to ectopic Notch activity in the wing disc, with and without the cooperating factor Twist. Initially, Nicd alone was produced ectopically in a stripe across the wing pouch using the patched-Gal4 driver (ptc-Gal4; Fig. 5A-F). However, none of the genes showed any marked change in expression under these conditions. Subsequently, as many AMP Notch targets require the transcription factor Twist (Twi) to be induced by Notch (Bernard et al., 2010), we tested the effect of co-expressing Nicd and Twi together and of Twi alone. This combination induced ectopic expression of all three genes within the ptc expression domain (Fig. 5J-L) while none was clearly up-regulated when Twi was expressed alone (Fig. 5G-I). This was even the case for Trio, suggesting that Twi does cooperate with Notch at this target, despite the fact that the NRE binds Su(H) even in cells that lack Twi. Together these results suggest that *Reck*, *talin* and *trio* are Notch regulated in vivo when Twi is present in the same cells. Although this regulation is likely direct, based on the results from the reporters, it remains possible that there are also indirect mechanisms involved.
DISCUSSION

Notch signalling is widely implicated in the control of cell fate during development but also has been shown to influence cell architecture and behaviour in different morphogenetic processes. In most cases, Notch is proposed to co-ordinate cell morphogenesis by regulating the expression of key transcription factors, rather than by directly regulating the effector genes that implement the cell behaviours (Niessen et al., 2008; Saad et al., 2010; Schober et al., 2005; Wang et al., 2007). One well-characterized example is epithelial-to-mesenchymal transition (EMT), a process that can be triggered by Notch signalling (sometimes in combination with other pathways such as TGFβ (Espinoza and Miele, 2013; Wang et al., 2010)) through its regulation of the key transcription factors Snail and Slug (Niessen et al., 2008; Saad et al., 2010). Our results suggest however that genes involved in implementing cell morphology are also directly regulated by Notch. Building on previous genome-wide analyses of Notch regulated genes, which revealed a wide spectrum of functional targets, we have found that Reck, talin and trio all have some characteristics of direct Notch targets in the muscle progenitors. Genome-wide analysis of CSL binding in mouse and human T-lymphoblastic leukemia cells also identified several genes implicated in cell architecture regulation, although those differ from the genes analyzed here (Wang et al., 2011). Direct control of genes with roles in co-ordinating cell morphology/behaviour may thus be a general feature of Notch activity in different morphogenetic processes.

Two of the three genes, trio and talin, are very widely expressed. Thus, a large proportion of their expression likely occurs independent of Notch. However, our identification of Su(H) responsive enhancers associated with each of these genes suggests that Notch activity can modulate their expression at specific stages and/or in specific cell types. This highlights the existence therefore of different categories of Notch regulated genes. Most commonly the focus is on cell-fate determining genes, which are specifically switched on in a cell only when Notch activity is present. The regulation of trio and talin suggests that Notch activity also augments the expression of genes that are already transcribed, modifying their expression rather than initiating it de novo. Such subtle changes of gene expression would not have been uncovered by conventional approaches, demonstrating the utility of genome-wide studies in uncovering the full spectrum of target genes. In the AMPs, trio is important for sustaining the normal cell morphology, and may do so by regulating the interaction of AMPs with their niche (Lin et al., 2013) or their transition to a migrating population at the onset of pupation (Kashef et al., 2009; Moore et al., 2013). However to distinguish the contribution that Notch regulation makes to trio and talin functions it will ultimately be necessary to eliminate the NREs from the endogenous genes.
In contrast, Reck exhibits a much more restricted expression pattern, being specifically up-regulated in the late stage AMPs where Notch is active. The identified NRE directs expression in these cells, consistent with Reck expression being controlled by Notch activity in AMPs. Intriguingly, in some mammalian cells (e.g. cortical progenitors,) expression of RECK has been found to activate Notch signalling, by directly inhibiting ADAM10-dependent processing of Notch ligands (Muraguchi et al., 2007). As the swarming myoblasts appear to undergo bi-directional Notch signalling, it is possible that Reck could be involved in subtle fine-tuning of signalling between cells. However, so far there is no evidence to indicate that Reck can inhibit Kuzbanian, the Drosophila Adam10 homologue. Instead Reck function has been linked to inhibition of the matrix metalloproteinase MMP1, through its ability, in conjunction with another metalloprotease inhibitor, to suppress invasion of tumours that had up-regulated MMP1 (Srivastava et al., 2007). Similar characteristics are well documented in mammals, where RECK functions as a tumour suppressor by inhibiting migration, invasion, and angiogenesis (Meng et al., 2008; Nagini, 2012; Noda et al., 2003; Takahashi et al., 1998).

Although we have not been able to detect any gross defects in the organization of indirect flight muscles, suggesting that AMP migration is not severely affected when Reck is knocked-down, as the RNA is only upregulated in late stage AMPs it is more likely to be involved in positively promoting myoblast migration, similar to its role in zebrafish neural crest cells (Prendergast et al., 2012), than in suppressing migration.

Besides the three genes whose regulation we have analysed in some details, at least nine others appear to be required in adult myogenesis, based on the phenotypes seen when their expression in AMPs is ablated. Two of these, corn and unc-5, were previously shown to be expressed in AMPs. For unc-5, the Su(H) bound region has been tested and shown to function as an NRE in the AMPs. Thus, the evidence suggests that other putative targets will be regulated by Notch activity and will contribute to the functional output in regulating the AMPs cell behaviours. The model that emerges is that Notch activity not only regulates transcription factors important in conferring cell fate identify but also directly affects the expression levels of genes encoding proteins that implement cell fates, such as those with roles in regulating cellular architectures and behaviours.
MATERIALS AND METHODS

Drosophila stocks and genetics.

Fly stocks used for RNAi experiments are from BDSC (Bloomington, Indiana, USA), DRGC (Kyoto, Japan) or VDRC (Vienna, Austria). Individual line numbers are indicated in table S1. We also used the UAS-twii- RNAi2x line to target twist expression (Wong et al., 2008). Gene ablation was obtained by crossing UAS-RNAi lines with the following drivers: 1151-Gal4 (Anant et al., 1998), mef2-Gal4 (Ranganayakulu et al., 1996) and E(Spl)m6-Gal4 (Gift from Dr. Alexis Lalouette, Université Paris-Denis Diderot, France (unpublished)), combined with an UAS-Dcr-2 (BDSC, Bl24650, (Dietzl et al., 2007)) to enhance RNAi effect. Crosses were culture at 25°C and progeny was assayed for its ability to fly. In order to limit RNAi expression to a defined period of larval and pupal development, the 1151-Gal4 was combined with a tub-Gal80 ts (McGuire et al., 2003). Crosses were cultured at 19°C and individually staged larvae or pupae (see table 3) were shifted to 29°C (non-permissive temperature for Gal80ts). Adults were then assayed for their ability to fly.

To assay AMP cell morphology following knock-down of Reck, talin and trio, wing imaginal discs from wandering larvae were dissected and stained for beta3-tubulin expression. For the phenotype observed with trio, discs were scored on the basis of whether the AMP cells were abnormally dispersed, with gaps evident.

For Notch and Twist gain of function experiments the patched[559.1]-Gal4 driver combined with tub-Gal80 ts (ptc-Gal4-Gal80ts) was used to drive UAS-Ni79.2 (Nicd) and/or UAS-Twist expression (Baylies and Bate, 1996). Crosses were cultured at 19°C for 7 days, then shifted to 29°C for 48 h before dissection and staining.

The trio-LacZ reporter line was previously described (Bl 8594)(Bateman et al., 2000).

Flight assay.

For each RNAi line tested, at least 40 adult flies aged 2-8 days were assayed for their ability to fly. For this, flies were dump dropped from their vials at approximately 50 cm from the bench and numbers of flies that fell on the bench were scored (i.e. flies that could not fly away). This test was repeated twice from independent crosses and the results were averaged. Depending on the percentage of flies with flight defect, each gene was categorised as “WT” (less than 5%), “weak” (between 5% and 33%), “mild” (between 33% and 66%) and “strong” (more than 66%). When lines targeting the same gene were in different categories, the gene was assigned to the strongest category if all lines were in categories not different by more
than one degree (e.g. “strong” and “mild” or “mild” and “weak”), except with “WT” and “weak” in which case the gene was assigned to the “WT-weak” category. Finally, genes with lines giving very different results (e.g. “weak” and “strong”) were classified as “uncertain”.

**Muscle preparation**

Adult flies were fixed in 4% paraformaldehyde overnight. Thoraces were cut sagittally, mounted in glycerol and viewed under polarized light.

**Luciferase experiments and GFP reporters.**

For luciferase assays, putative NRE fragments from *Reck*, *rhealatin* and *trio* were amplified from *Drosophila* genomic DNA using primers containing restriction enzyme sequences and cloned into a luciferase vector containing a minimal promoter from the hsp70 gene (pGL3::Min). Release 5 coordinates of the cloned fragments were *Reck*, chr3L:15000573-15003206 (long) and 15001662-15002553 (short); *rhealatin* chr3L:8542368-8545156 (long) and 8543963-8544835 (short); *trio* chr3L:1030426-1033023 (long) and 1030865-1031821 (short). Matches to Su(H) motifs were identified using Patser (Hertz and Stormo, 1999) with the position weight matrix described previously (Krejci et al., 2009).

Mutated Su(H) binding motifs were: in *Reck-NRE*, CATGGGAA > CATtGttA (at position 15002089) and GTCAACAG > GaaAaACG (15002161); in *rhealatin-NRE*, CATGGGAA > CATtGttA (8544448) and TGGGAGAA > TGGtAttA (8544370); in *trio-NRE*, TTCCCACG > TaaGaACG (1031441) and GTCCCACA > GaaCaACA (1031363). Cell culture conditions and transfections were as described previously (Nagel et al., 2005; Narasimha et al., 2008). At least three biological replicates were performed in all experiments. Significance of differences in luciferase measurements was assayed with unpaired, two-tailed Welch’s t-test using R (R Core Team, 2013).

To produce GFP reporters, long NREs (as defined above) were cloned in the pGreenRabbit vector (pGR) (Housden et al., 2012). Flies carrying the pGR transgenes were generated by Phi-C31 mediated site-directed integration on the 86Fb platform. To test whether GFP expression was dependent on Notch signalling, reporters were combined with *1151-Gal4* and *UAS-Notch-RNAi*. Crosses were cultured at 25°C for 4 days, then shifted to 29°C for 48 h before dissection of wandering third instar larvae and staining. In all, 12-20 wing imaginal discs obtained from independent crosses were imaged. Areas occupied by cells expressing Cut (AMPS marker) or expressing Cut and GFP were measured using ImageJ (Rasband, 1997-2012). Significance of differences in measurements was assayed with unpaired, two-tailed Welch’s t-test using R.
**Immunostaining and In Situ Hybridization.**

Antibody staining of wing imaginal discs was performed according to standard protocol. The following antibodies from DSHB (Developmental Studies Hybridoma Bank, Iow City, Io, USA) were used: rat anti-ECad (DCAD2. 1:20), rat anti-Ci (2A1. 1:20), mouse anti-Cut (2B10. 1:20), mouse anti beta-gal (40-1A. 1:20) and mouse anti-Trio (9.4A. 1:20). We also used rabbit anti-GFP (1:500, Life Technologies. Carlsbad, CA, USA); rabbit anti-Talin (1:500, gift from N. Brown. (Brown et al., 2002)); rabbit anti-Mef2 (1:1000, gift of B. Patterson (Lilly et al., 1995)); anti-beta3-tubulin (1: 5000, gift from R. Renkawitz-Pohl (Rudolf et al., 2012)). Samples were imaged using a Nikon Eclipse C1 confocal microscope. Images were processed with ImageJ and assembled with Adobe Photoshop.

*Reck* and *trio* expression were analysed by *in situ* hybridization with RNA probes synthesised from PCR amplified DNA fragments (circa 1.2 kb) corresponding to *Reck* exon 7 and 8, and *trio* exon 4 and 5 of the longest isoform. In both cases the two probes gave the same pattern. *In situ* hybridization was performed according to standard protocol. Fluorescent *in situ* hybridization was performed using Tyramide Signal Amplification (Perkin-Elmer, Waltham, Massachusetts, USA). Standard colorimetric staining was imaged using a Zeiss Axiophot microscope. Fluorescent samples were analyzed as above.

**Quantitative RT–PCR**

Wing imaginal discs from third instar control (1151-Gal4) and Notch depleted (1151>N-RNAi) larvae were dissected (20 discs for each genotype). Dorsal halves (corresponding to the notum, where the AMPs are located) were separated from the wing pouch and used for RNA extraction using TRIzol (Life Technologies). Genomic DNA was eliminated using Ambion’s DNA-free kit. cDNA was synthesized using random hexamers (Promega. Madison, Wi, USA) and M-MLV reverse transcriptase (Promega). cDNA levels were quantified by real-time PCR using QuantiTec Sybr Green PCR mix (Qiajen. Valencia, CA, USA) and the AbiPrism machine. The calibration curve was constructed from serial dilutions of genomic DNA, and values for all genes were normalized to the levels of *Elongation factor 2* (*Ef2*). The following primers were used. *Ef2*: Fwd GCCGATCTCTGCTCTAATAC, Rev ACGAGTATCCCTGGAGATGG, within exon 5; *Notch*: Fwd TGCGATGTTCAGACGATTTC, Rev CGTATCCCTGGGAGCAGTAG, within exon 5; *Reck*: Fwd TGGACCAAAACTCGACACTG, Rev TACTCCTAGGGACACATGC, within exon 8; *talin*: Fwd CAGCAGCAGTGAACTTGGAG, Rev CTGGGGTCATCGAGGTGAGTC, within exon 15; *trio* Fwd
ACCATGAAAAGGACGTGAC, Rev CTCTCCTGCTGATCCCTCTG, within exon 4 of the longest isoform.

**Acknowledgments**

We are grateful to Alexis Lalouette for the E(spl)m6-Gal4 line, to Renate Renkawitz-Pohl for the beta3-tubulin antibody, to Nick Brown for Talin antibody and to Bruce Paterson for Mef2 antibody. We also acknowledge the Bloomington Stock Center (BL), Vienna Drosophila RNAi Center (VDRC), The Kyoto Stock Center (DGRC) and The Developmental Studies Hybridoma Bank (DSHB) for providing *Drosophila* strains and antibodies. We thank members of the Bray lab for valuable discussions.

This work was supported by a programme grant from the Medical Research Council to SJB [G0800034], by fellowships to GP from Fondation pour la Recherche Medical and from Marie Curie (Intra European Fellowship [PIEF-GA-2009-236426]) and by an EMBO Long Term Fellowship to HB [ALTF 325-2013].

**Author Contributions**

Conceived and designed the experiments: GP, HB, SJB.
Performed the experiments: GP, HB, KM
Analysed the data: GP, HB, SJB.
Contributed reagents/materials/analysis tools: GP, KM.
Wrote the paper: GP, SJB.
REFERENCES


FIGURE LEGENDS

Figure 1. An RNAi assay identifies genes required for muscle formation.

(A) Pie chart showing the proportion of genes whose loss of function resulted in the different phenotypical classes (See Methods for more details about classes). Note that the 1151-Gal4 driver is expressed in AMPs but not in the larval muscles to avoid any confounding effects from defects in larval musculature (as might occur with mef2-Gal4).

(B) AMP organisation in wing imaginal discs from control (1151-Gal4) and trio-RNAi (1151-Gal4 UAS-trio-RNAi) larve detected with beta-3-tubulin (red). Cut (green) marks nuclei of AMPs, and DAPI (blue) labels all nuclei. Altered cell morphology and organization is evident in trio-RNAi compared to control (representative examples of intermediate severity are shown). White squares indicate regions shown at higher magnification.

(C) Dorsal view of control (1151-Gal4) and 1151-Gal4 driven Reck-RNAi adult flies. The defective position of the wings observed, “held out wings” phenotype, has often been associated with flight muscle defects (Greene et al., 2003; Zaffran et al., 1997).

Figure 2. Reck, talin and trio are expressed in AMP cells.

Expression profiles of Reck, talin and trio in the wing imaginal disc show that all three genes are expressed in AMPs.

(A-B) Reck In situ hybridization in young L3 larvae (A) and P1 pupae (B)

(C-D) talin expression profile revealed by immunostaining and co-stained with the AMP marker cut. (C. 20x and D. 40x, Maximum projections of z-stacks from confocal acquisitions are presented).

(E-H) trio expression profile shown by immunostaining (E, Maximum projections), In situ hybridization (F) and an enhancer-trap reporter line (G. 20x) also co-stained with the AMP marker mef2 (H. 40x, Maximum projections).

Figure 3. Su(H) ChIP identifies Notch Responsive Enhancers in Reck, talin and trio loci.

(A - C) Genomic region surrounding Drosophila Reck (A), talin (B) and trio (C) genes. Black lines and boxes (exons) represent transcribed regions. Graphs show matches to Su(H) PWM (dark red bars; height of bar indicates Patser score 5-9.79), Twi PWM (dark blue bars; Patser score 5-9.3), Su(H) (red; enrichment (AvgM log2)) and Twi (blue) ChIP-enriched
regions in DmD8 cells (Bernard et al., 2010; Krejci et al., 2009). Brown and green rectangles
represent long (brown) and short (green) fragments tested for their Notch signalling activation
sensitivity. Asterisks indicate Su(H) binding sites mutated in subsequent experiments.

(D) Response of the indicated long (brown) and short (green) fragment to Notch
signalling activation in transient transfection assays in DmD8 cells. Plain bars represent
wildtype fragments and striped bars fragments in which Su(H) sites were mutated. E(spl)m3
which is inducible by Notch in all tested condition (Krejci and Bray, 2007) was used as
positive control. Errors bars represent s.d. (** indicates p<0.05 and *** indicates p<0.001).

Figure 4. Reck and talin NREs drive Notch dependent GFP expression in AMPs
in vivo.

(A-B’) Reck (A and higher magnification in A’) and talin (B and higher magnification
in B’) L-NREs drive GFP expression in a subset of AMPs as shown by GFP colocalisations
with the AMP marker Cut. White squares in A and B indicate regions shown at higher
magnification in A’ and B’. Single optical sections from confocal acquisitions are presented.

(C-F’) Expression from reporters Reck-L-NRE-GFP (C-C’, E-E’) and talin-L-NRE-
GFP (D-D’, F-F’) in wild type (C-D’) and Notch depleted conditions (E-F’). Cut expression
was used to mark the AMPs. Green dotted lines in C’, D’, E’ and F’ outline Cut and GFP
expressing cells. Maximum projections of z-stacks from confocal acquisitions are presented.

(G) Boxplot representing the percentage of AMPs (Cut expressing cells) expressing
GFP from Reck-L-NRE-GFP and talin-L-NRE-GFP reporters in wild type (CTRL) and Notch
depleted (N-RNAi) conditions. The percentage of AMPs expressing GFP was estimated by
manually measuring areas occupied by cells expressing Cut or Cut and GFP. (*** indicates
p<0.001).

Figure 5. Notch can induce ectopic expression of Reck, talin and trio in the
presence of Twist.

(A-L) ptc-Gal4; Tub-Gal80° was used to drive expression of Nicd and / or Twist in
the wing pouch. Expression profile of Reck (A, D, G, J, In situ hybridization), talin (B, E, H,
K. immunostaining) and trio (C, F, I, L. immunostaining) in a wild type wing pouch (A-C), in
Nicd expressing discs (D-F), Twist expressing discs (G-I) and Nicd + Twist expressing discs
(J-L). Note the ectopic expression of Reck (J, black arrows) and the upregulation of talin and
trio (K, L. white arrows) induced by Notch in the presence of Twist. Ci staining was used to
indicate the limit between anterior and posterior domains, along which ptc-Gal4 is expressed
(Indicated with orange lines).
<table>
<thead>
<tr>
<th>FBgn ID</th>
<th>CG Id</th>
<th>Name</th>
<th>Function</th>
<th>GO TERM</th>
<th>Su(H)</th>
<th>Phenotype</th>
<th>Schnorrer et al.</th>
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<td>FBgn0001316</td>
<td>CG17046</td>
<td><em>klarsicht</em></td>
<td>actin binding</td>
<td>GO:0003779</td>
<td>D8, Su(H), Nicd, Kc</td>
<td>uncertain wt</td>
<td>Su(H), Nicd, Kc</td>
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<td>FBgn0024277</td>
<td>CG18214</td>
<td><em>trio</em></td>
<td>Rho guanyl-nucleotide exchange activity</td>
<td>GO:0005089</td>
<td>D8, Su(H), Nicd, Kc</td>
<td>strong flightless</td>
<td>/ lethal (AP)</td>
</tr>
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<td>FBgn0014133</td>
<td>CG1822</td>
<td><em>bifocal</em></td>
<td>actin binding</td>
<td>GO:0003779</td>
<td>D8, Su(H), Nicd</td>
<td>wt</td>
<td>nd</td>
</tr>
<tr>
<td>FBgn0011661</td>
<td>CG10701</td>
<td><em>Moesin</em></td>
<td>cytoskeletal protein binding</td>
<td>GO:0008092</td>
<td>Su(H), Nicd, Kc</td>
<td>weak flightless</td>
<td>/ wt</td>
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<td>FBgn0036101</td>
<td>CG6449</td>
<td><em>Ninjurin A</em></td>
<td>cell adhesion</td>
<td>GO:0007155</td>
<td>D8, Su(H), Nicd</td>
<td>wt</td>
<td>flightless</td>
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<td>FBgn0013726</td>
<td>CG8705</td>
<td><em>peanut</em></td>
<td>actin binding, microtubule binding</td>
<td>GO:0003779, GO:0008017</td>
<td>D8, Su(H), Nicd</td>
<td>wt</td>
<td>nd</td>
</tr>
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<td>FBgn0035802</td>
<td>CG33275</td>
<td><em>CG33275</em></td>
<td>Rho guanyl-nucleotide exchange activity</td>
<td>GO:0005089</td>
<td>Su(H), Nicd</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>FBgn0030955</td>
<td>CG6891</td>
<td><em>CG6891</em></td>
<td>actin binding</td>
<td>GO:0003779</td>
<td>Su(H), Nicd</td>
<td>wt - weak</td>
<td>nd</td>
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<td>FBgn0014141</td>
<td>CG3937</td>
<td><em>cheerio</em></td>
<td>actin binding</td>
<td>GO:0003779</td>
<td>Su(H), Nicd</td>
<td>weak flightless</td>
<td>/ wt</td>
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<td><em>chickadee</em></td>
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<td>Su(H), Nicd</td>
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<td><em>cornetto</em></td>
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<td>D8, Nicd</td>
<td>weak flightless</td>
<td>/ wt</td>
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<td>FBgn0011225</td>
<td>CG5695</td>
<td><em>jaguar</em></td>
<td>actin binding, microtubule binding</td>
<td>GO:0003779, GO:0008017, GO:0032027</td>
<td>Su(H), Nicd</td>
<td>strong lethal (P)</td>
<td>/ wt</td>
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<td><em>singed</em></td>
<td>actin binding</td>
<td>GO:0003779</td>
<td>Su(H), Nicd</td>
<td>wt - weak</td>
<td>/ wt</td>
</tr>
<tr>
<td>FBgn0034013</td>
<td>CG8166</td>
<td><em>unc-5</em></td>
<td>netrin receptor activity</td>
<td>GO:0005042</td>
<td>D8, Su(H)</td>
<td>mild flightless</td>
<td>/ wt</td>
</tr>
<tr>
<td>FBgn0000083</td>
<td>CG5730</td>
<td><em>Annexin B9</em></td>
<td>actin binding</td>
<td>GO:0003779</td>
<td>Su(H)</td>
<td>wt</td>
<td></td>
</tr>
<tr>
<td>FBgn0051536</td>
<td>CG31536</td>
<td><em>Cdepi</em></td>
<td>Rho guanyl-nucleotide exchange activity</td>
<td>GO:0005089</td>
<td>Su(H)</td>
<td>uncertain</td>
<td>locomotion</td>
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<tr>
<td>FBgn0011202</td>
<td>CG1768</td>
<td><em>diaphanous</em></td>
<td>actin binding, Rho GTPase binding</td>
<td>GO:0003779, GO:0017048</td>
<td>Su(H)</td>
<td>mild flightless</td>
<td>/ wt</td>
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<td>FBgn0260866</td>
<td>CG12489</td>
<td><em>defense repressor I</em></td>
<td>zinc ion binding</td>
<td>GO:0008270</td>
<td>D8</td>
<td>wt</td>
<td>/ wt</td>
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<td>FBgn0000578</td>
<td>CG15112</td>
<td><em>enabled</em></td>
<td>actin binding</td>
<td>GO:0003779</td>
<td>Su(H)</td>
<td>wt - weak</td>
<td>/ wt</td>
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</table>
FBgn0050115   CG30115   GEFmeso   | Rho guanyl-nucleotide exchange factor activity | GO:0005089   Nicd   | wt - weak   | nd

FBgn0036463   CG5392   Reck*   | serine-type endopeptidase inhibitor activity | GO:0004867   D8   | strong   | nd

FBgn0260442   CG6831   rhea / talin   | actin binding, structural constituent of cytoskeleton | GO:0003779, GO:0005200   D8   | lethal   | lethal (L)

FBgn0086906   CG1915   sallimus   | myosin light chain kinase activity, actin binding | GO:0004687, GO:0003779   Su(H)   | lethal   | lethal (E)

FBgn0051352   CG31352   Unc-115a   | actin binding | GO:0003779   Su(H)   | weak   | wt

FBgn0261563   CG42677   wing blister   | receptor binding, regulation of cell adhesion, cell migration | GO:0005102, GO:0030155, GO:0030334   Su(H)   | uncertain   | wt

Cdep*: Chondrocyte-derived ezrin-like domain containing protein.
Reck*: Reversion-inducing-cysteine-rich protein with kazal motifs.

Candidates are sorted by the number of datasets in which Su(H) was found bound to their loci. (Su(H) column: D8: DmD8 cells (Krejci et al., 2009); Su(H) and Nicd: Wing imaginal discs overexpressing Su(H) and Nicd respectively (Djiane et al., 2013); Kc: Kc cells (Terriente-Felix et al., 2013).

The phenotypical category obtained in our assay and the phenotype observed by Schnorrer et al. (Schnorrer et al., 2010) are indicated (AP: Adult-pharate, E: Embryonic, L: Larval, P: Pharate).
Table 2. Percentage of flight defects obtained with three muscle drivers.

<table>
<thead>
<tr>
<th>gene</th>
<th>line</th>
<th>1151-Gal4</th>
<th></th>
<th>mef2-Gal4</th>
<th></th>
<th>E(spl)m6-Gal4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>s.d.</td>
<td>%</td>
<td>s.d.</td>
<td>%</td>
<td>s.d.</td>
</tr>
<tr>
<td>Reck</td>
<td>52427 (Vi)</td>
<td>94.22</td>
<td>1.65</td>
<td>95.12</td>
<td>1.67</td>
<td>82.04</td>
<td>2.26</td>
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<td></td>
<td>5392-R2 (Ky)</td>
<td>81.56</td>
<td>1.12</td>
<td>80.69</td>
<td>2.83</td>
<td>67.29</td>
<td>1.89</td>
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<tr>
<td>talin</td>
<td>40399 (Vi)</td>
<td>lethal</td>
<td>na</td>
<td>lethal (E)</td>
<td>na</td>
<td>lethal</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>6831-R2 (Vi)</td>
<td>lethal</td>
<td>na</td>
<td>lethal (E)</td>
<td>na</td>
<td>lethal</td>
<td>na</td>
</tr>
<tr>
<td>trio</td>
<td>40138 (Vi)</td>
<td>93.89</td>
<td>0.48</td>
<td>97.60</td>
<td>0.77</td>
<td>89.53</td>
<td>1.21</td>
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<td>18314-R1 (Ky)</td>
<td>82.49</td>
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<td>82.13</td>
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<tr>
<td>Notch</td>
<td>7078 (Bl)</td>
<td>94.96</td>
<td>1.99</td>
<td>lethal (E)</td>
<td>na</td>
<td>95.31</td>
<td>6.63</td>
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<tr>
<td>control w/o RNAi</td>
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<td>4.85</td>
<td>0.33</td>
<td>3.42</td>
<td>0.61</td>
<td>3.70</td>
<td>0.74</td>
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</table>

(E) lethality was observed at embryonic or early larval stages.

Table 3. Percentage of flight defects (or lethality) resulting from the induction of RNAi transcription at different stages.

<table>
<thead>
<tr>
<th>gene</th>
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<th>L3a (3 dpf.)</th>
<th></th>
<th>L3b (4 dpf.)</th>
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<th>P1-P3</th>
<th>always at 18°C</th>
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<tr>
<td></td>
<td></td>
<td>%</td>
<td>s.d.</td>
<td>%</td>
<td>s.d.</td>
<td>%</td>
<td>%</td>
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<tr>
<td>Reck</td>
<td>52427 (Vi)</td>
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<td>na</td>
<td>100</td>
<td>na</td>
<td>34.25</td>
<td>4.93</td>
</tr>
<tr>
<td></td>
<td>talin</td>
<td>lethal</td>
<td>100</td>
<td>na</td>
<td>100</td>
<td>34.25</td>
<td>4.93</td>
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<tr>
<td>trio</td>
<td>40138 (Vi)</td>
<td>flightless</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42.25</td>
<td>4.94</td>
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<tr>
<td>Notch</td>
<td>7078 (Bl)</td>
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<td>na</td>
<td>100</td>
<td>na</td>
<td>86.3</td>
<td>1.32</td>
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<td>ref.*</td>
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<td>na</td>
<td>nd</td>
<td>nd</td>
<td>4.26</td>
<td>0.25</td>
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<tr>
<td>control w/o RNAi</td>
<td>yw</td>
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<td>1.22</td>
<td>6.64</td>
<td>1.08</td>
<td>2.89</td>
<td>1.14</td>
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</table>

Larvae combining UAS-RNAi, 1151-Gal4 and tub-Gal80ts were raised at 18°C and shifted to 29°C (allowing RNAi expression) at the indicated stage. The observed percentage (%) of adults with flight defect (or lethality) and standard deviation (s.d.) are indicated. Dpf: days post fertilisation. L3a: Early third instar larval stage. L3b : Late third instar larval stage (wandering larvae). P1-P3 : Pupal stage 1 to 3 (0-6 hours after puparium formation). Ref.* : (Wong et al., 2008).