Amalgam plays a dual role in controlling the number of leg muscle progenitors and regulating their interactions with developing tendon

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ABSTRACT

Formation of functional organs requires cell-cell communication between different cell lineages, and failure in this communication can result in severe developmental defects. Hundreds of possible interacting pairs of proteins are known, but identifying the interacting partners that ensure specific interaction between two given cell types remains challenging. Here we use the *Drosophila* leg model to uncover molecular mediators of cell-cell communications that ensure coordinated development between tendon and muscle precursors. By analyzing gene expression signatures of appendicular muscle and tendon precursor cells, we identify *Amalgam (Ama*) and *Neurotactin (Nrt)* as candidates ensuring early interactions between these two cell populations. Ama encodes secreted proteins and is expressed in the leg myoblasts, whereas Nrt encodes membrane-bound proteins and is expressed in adjacent tendon precursors. In Ama and Nrt mutants, myoblast-tendon cell-cell association is lost, leading to tendon developmental defects. We also show that Ama acts downstream of the FGFR pathway to maintain the myoblast population by promoting cell survival and proliferation in a Nrt-independent manner. Together, our data characterize molecular actors in an early interaction between leg muscle and tendon precursors. These results demonstrate early reciprocal communication at the molecular level between two major components of the appendicular musculoskeletal system, ensuring specificity of myoblasts to tendon precursor association.

INTRODUCTION

Development of organs in a multicellular organism requires the orchestration of cell-cell interactions that control the assembly of various cell types. Altered cellular communication or inappropriate responses to intercellular signals can lead to major developmental defects (Mattes and Scholpp 2018). Research on multicellular organism development generally focuses on one specific tissue. Classically, these studies highlight how the activation of signalling pathways, in a cell-autonomous or non cell-autonomous manner, influences the fate or behavior of a particular cell type. However, bi-directional communication between

different cell types, and how these cells are integrated into a functional physiological unit, have been less investigated.

The limb musculoskeletal system is a remarkable model to study the integration of multiple cell types. It is composed of muscle fibers, motor neurons and connective tissue (CT) cells. CTs include tendons connecting muscles to bones and irregular muscle CT cells (MCT) surrounding and connecting muscle fibers. During vertebrate development, CT progenitors from the lateral plate mesoderm and myogenic cells from the myotome undergo a coordinated migration into the limb bud and differentiate coordinately to build the appendicular musculoskeletal organ. This process involves reciprocal interactions between muscle and CT progenitors (Hasson 2011, Nassari *et al.* 2017, Laurichesse and Soler 2020).

Drosophila leg musculature develops from the appendicular muscle precursors that lie on the surface of the leg disc epithelium in close vicinity of the leg tendon precursors that derive from the leg disc epithelium. At the onset of metamorphosis, tendon precursors undergo a collective cell migration and form long internal structures inside the developing leg, whereas myoblasts aggregate and engage in a coordinated migration following the invaginating tendon precursors. Consequently the perturbation of tendon formation during the early steps of leg development affects the spatial localization of the associated myoblasts (reviewed in Laurichesse and Soler 2020). Interestingly, *Drosophila* leg myoblasts express *ladybird (lb)*, the vertebrate ortholog of *Lbx1,* which is also expressed in vertebrate myoblasts and required for their migration into the limb buds (Brohmann *et al.* 2000, Gross *et al.* 2000, Maqbool *et al.* 2006). Moreover, appendicular tendon specification and invagination/migration rely on Stripe (Sr), Dar1 and Odd-skipped (Odd), three transcription factors whose vertebrate orthologs are markers of limb tendon and MCT cells (Vallecillo-García *et al.* 2017, Orgeur *et al.* 2018). Strikingly, in limb of chick embryo, a subset of MCT cells expressing Odd skipped-related 1 (Osr1), the ortholog of Odd, were observed in close association with migrating Lbx1+ myogenic progenitors (Vallecillo-García *et al.* 2017). In *Osr1* mutant, the pool and distribution of myogenic cell is altered, suggesting that muscle progenitors require Osr1+ MCT cells to survive/proliferate and/or to reach the correct position (Vallecillo-García *et al.* 2017).

Thus, convergent observations in vertebrates and invertebrates suggest the existence of a common genetic circuitry that controls muscle and CT progenitor interactions, and coordination during appendicular development. The molecular signals that ensure the specifity of interactions between subpopulations of myoblasts and their respective tendon precursors remain largely unknown. This prompted us to use *Drosophila* model to search for candidates affecting early interactions between muscle and tendon precursors.

Nrt is a transmembrane protein of the serine esterase-like family and belongs to the family of neuronal cell adhesion molecules (de la Escalera *et al.* 1990, Hortsch *et al.* 1990, Frémion *et al.* 2000, Liebl *et al.* 2003, Speicher *et al.* 1998). Ama is a secreted protein of the

immunoglobulin superfamily (Seeger *et al.* 1988) that plays a role as a cell adhesion molecule involved in axon guidance (Frémion *et al.* 2000, Liebl *et al.* 2003) by acting through its ligand, Nrt. In the central nervous system, Ama/Nrt also regulate neuroblast specification (Becker *et al.* 2016), and Ama affects glial cell proliferation/survival and migration through the receptor tyrosine kinase signaling (RTK) by modulating the expression of Sprouty, a negative regulator of the RTK pathway (Hacohen *et al.* 1998, Reich *et al.* 1999, Ariss *et al.* 2020). More recently, two independent single-cell transcriptomic studies indicated that Ama and Nrt are expressed in a subset of flight muscle progenitors. In the wing disc, depletion of Ama results in a reduced pool of wing disc-associated myoblasts (Zappia *et al.* 2020), and Nrt is critical for proper direct flight muscle development (Everetts *et al.* 2021).

Here we generate transcriptomic datasets from myogenic precursors and analyzed them together with our previously published datasets on isolated tendon precursors (Laurichesse *et al.* 2021) to identify Neurotactin (Nrt) and its binding partner, Amalgam (Ama) as candidates selectively expressed in a specific tendon cluster and in leg myoblasts, respectively. We show that knockdown of Ama results in a dramatic decrease of the leg disc myoblast population independently of Nrt. However, later during metamorphosis, Ama and Nrt are both required for keeping tendon and myogenic precursor cells closely associated, thus ensuring correct development of appendicular muscles. These results highlight an unexpected double role of Ama during leg muscle formation in both maintaining the pool of leg muscle precursor cells and coordinating tendon-muscle precursor growth.

RESULTS AND DISCUSSION

Transcriptomics data identify Ama and Nrt as a candidate pair for cell-cell interaction

We previously reported transcriptional signatures of leg tendon precursor cells (Laurichesse *et al.* 2021) at the developmental time point of 0 h after pupae formation (APF) when sub-populations of myoblasts localize around clusters of tendon precursors. Here, we analyzed the transcriptome of leg disc myoblasts at the same time of development to identify potential interacting pairs. After fluorescence-activated cell sorting and RNA sequencing of the myoblasts (see Material and Methods and GSE245192), we performed differential gene expression analysis and identified a set of 2,236 differentially expressed (DE) genes in myoblasts compared to all other cells in the leg disc (Fig. 1A). Amongst enriched genes (FC>1.5), we consistently found genes specifically expressed in leg myoblast or involved in leg muscle development, such as *twist*, *htl*, *zfh1, him, mef2, vg*, *cut* (Bate *et al.* 1991, Broadie and Bate 1991, Sudarsan *et al.* 2001, Soler *et al.* 2004, Maqbool *et al.* 2006, Soler and Taylor 2009, Boukhatmi and Bray 2018). Gene ontology (GO) analysis of enriched genes showed an over-representation of GO terms related to muscle development (Fig. 1B). We then searched for corresponding interacting partners expressed in muscle and tendon precursors. Considering a RPKM>5, we identified up to 290 pairs of cell surface or secreted proteins known to interact

with each other. To identify pairs of interactors more likely to participate in specific interactions between tendon cells and myoblasts, we narrowed down this list to genes exhibiting a more specific expression (FC > 1.2) in tendon cells and myoblasts compared to other disc cells (Fig. 1C). Amongst the most enriched genes, we found *Nrt* encoding a transmembrane protein (RPKM=234, FC=2.5) specifically in tendon cells and *Ama* encoding its binding partner. Ama was highly enriched both in myoblasts (RPKM=385, FC=3) and in tendon cells (RPKM=221, FC=1.7). These data prompted us to analyze in detail the expression pattern of *Ama* and *Nrt* during leg disc development.

Ama and Nrt show a complementary expression pattern in muscle and tendon precursors of the leg disc

As tendon precursors in leg disc are specified from third larval instar (L3) and elongate during early metamorphosis, we performed immunostaining using anti-Nrt antibody on leg discs from L3 to 5 h APF expressing Lifeact.GFP in developing tendons (Sr>Lifeact.GFP line; Laurichesse *et al.* 2021) (Fig. 2A-D; I-L). Remarkably, in L3 stage, *Nrt* is expressed in only one cluster of tendon cells, in the dorsal femur, corresponding to the future tibia levator tendon (*tilt*) (for nomenclature see Soler et al. 2004) (Fig. 2A-B). This tendon precursor-associated Nrt expression remains restricted to the dorsal femur in the next stages of development (Fig. 2C-D; I-L). In addition, Nrt is detected in the chordotonal organ (Fig. 2A-D; I-L) and in the two most distal neuronal precursors that project axons in tarsi (Jan *et al.* 1985). To follow *Ama* expression we generated an *Ama::EGFP* knock-in line. In stages preceding tendon cell specification, Ama::EGFP protein can already be detected in the vicinity of leg disc myoblasts (Suppl. Fig.1A-F). From L3 stage to 5 h APF, Ama is associated with muscle precursors located in the presumptive femur, tibia, and tarsi segments (Fig. 2E-H; M-P), and is barely detectable around myoblasts in more proximal regions, at the periphery of the leg disc. This observation suggests that distal myoblasts could be the major source of secreted Ama protein. Noticeably, as early as L3 stage, Ama protein strongly accumulates in the dorsal femur at the interface with Nrt-expressing tilt tendon (arrowheads in Fig. 2E-H; M-P). We previously showed that myoblasts align along this tendon, and that altering tendon development affects their spatial distribution (Soler *et al.* 2016). To precisely document the expression of *Ama* and *Nrt* in this specific region, we stained 5h APF leg discs from Sr>CAAXmCherry ; *Ama::EGFP* line for the myoblast (Twist) and tendon (mCherry) markers and for Ama (EGFP) and Nrt (Fig. 2Q-T). High magnification of dorsal femur region shows that myoblasts are indeed closely associated with the invaginating tendon (Fig. 2Q, T). We also confirmed that Nrt protein is specifically expressed by the developing tendon (Fig. 2R), whereas Ama::EGFP staining accumulates at the interface between the myoblasts and the basal membrane of the tendon cells, though appears more diffuse, as expected for a secreted protein (Fig. 2S).

To precisely determine the origin of *Ama* expression, we performed a series of *in situ* hybridization to detect *Ama* RNA in leg discs (Fig. 3). We identified a new myoblast-specific

Gal4 line R32D05-Gal4 (Suppl. Fig. 1G-I; Jenett *et al.* 2012) and crossed it to UAS-GFP. In L3 larval leg disc of R32D05>GFP line, *Ama* RNA colocalizes with most GFP+ myoblasts as well as in three distinct clusters in tarsal segments, revealing an additional source of Ama in most distal segments (Fig. 3A-C). In the dorsal femur, in the vicinity of Nrt+ tendon, myoblasts show a particularly high level of *Ama* RNA. To confirm that *Ama* expression is myoblast-specific, we crossed a UAS-AmaRNAi line with R32D05-Gal4>UAS-GFP line (Jenett *et al.* 2012). Ama RNAi expression in myoblasts led to a strong reduction of *Ama* RNA level in leg disc, except in the three clusters in the tarsi where R32D05-Gal4 driver is not expressed (Fig. 3D-F). In the dorsal femur, *Ama* expression is completely abolished indicating that at L3 stage, myoblasts are the only source of Ama in this region. However, at later stage of development (5h APF), *Ama* RNA was clearly detectable in both myoblasts and in the tilt tendon in control leg disc (Suppl. Fig. 2A-C; G-I). Accordingly, when we used specific Gal4 drivers to induce UAS-AmaRNAi either in myoblasts (R32D05-Gal4) or tendon (Sr-Gal4), *Ama* expression was abolished only in myoblasts or only in the tendon (Suppl. Fig. 2D-F; J-L). This result indicates that, a few hours after pupae formation, the tendon in the dorsal femur also expresses *Ama.* Aggregation assays and biophysical analysis have shown that Ama possesses homophilic adhesion properties (Liebl *et al.* 2003, Zeev-Ben-Mordehai *et al.* 2009b, 2009a). Thus, this later *Ama* expression by the tendon cells might reinforce the aggregation of the myoblasts in the vicinity of the developing tendon.

Overall, our expression analyses indicate that most myoblasts express *Ama,* while *Nrt* is restricted to a single developing tendon (tilt). Ama expression is also initiated earlier in larval development than that of Nrt and could be detected in leg myoblasts at L2 larval stage (Suppl. Fig. 1A-F). Thus, spatial and temporal expression patterns of Ama and Nrt suggest that during appendicular myogenesis Ama could act not only by interacting with Nrt but also in a Nrt-independent way.

Ama controls myoblast number in an NRT-independent way

Ama was previously shown to regulate the pool of glial cells and flight muscle progenitors (Zappia *et al.* 2020, Ariss *et al.* 2020). Interestingly, Ama is expressed in leg disc myoblasts from the early larval stage onwards (Suppl. Fig. 1A-C). Ama downregulation in myoblasts in R32D05-Gal4>UAS-GFP leg discs (Fig. 3) leads to a strong reduction in GFP fluorescence, suggesting that the number of myoblasts could have been reduced. Because the number of total leg disc myoblasts and their proliferative rate have never been precisely determined, we first counted them at different stages of development using a Twist antibody. Myoblast numbers grow from a pool of around ten per disc primordia at the end of embryonic stage to 36 (+/-10) at mid-L2 stage and to 653(+/-82) by the end of L3 stage.

To thoroughly assess the effect of Ama knockdown on the pool of myoblasts, we used a Twist antibody to compare the number of muscle progenitors in L3 leg discs from R32D05-

Gal4>UAS-AmaRNAi, R32D05-Gal4>UAS-mcherryRNAi and W^{1118} strains. Myoblast-specific RNAi-knockdown of *Ama* leads to a severe reduction in muscle precursors with almost no Twist+ myoblasts (<10/disc) when compared to both controls (Fig. 4A-B and E). No significant differences could be observed between R32D05-Gal4>UAS-mcherryRNAi and W^{1118} controls, with 643 and 653 myoblasts on average, respectively by the end of L3. Thus, Ama depletion results in a severe reduction of Twist+ myoblasts associated with the leg disc. As a decrease in the number of cells may be due to a defect in cell proliferation and/or to an increase in cell death, we tested these two variables by immunostaining L3 leg discs with antibodies against either the mitotic marker phospho-histone H3 (pH3) or the apoptotic marker caspase-activated Dcp1. Because these two antibodies were raised in rabbit, as the Twist antibody, we performed the immunostainings on R32D05-Gal4>UAS-AmaRNAi and R32D05-Gal4>UAS-mcherryRNAi leg discs expressing UAS-GFP to visualize the myoblasts (Fig. 4F-J). Then, we quantified the number of GFP cells that were pH3+ or Dcp1+. In control leg discs, 1.8% of cells were pH3+/GFP+. After *Ama* RNAi induction, this ratio decreased at 0.7% (Fig. 4F-H). 2.5% of GFP+ cells were also Dcp1+ in controls, indicating the cells were apoptotic. This ratio increased to 13.8% when *Ama* was knocked down. (Fig. 4H-J). The low number of total myoblasts in L3 leg discs after Ama knockdown (<10) can make these outcomes difficult to interpret. Thus, to increase the number of myoblasts at the time of counting (L3), we delayed the expression of the *UAS-AmaRNAi* transgene using $Gal80^{ts}$, a thermosensitive form of the Gal4-inhibitor Gal80 (McGuire *et al.* 2003). In this way, we induced *UAS-AmaRNAi* expression from L2 stage, and we could count an average of 100 GFP+ cells in L3 stage. The percentage of mitotic cells was still significantly lower compared to controls, and the percentage of apoptotic cells was higher (Fig. 4H), indicating that Ama depletion affects both viability and the proliferation rate of leg disc myoblasts.

A previous study suggested that Ama-depleted flight muscle precursors were unable to proliferate but the authors could not assay a defect in proliferation rate (Zappia *et al.* 2020). Moreover, Nrt was expressed in flight muscle precursors and its depletion resulted in a flight muscle defect, suggesting that Ama may interact with Nrt to regulate the pool of flight muscle myoblasts (Zappia *et al.* 2020, Everetts *et al.* 2021). To test this possibility in leg myoblasts, we counted the number of Twist+ cells in leg discs of Nrt^1/Nrt^2 transheterozygous mutants. We observed no differences compared to W^{1118} or with heterozygous Nrt^1/Nrt^+ and Nrt^2/Nrt^+ leg discs (Fig. 4C-E). This result is consistent with the absence of *Nrt* expression in myoblasts at L3 stage (Fig. 2). Moreover, myoblast depletion can already be observed in Ama-RNAi expressing leg discs of earlier stage L2 (Suppl. Fig. 3A-F), a stage when Nrt could not be detected in leg disc even in tendon cells, which are specified at the beginning of L3 stage (Suppl. Fig. 3G-I). We conclude that Ama controls the number of leg myoblasts in an Nrt-independent way during larval stages.

Ama, potential downstream effector of FGF pathway in the regulation of myoblast number

A previous study proposed that Ama could modulate the Receptor Tyrosine Kinase (RTK) pathway activity during *Drosophila* brain development by regulating Sprouty (Sty) expression, a negative regulator of this pathway (Ariss *et al.* 2020). In this study, when Ama was specifically attenuated in glial cells, single-cell RNA-sequencing showed a strong downregulation of cell cycle genes such as *PCNA* and *Cyclins,* as well as high expression of the pro-apoptotic gene *hid,* supporting a role of Ama in regulating cell survival and proliferation. Interestingly, we had previously shown that the RTK pathway, FGFR, is involved in regulating the number of leg disc myoblasts (Maqbool *et al.* 2006). To test whether Ama could regulate myoblast number through the modulation of the RTK pathway, we conducted a set of gain- and loss-of-function experiments using the R32D05-Gal4 line. First, we counted the total number of Twist+ myoblasts in leg disc expressing an active form of the downstream effector of the RTK pathway, ERK, also named Rolled (UAS-rl^{sem}), a dominant-negative form (UAS-Htl^{DN}) or a constitutively active form (UAS-Htl^{CA}) of the FGF receptor Heartless (Htl) (Fig. 4K). Compared to control (mean=643 myoblasts/disc, n=37), we found that the number of myoblasts was significantly increased in rl^{sem}- (mean=1032, n=14) and in Htl^{CA}- (mean=1706, n=27) expressing leg discs, and reduced after Htl^{DN} induction in myoblasts (mean=410, n=19). This result confirmed that RTK pathways, and notably the FGFR pathway, are involved in controlling the number of total myoblasts. Over-expression of Sty (UAS-Sty) resulted in a significant decrease of myoblast number (mean=502, n=19), whereas its reduction (UAS-StyRNAi) lead to more myoblasts (mean=839, n=18), in accordance with its role of inhibiting FGFR pathway (Hacohen *et al.* 1998).

As Ama negatively regulates Sty expression in glial cells, we asked whether Ama could counteract Sty-induced changes in myoblast numbers in the developing leg. We found that Ama overexpression (Ama-HA) alone cannot rescue the reduced number of myoblasts caused by Sty overexpression (UAS-lacZ;UAS-Sty) (Fig. 4L). Similarly, no rescue was observed when co-expressing AmaRNAi with StyRNAi compared to UAS-lacZ;UAS-AmaRNAi (Fig. 4L). These results indicate that the hypothesis that Ama stimulates the FGFR pathway by negatively regulating *Sty* expression is not sufficient to explain the role of Ama in controlling leg myoblast number (see Material and Methods for myoblast counting details). Nevertheless, we continued to investigate possible connections between Ama and the FGFR pathway. The expression of the constitutively active form of ERK could rescue the phenotype of Ama depletion in glial cells (Ariss *et al.* 2020), therefore we tried to rescue the UAS-AmaRNAi phenotype by co-expressing the UAS-rl^{sem}. However, rl^{sem} over-expression was insufficient to rescue myoblast depletion (Fig. 4L). Since we could not show an upstream effect of Ama on the FGFR pathway, we asked if Ama could act downstream. Strikingly, leg discs co-expressing UAS-AmaHA and UAS-Htl^{DN} showed a significant higher number of myoblasts compared to the ones expressing UAS-Htl^{DN}; UAS-mcherryRNAi. This suggests that Ama could, in fact, be a target of the FGFR pathway. Lastly, when Ama was overexpressed in a context where Sty expression was decreased (UAS-StyRNAi; UAS-AmaHA), the increase in the number of myoblasts was even higher than when only Sty expression was reduced (Fig. 4L). However, the

mere overexpression of Ama (UAS-AmaHA) without modifying Sty expression had no impact on the number of myoblasts (Fig. 4K).

Thus, our results indicate that Ama does not promote myoblast proliferation by alleviating Sprouty's inhibitory effect on the RTK pathway, in contrast to the previously described effect on glial cells (Ariss *et al.* 2020). However, the fact that Ama over-expression and Sty knockdown show an additive effect on myoblasts number and that Ama overexpression could improve loss of myoblasts in the Htl^{DN} context supports a model in which Ama acts downstream of FGF/RTK pathway. Another, not exclusive, possibility is that Ama enhances myoblast responsiveness to proliferation-inducing FGF signals. This view is supported by two studies. First, Ama knockdown was found to reduce the suppressive effect on retinal degeneration of the E3 ligase SORDD1 (Xu *et al.* 2020). Second, a two-hybrid screen found Ama interaction with Syndecan (Nguyen *et al.* 2016), a proteoglycan acting as a co-receptor that modulates ligand availability for FGF receptor (Stork *et al.* 2014).

Ama and its receptor Nrt are required for myoblast-tendon adhesion

Given the restricted *Nrt* expression in one unique tendon precursor, the tilt, and the known role of Nrt as an adhesion molecule (de la Escalera *et al.* 1990, Hortsch *et al.* 1990, Frémion *et al.* 2000), we hypothesized that Ama and Nrt interact to ensure adhesion between myoblasts and the tilt. To test this hypothesis, we used an alternative myoblast specific driver, R15B03-Gal4 (Suppl. Fig.4A-F), to induce Ama attenuation (UAS-AmaRNAi) after the extensive larval phases of proliferation. We combined the R15B03-Gal4 driver with R79D08-lexA that induced lexAop-GFP transgene in tendon cells (Suppl. Fig.4G-I), allowing us to observe the developing tendons while inducing UAS-AmaRNAi in myoblasts from late L3 stage. We then analyzed the consequences of late myoblast-specific *Ama* knockdown on myoblast distribution at 5 h APF, a time when myoblasts are properly aligned along the elongating tilt tendon (Soler *et al.* 2016, Fig. 5A and A'). Late myoblast-specific *Ama* knockdown results in an apparent misdistribution of the myoblasts around the tilt tendon (Fig. 5B-B' compared to 5A-A'). To quantify the myoblast position variability relative to the tilt, we measured the shortest distance between each myoblast and the surface of the tilt and calculated the myoblast-to-tendon mean distance (dMT) for each disc (see Material and Methods). In controls, myoblasts are located at an overall average dMT of 4 μm from the tilt across all discs, whereas in AmaRNAi-expressing leg discs, this value increased slightly but significantly to 5.4 μm, suggesting that the reduction of Ama level could affect myoblast-tendon adhesion (Fig. 5D). To better assess whether *Ama* knockdown increases the probability of the myoblasts being located farther away from the tilt than in control, we calculated the percentage of discs for which the dMT was greater than 4 μm (average dMT across all control discs). 68% of the discs expressing R15B03-Gal4>AmaRNAi had a dMT greater than 4 μm, compared to 45% of the control discs (Fig. 5E). Thus, reducing level of Ama expressed by the myoblasts significantly increases the proportion of discs in which myoblasts are not properly associated with the tilt. In parallel, we

generated a strain carrying the *Nrt*^{2} allele and the R79D08-lexA>lexAop-GFP transgenes that we crossed with the line carrying the *Nrt*^{1} allele. F1 were then analyzed the same way as Ama knockdowns for myoblast positioning relative to the tilt. R79D08-lexA>lexAop-GFP; *Nrt*^{2}/*Nrt*^{1} transheterozygous mutants exhibit an average dMT of 6.2 μm, significantly elevated compared to the average dMT of R79D08-lexA>lexAop-GFP of 4.3 μm and R79D08-lexA>lexAop-GFP; *Nrt*^{2}/*Nrt*$^{+}$ controls (4.8 μm) (Fig. 5D). As expected, the percentage of discs with a dMT greater than the average dMT of control discs (4.3 μm) was much higher in transheterozygous mutants (84%) compared to controls (48% and 45%, respectively - Fig. 5E). Thus, in both *Ama* and *Nrt* knockdown contexts, myoblasts lose their ability to aggregate and to align along their corresponding tendon. These results support the hypothesis that Ama/Nrt heterophilic interactions play a role in myoblasts for tendon precursor adhesion. Given that Ama is a secreted protein (Seeger *et al.* 1988) and Nrt is only present on the surface of the tendon, it is likely that Ama interacts with another membrane-associated protein at the surface of the myoblasts. Alternatively, Ama is capable of homophilic adhesion (Liebl *et al.* 2003), and analysis of its quaternary structure indicates that it can simultaneously interact with Nrt and form homodimeric structures (Zeev-Ben-Mordehai *et al.* 2009a). Moreover, the presence of a short hydrophobic domain at its COOH-terminal end suggests that Ama could tether to the membrane via a glycosyl-phosphotidylinositol (GPI) anchor (Seeger *et al.* 1988) similar to other cell adhesion molecules (Saha *et al.* 2016).

Thus, Ama could mediate tendon-myoblast adhesion by interacting with Nrt on the tendon side and myoblast-myoblast adhesion on the other side, directly or through another unidentified transmembrane receptor. Ama is orthologous to several Ig-like neuronal cell adhesion molecules in vertebrates, including the IgLON 4 and 5 GPI-anchored membrane proteins that regulate myoblast adhesion during myogenesis and muscle regeneration by providing an essential microenvironment that ensures muscle stem cell survival (Lim *et al.* 2021, 2022). Thus, the conservation of Ama's function could highlight a pivotal role in sustaining muscle tissue homeostasis and development across evolution.

Ama and Nrt contribute to long tendon morphogenesis

In addition to the loss of myoblast adhesion observed in Nrt mutants and R15B03-Gal4>AmaRNAi discs, we also noticed a general alteration in the morphology of the tilt. In particular, the tilt appears shorter in mutants compared to controls (Fig. 5B' and C'). Suggesting that in addition to their role in myoblast positioning, Ama-Nrt interaction would also contribute to tendon morphogenesis. This would support the hypothesis of a reciprocal influence between these two cell types for coordinating their developmental fate.
Given the implications of Ama and Nrt in glial cell migration and axonal pathfinding (Liebl *et al.* 2003, Liu *et al.* 2020, Ariss *et al.* 2020), and because leg long tendons form by collective migration of epithelial cells (Laurichesse *et al.* 2021), we wondered if loss of Ama or Nrt could affect tilt elongation. We measured the length of the tilt relatively to the length of the femur

(see Materials and Methods), and found that tilts in discs with *Ama* knockdown in myoblasts are shorter than in control (Fig. 5G). In a similar manner, the combination of transheterozygous Nrt^1/Nrt^2 mutants exhibit a shorter tilt compared to controls (Fig. 5G). Interestingly, in both cases, the overall tendon volume is not affected. This result implies that the tendon shortening is somehow compensated for by a widening of the structure, resulting in a clearly visible deformation of the tendon (Fig. 5F). Thus, Ama secretion by the myoblasts, likely interacting with Nrt at the tendon surface, is essential for proper elongation and morphogenesis of the tilt long tendon.

Altogether, these results support a model in which Ama::Nrt interaction is important for ensuring specificity of myoblast to tendon adhesion. These results also suggest that, in return, Ama could provide a guiding cue through Nrt that would be necessary for the correct growth of the tendon. It has been shown that Ama and Nrt are dominant enhancers of the axon misguidance phenotype observed in an Abelson tyrosine kinase (Abl) mutant (Liebl *et al.* 2003). Because Abl regulates modulators of the actin cytoskeleton (Liebl *et al.* 2000, Grevengoed *et al.* 2003, Kannan *et al.* 2017) is tempting to speculate that activation of this pathway by Ama/Nrt could contribute to tendon growth in coordination with myoblast adhesion.

CONCLUSION

Overall, we identify *Ama* and *Nrt* as a part of transcriptional signature of developing leg muscle and tendon precursors. We show that Ama is a key player with a double role during appendicular myogenesis. First, during early larval development, Ama acts in a Nrt-independent way to promote myoblast proliferation and to establish the appropriate number of leg myoblasts. Our genetic analyses indicate that Ama acts downstream of FGFR pathway to fulfill this function. Second, slightly later in development, Ama secreted by myoblasts interacts with Nrt expressed at the surface of one of leg tendon (tilt) precursors. This interaction is crucial for myoblast-tendon specific adhesion, tilt tendon elongation, and consequently, coordinated development of tilt-associated appendicular muscle. Thus, Ama and Nrt represent a pair of molecules that ensure early cross talk between two key components of developing muscle, the tendon precursors and myoblasts. This result points to the importance of reciprocal cell-cell communication during early steps of muscle development. We also uncover a dual role for Ama in regulating both myoblast number and cell adhesion, indicating that within a cell population the use of a single molecule can be reoriented by modulating its mode of interaction. Giving the well-characterized cellular and molecular convergences observed during muscle development between vertebrates and *Drosophila*, our findings could be relevant to analogous processes in vertebrates.

MATERIAL AND METHODS

Fly strains and genetics

The following fly stocks were used: enhancer traps line sr-gal4^{md710} (Usui et al., 2004, BDSC 2663); UAS-Lifeact.GFP (BDSC 35544); UAS-mCherryCAAX (BDSC 59021); R32D05-Gal4 (BDSC 49712); UAS-stingerGFP (BDSC 84278); UAS-mCherryRNAi (BDSC 35785); UAS-AmaRNAi (BDSC 33416); W^{1118}; Nrt^{1}/TM6B (BDSC 25032); Nrt^{2}/TM6B (BDSC 25033); tubGal80^{ts} (BDSC 7108); UAS-rlSem (BDSC 59006); UAS-Htl-DN (BDSC 5366); UAS-Htl-CA (BDSC 5367); UAS-StyRNAi (VDRC 330208); UAS-Sty (BDSC 20670); UAS-lacZ (BDSC 1776); R15B03-Gal4 (BDSC 49261); R79D08-lexA (BDSC 54734); lexAop-mCD8::GFP (BDSC 66545); UAS-mCherryNLS (BDSC 38425). For experiments using tubGal80^{ts}, crosses were kept at 18°C and then shifted at the appropriate stage (L2) to 30°C. All other experiments were performed at 25°C.

Cell sorting and RNA extraction

The fluorescence-activated cell sorting and RNA sequencing of 0 h APF leg disc myoblasts were conducted following the same protocol as described in Laurichesse et al. (2021). For myoblast sorting, we used the targeted expression system lexA/lexAop to express the lexAopGFP transgene specifically in leg disc myoblasts (R32D05-lexA>lexAopGFP).

RNAseq and transcriptomic data analysis

Extracted total RNA from GFP+ myoblasts and input (IP) cells (sample of all leg disc cells) from three different replicates were sent to Heidelberg genomic platform EMBL for high-throughput mRNA sequencing (NextSeq 500/Illumina). They generated mRNA libraries with NEB RNA Ultra kit (New England Biolabs E7770L) and dToligos probe were used to target mRNA for cDNA synthesis. Single-end multiplex was performed. Bioinformatic analysis was performed using FastQC and Cutadapt (Kechin et al. 2017) for reads quality control and filtration. Reads alignment and counting were performed using Hisat2 (Kim et al. 2019) and HTseq (Anders et al. 2015) (reference genome Dm6 Ensembl release 70). Values were normalized for each gene (RPKM) using Cufflinks (Trapnell et al. 2012) and differential expression between IP and GFP+ samples were determined using R script DEseq2 V :3.13 software. Fold change (FC) between GFP+ and IP samples was computed using their normalized raw counts. GEO accession number: GSE245192

Ama transgenes

UAS-Ama-HA transgene

The UAS-Ama C-terminal tagged with FLAG-HA (UFO01101 - DGRC Stock 1621050; https://dgrc.bio.indiana.edu//stock/1621050; RRID:DGRC_1621050) clone was obtained from the Universal Proteomics Resources, a Berkeley Drosophila Genome Project (BDGP) (https://www.fruitfly.org/EST/proteomics.shtml) and contains a *white* gene selectable marker and an *attB* site. The PhiC31 transformation services in BestGene (http://www.thebestgene.com/) were performed on *y^{1} w^{67c23}; P{CaryP}attP2* line (BDSC 8622) with an

estimated CytoSite 68A4 (chromosome 3) and on *y^1 w^{67c23}; P{CaryP}attP40* line with an estimated CytoSite 25C6 (chromosome 2) using the UAS-Ama construct above.

Ama::EGFP transgene

W^{1118} line expressing EGFP C-terminal tagged endogenous Ama protein was generated by CRISPR-mediated-genome editing by homology repair using the following guide 5'-CTTCGAGGGCACTGGGATGACGGTC-3' by Wellgenetics Inc. (Taiwan). Detailed protocol on request.

Immuno-Histochemistry and Confocal Microscopy

The following primary antibodies were used: mouse anti-Nrt (1:100 - BP106- Developmental Studies Hybridoma Bank DSHB); rabbit anti-Twist (1:300 - our lab); rabbit anti-Dcp1 (1:100 - Cell Signaling); rabbit anti-pH3 (1:500 - Merk Millipore); goat anti-GFP (1:500 – ab5450- Abcam). Secondary antibodies donkey anti-rabbit, donkey anti-mouse conjugated to cy3- or cy5- fluorochromes (Jackson Immunoresearch), donkey anti-rabbit Dylight 405 (Jackson Immunoresearch), donkey anti-rabbit Alexa Fluor 488 (ThermoFisher) and donkey anti-goat Alexa Fluor 488 (Invitrogen) were used (1:500). Immunohistochemistry experiments were performed on samples fixed in 4% formaldehyde (FA-methanol free) for 20 min, washed in 0.5% PBS-Triton and blocked in 30% horse serum before immunostaining with primary antibodies, at 4°C overnight. The samples were washed in 0.5% PBS-Triton and incubated with secondary antibodies, at room temperature during 2h. After three washes in 1XPBS, samples are mounted in Vectashield medium for analysis. Immunostained samples were imaged on LSM800 Zeiss confocal microscope, and images were analyzed using Imaris 9.8.2 and ImageJ-fiji 2.9.0/1.53t softwares.

***In situ* hybridization**

Leg imaginal discs were dissected in 1XPBS and fixed by 4% formaldehyde (FA-methanol free) for 20min. Samples were washed three times in 1XPBS and incubated in methanol over-night (o/n) at -20°C. Samples were progressively rehydrated in 75% MetOH-25% PBS for 5min; 50% MetOH-50% PBS for 5min; 25% MetOH-75% PBS for 5min; 10% MetOH- 90% PBS for 5min and samples were quenched with 0.3% H2O2 for 15min. The samples were washed three times in 1XPBS for 5min each and were permeabilized by 80% acetone for 10min at -20°C, and washed three times in PBS-Tween (PBSTw) for 5min at room temperature. The samples were refixed by 4% FA for 20min and washed three times for 5min each. Then, samples were incubated in 50% Hybridization Buffer (50% formamide, 2x SSC, 1 mg/ml Torula RNA, 0.05 mg/ml Heparin, 2% Roche blocking reagent, 0.1% CHAPS, 5mM EDTA, 0.1% Tween 20) – 50% PBSTw for 5min, 75% HB-25%PBSTw for 5min, 90% HB-10%PBSTw for 5min, and incubated during 1.5h at 60°C in 100% HB Buffer. The samples were incubated o/n at 60°C with anti-sense DIG-labeled RNA probes against *Ama* diluted in HB (1:100). The samples were washed in 100% HB for 1h at 60°C, in 75%HB-25%PBSTw for 15min at 60°C, in 50%HB-50%PBSTw for 15min at 60°C, in 25%HB-75%PBSTw for 15min at 60°C, in 10%HB-90%PBSTw at 60°C for 15min and were

washed three times in PBSTw 5min for each at room temperature. Then, the samples were incubated for 1.5h in PBSTw-1%BSA before being incubated with anti-DIG-POD antibody (Roche, 1:2000) and other primary antibodies o/n at 4°C. After 5 washes in PBSTw, Tyramide Signal Amplification (TSA™) were performed in 100ul Amplification Buffer + 2ul TSA^+ Cy3 (PerkinElmer, 1 :50) for 5min at room temperature. The sample were washed six times for 5min each in PBSTw and were incubated with secondary antibodies for 2h at room temperature. After three washes in 1XPBS, samples are mounted in Vectashield medium for analysis.

Quantification and statistical analysis

Myoblast counting

Images were processed with Imaris 9.8.2 software and GFP+, Twist+, Dcp1+ and $pH3^+$ cells were detected automatically using the *"Spots"* function. The automatic cell counting was subject to manual adjustment. Myoblast proliferation and apoptosis were calculated by determining the percentage of pH3+/GFP+ cells and Dcp1+/GFP+ cells, respectively. It is noteworthy that the use of two UAS-transgenes (UAS-lacZ; UAS-Sty) is sufficient to rescue the reduction of myoblast number induced by UAS-Sty expression alone, emphasizing the importance of adding a second UAS-transgene as a control.

Myoblasts to tendon distance

Tilt surface was automatically determined using the "*Surface*" function of Imaris Software, then a distance map was generated using the *"Distance Transformation"* function. The distance in µm between the tilt surface and each myoblast, detected using the *"Spot"* function as described above, was calculated with the *"intensity center spot"* function.

Tendon length and volume measurement

Contour surfaces of the tilt and of the femur were determined either automatically (for the tilt) or manually (for femur) using Imaris *"Surface"* function to extract 3D objects and calculate their volumes. Tilt volume was determined by normalizing the volume of the tilt over the volume of the femur. 3D objects were then used to measure tilt and femur lengths using *"skeletonize" (2D-3D)* plugin of ImageJ-Fiji 2.9.0/1.53t software. Tilt length was then determined by normalizing the length of the tilt over the length of the femur.

Statistical analyses were carried out using Prism 9.2.0 and statistical significances were determined using Mann-Whitney tests or Fisher's exact test/Chi-square test. Differences were considered significant if $p\text{-value}<0.05$.

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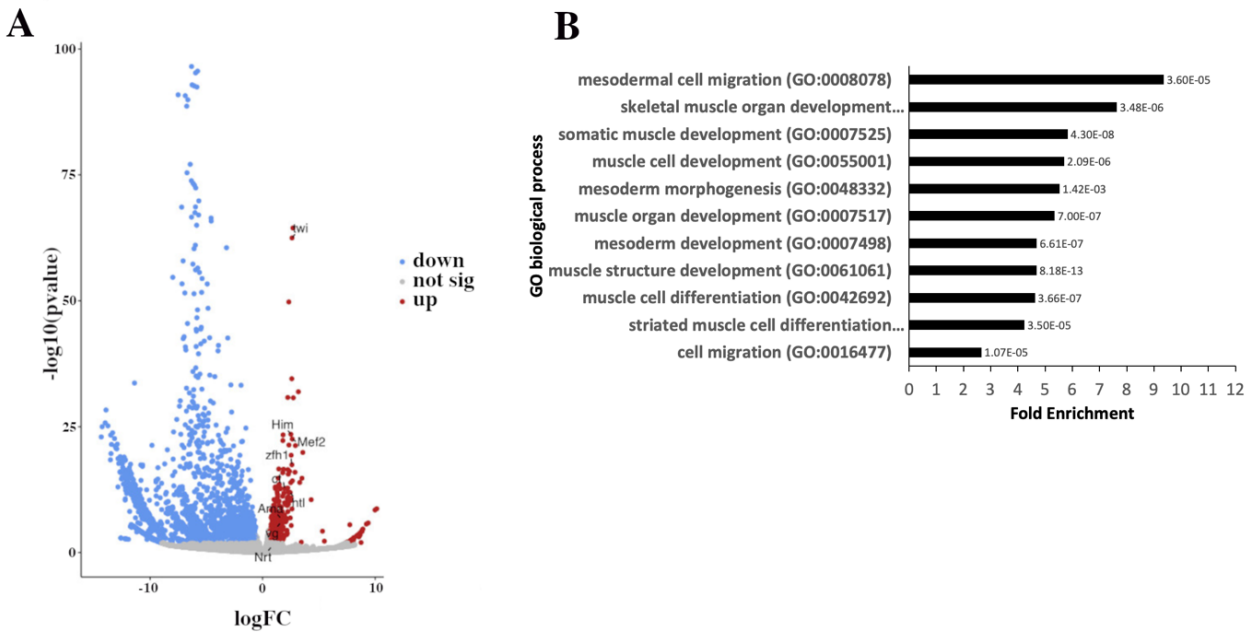


C

CSS expressed in tendon cells (RPKM>5 ; FC>1.2)	interacting partners expressed in myoblasts (RPKM>5 ; FC>1.2)
Ama	Ama
Nrt	Ama
Appl	Fas2
CG33543	Fas2
fipi	Fas2
Ror	fz2
if	kon
mew	kon
Ror	otk
Mmp2	shg
beat-Ia	side
Ror	Wnt4

Figure 1: Differentially-expressed (DE) genes and interactome data in leg disc myoblasts

(A) Volcano plot showing DE genes, including 533 enriched genes (red) and 1703 down-regulated genes (blue). Those not significantly changed (fold change < 1.5; $P > 0.05$) are in grey. Examples of genes known to be specifically expressed in myoblasts are annotated, as are *Nrt* and *Ama.* For graphical representation, outliers (-log10(Pvalue) >100 and logFC>20) were excluded. Generated using VolcanoPlot tool (Galaxy V.0.0.5).

(B) Gene ontology (GO) analysis of enriched genes in leg myoblasts. Graph shows selected GO terms for biological process, GO term accession numbers, fold enrichment, and *P*-value. Data were obtained by GO overrepresentation test using PANTHER.

(C) List of identified pairs of interacting partners expressed in myoblasts and tendon cells (Flybase protein interactions browser). Among the 290 pairs of interacting genes expressed in myoblasts and tendon cells (RPKM>5), 12 of them have corresponding interacting genes with a Fold Change >1.2

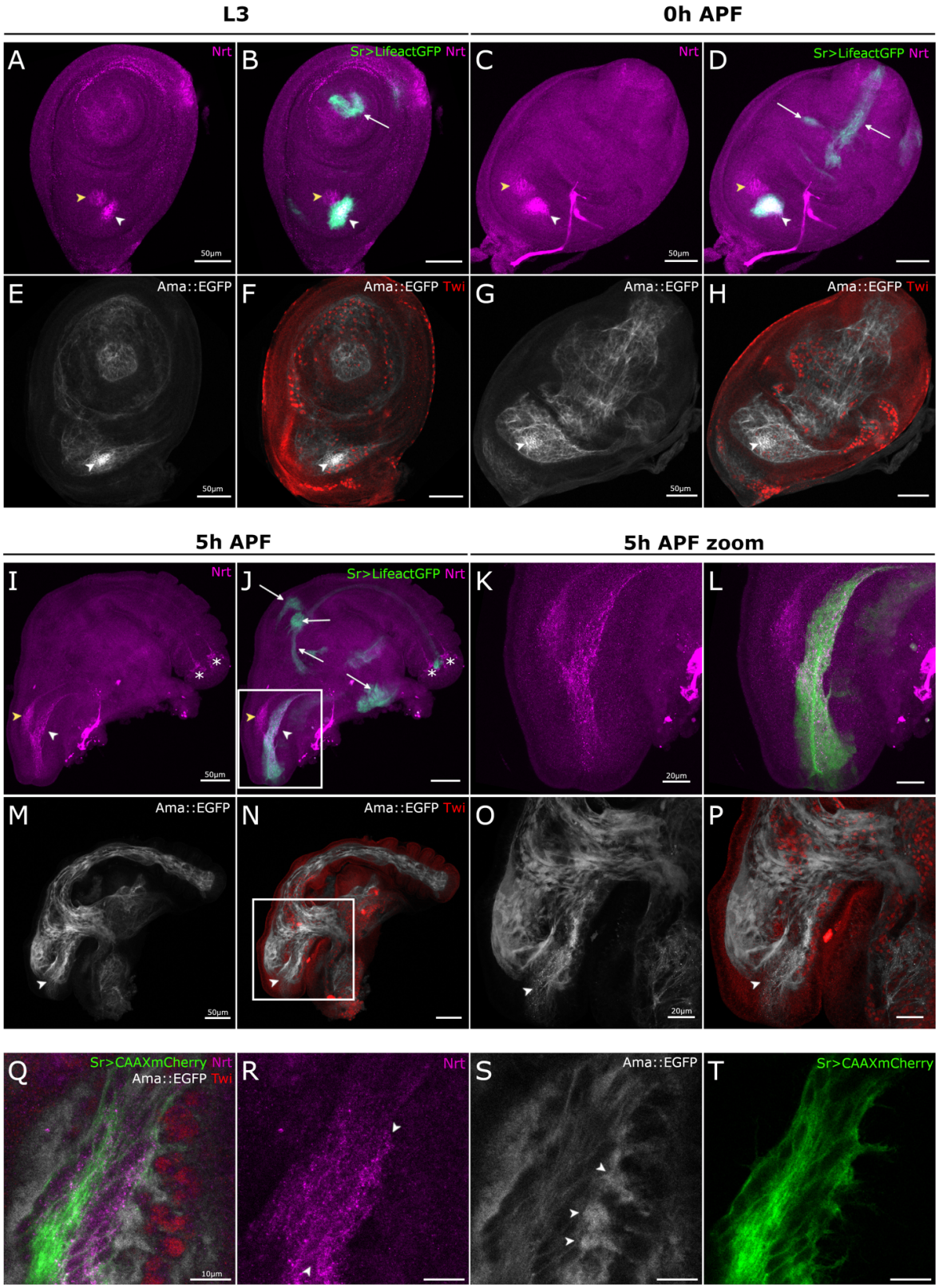
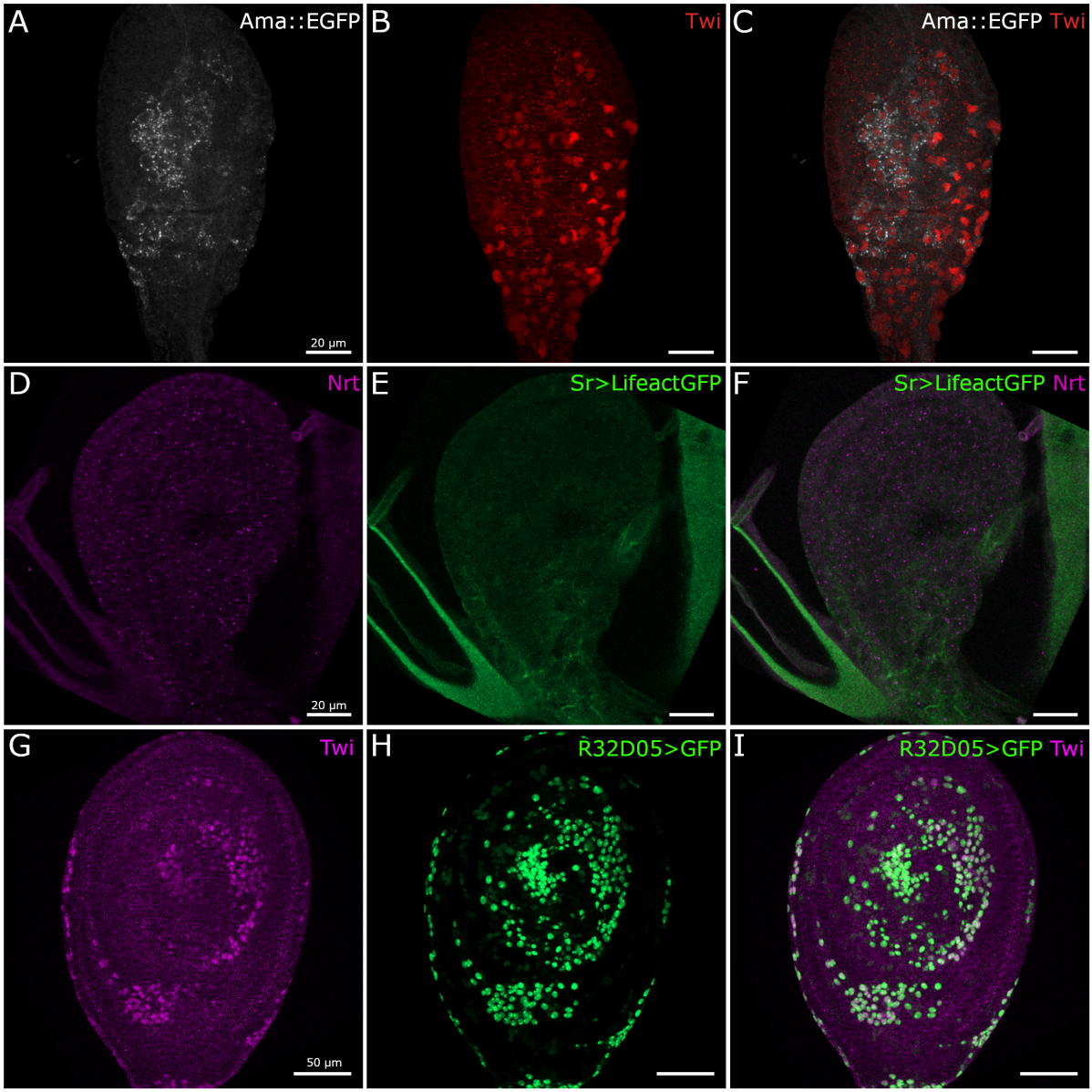


Figure 2: *Nrt* and *Ama* are expressed in muscle and tendon precursors during leg disc development

(A-P) Confocal optical sections of Sr-Gal4>UAS-LifeactGFP (green) and Ama::EGFP (gray) leg discs at different stages of development immunostained for Nrt (magenta) and for Twist (Twi - red), respectively. **(A-D)** In L3 and 0h APF leg discs, Nrt is localized in the tilt tendon in the dorsal femur (white arrowhead) but not in the other tendon precursors in green (white arrows); note Nrt presence in the chordotonal organ (yellow arrow head). **(E-H)** In L3 and 0h APF leg discs, Ama::EGFP protein is found surrounding most of distal myoblasts immunostained by Twi (red); but not around the most proximal ones at the periphery of leg

disc. Ama::EGFP accumulates in the region of dorsal femur close to tilt tendon precursors (white arrowhead). **(I-L)** At 5h APF, Nrt remains localized in the tilt that elongates in the dorsal femur (white arrowhead) and in the chordotonal organ (yellow arrowhead). Nrt is not visible in any tendons of the other segments (white arrows) but can be detected in the two most distal neuronal precursors projecting axons in tarsi (asterisks, Jan et al. 1985). **(K, L)** Higher magnification of the dorsal femur region from (J). **(M, N)** At 5h APF, Ama::EGFP is detected in the entire leg disc cavity where myoblasts stand and tendons invaginate and elongate (Soler et al. 2004). **(O-P)** Higher magnification of the dorsal femur region shows Ama::EGFP accumulation close to Nrt-positive tilt precursors (arrowhead). **(Q-T)** Close up view of myoblast-tendon interface in the dorsal femur of a 5h APF leg disc expressing *Sr-Gal4>CAAXmCherry* and *Ama::EGFP* immunostained with Twi and Nrt antibodies. Ama::EGFP (gray) localizes all around myoblasts (red) and tendon cells (green), whereas Nrt (magenta) is specifically found in tendon cells. Note the enrichment of Nrt at the tendon membrane (between arrowheads in R) facing the myoblasts where Ama::EGFP accumulates (arrowheads in S).



Suppl figure 1: ***Nrt* and *Ama* expression patterns in L2 leg disc and *R32D05-Gal4* expression pattern in L3 leg discs**

(A-C) Confocal optical sections of Ama::EGFP (gray) L2 leg discs immunostained for Twist (red). Ama::EGFP protein is found surrounding most of the myoblasts. **(D-F)** Confocal optical sections of Sr-Gal4>UAS-LifeactGFP (green) L2 leg discs immunostained for Nrt (magenta). Tendon precursors are not yet specified at this stage and no Nrt presence can be detected. **(G-I)** R32D05-Gal4 drives expression in all leg disc myoblasts. *Gal4* expression under the control of DNA sequences from regulatory regions of *string* gene drives the expression of *GFP* (green) that colocalizes with Twi-positive myoblasts (magenta). This expression was already observed in early L2 stage (see suppl. Fig.3).

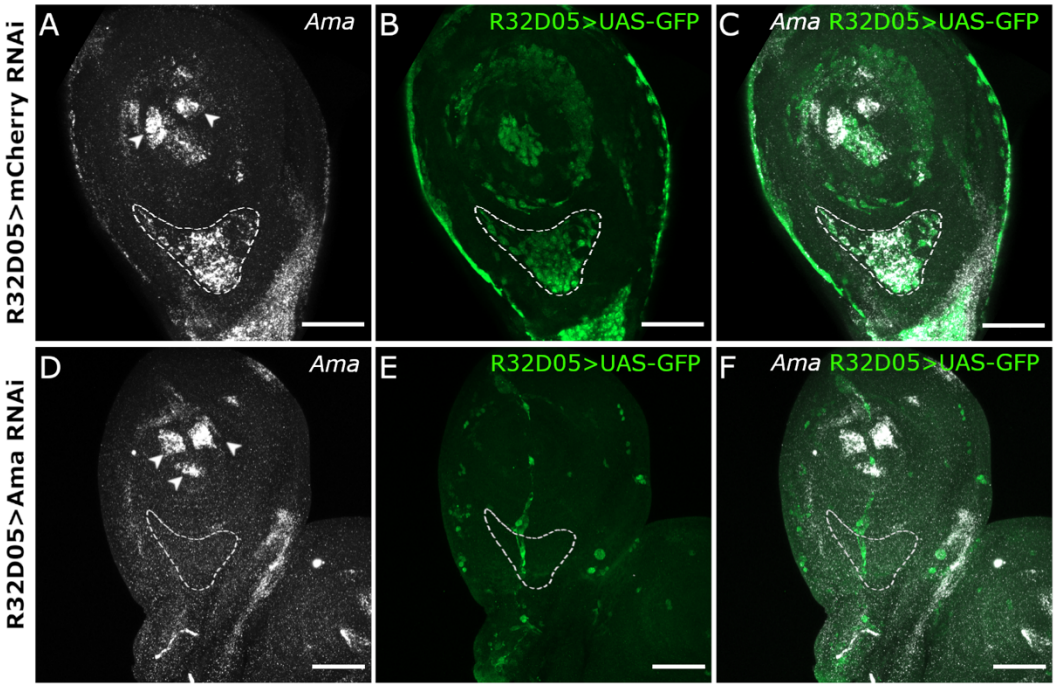
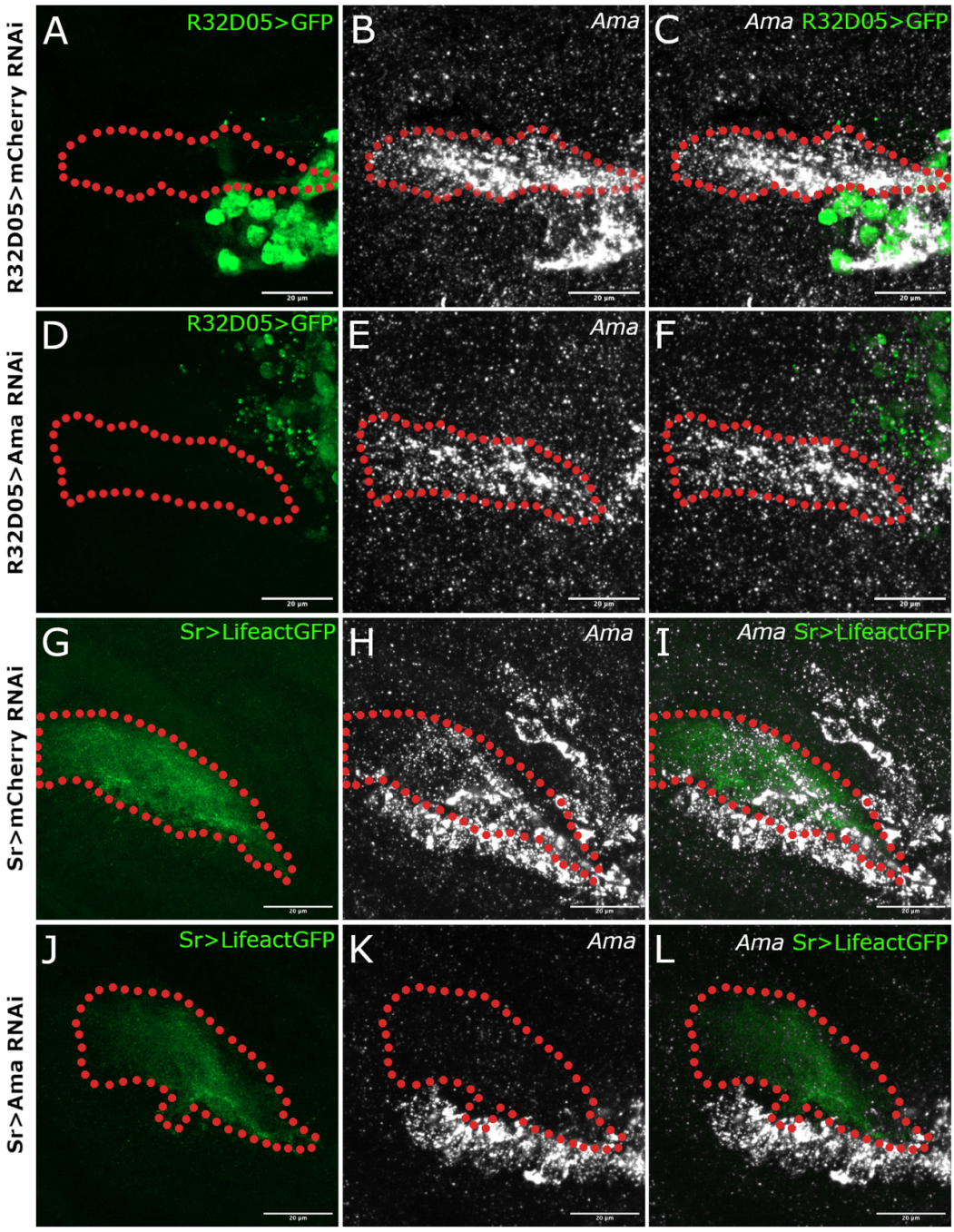


Figure 3: Myoblasts are a main source of Ama in third larval instar

Expression of *Ama* revealed by *in situ* hybridizations on L3 leg discs from R32D05-Gal4>UAS-GFP larvae. **(A-C)** In control leg disc expressing *UAS-mCherryRNAi* in the myoblasts, *Ama* mRNA localized in myoblasts (green) with a sustained expression in the dorsal femur (outlined area). Note the 2 additional sources of *Ama* mRNA in the most distal tarsus that are not GFP-positive (arrowheads in A). **(D-F)** In leg disc expressing *UAS-AmaRNAi* specifically in the myoblasts, *Ama* expression is nearly abolished in the region of the tilt in the dorsal femur (outlined area); confirming that, in this region, myoblasts are the main source of Ama transcripts at this stage. As an internal control, *Ama* remains strongly expressed in the three non-GFP clusters of the distal tarsus (arrowheads). Scale bar 50 μm.



Suppl. Figure 2: During early metamorphosis *Ama* is expressed in both myoblasts and the tilt tendon

(A-F) *in situ* hybridizations of *Ama* (gray) in dorsal femur region from R32D05-Gal4>UAS-GFP (green) 5h APF leg discs. **(A-C)** In control disc expressing *UAS-mCherryRNAi* in myoblasts, *Ama* expression is detected in both myoblasts (in green) and the elongating tilt tendon (red outlined). **(D-F)** When AmaRNAi is specifically driven in the myoblasts using R32D05-Gal4, *Ama* expression remains visible only in the elongating tendon, indicating that, at this stage, both myoblasts and the tilt express *Ama*. **(G-L)** *in situ* hybridizations of *Ama* (gray) in dorsal femur region from Sr-Gal4>UAS-LifeactGFP (green) 5h APF leg discs. **(G-I)** In control disc expressing *UAS-mCherryRNAi* in tendon cells, *Ama* expression is detected in both the elongating tilt tendon (green, red outlined) and the surrounding myoblasts. **(J-L)** When AmaRNAi is specifically driven in the tendon cells using Sr-Gal4, *Ama* expression is lost in the tilt but remains visible in the surrounding myoblasts, confirming that, at this stage, both myoblasts and the tilt express *Ama.*

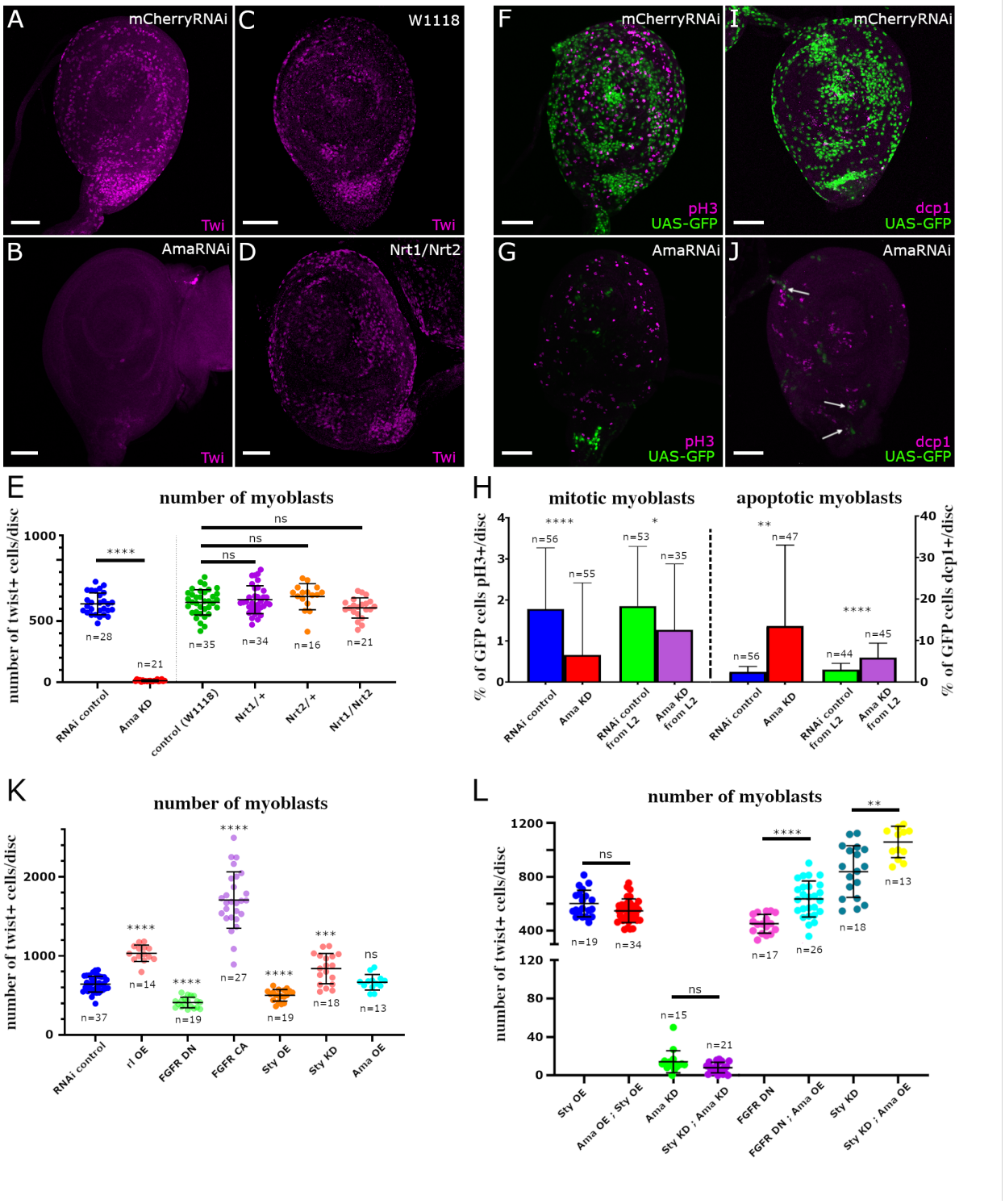
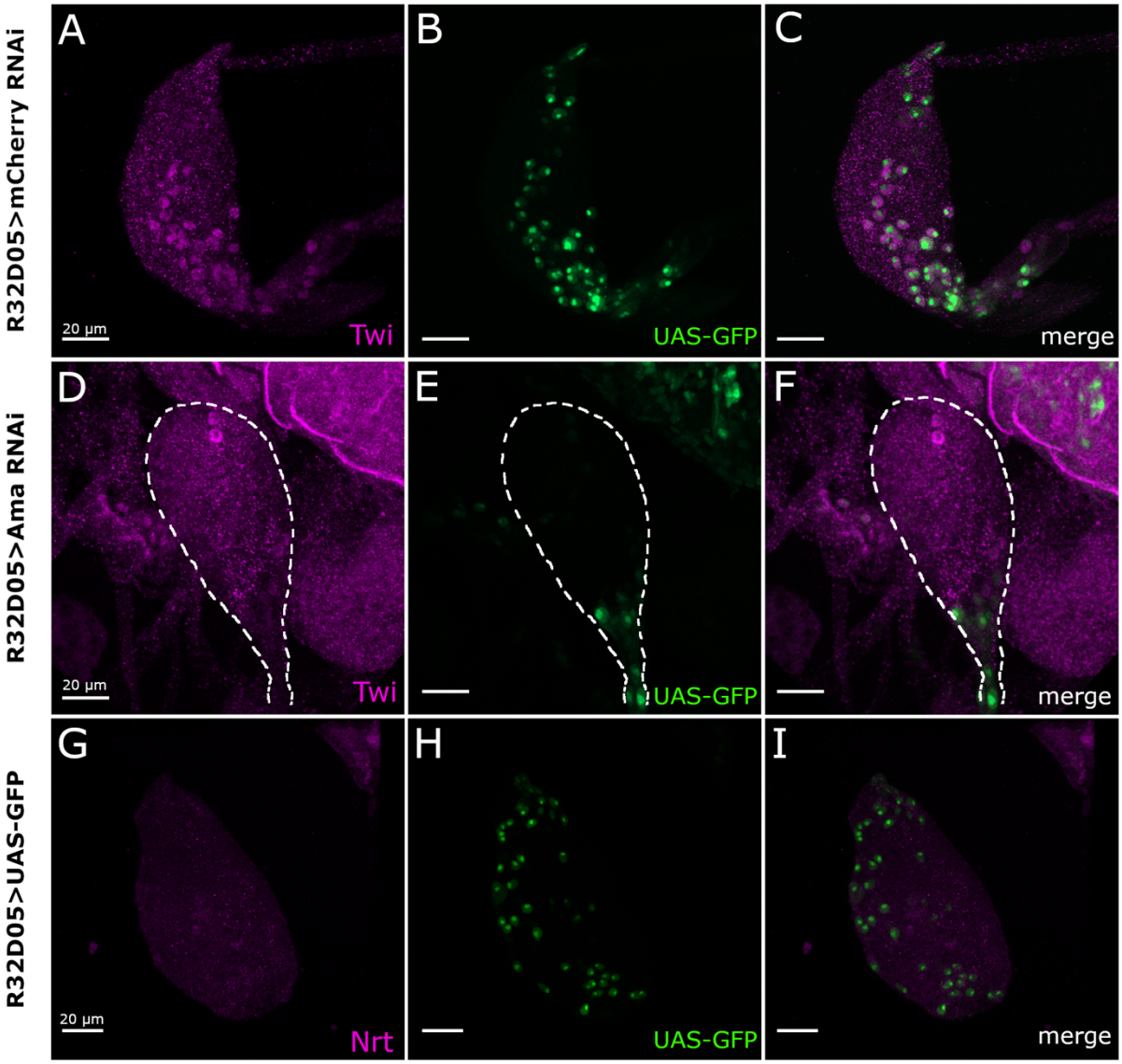


Figure 4: Ama controls the pool of myoblast number independently of Nrt

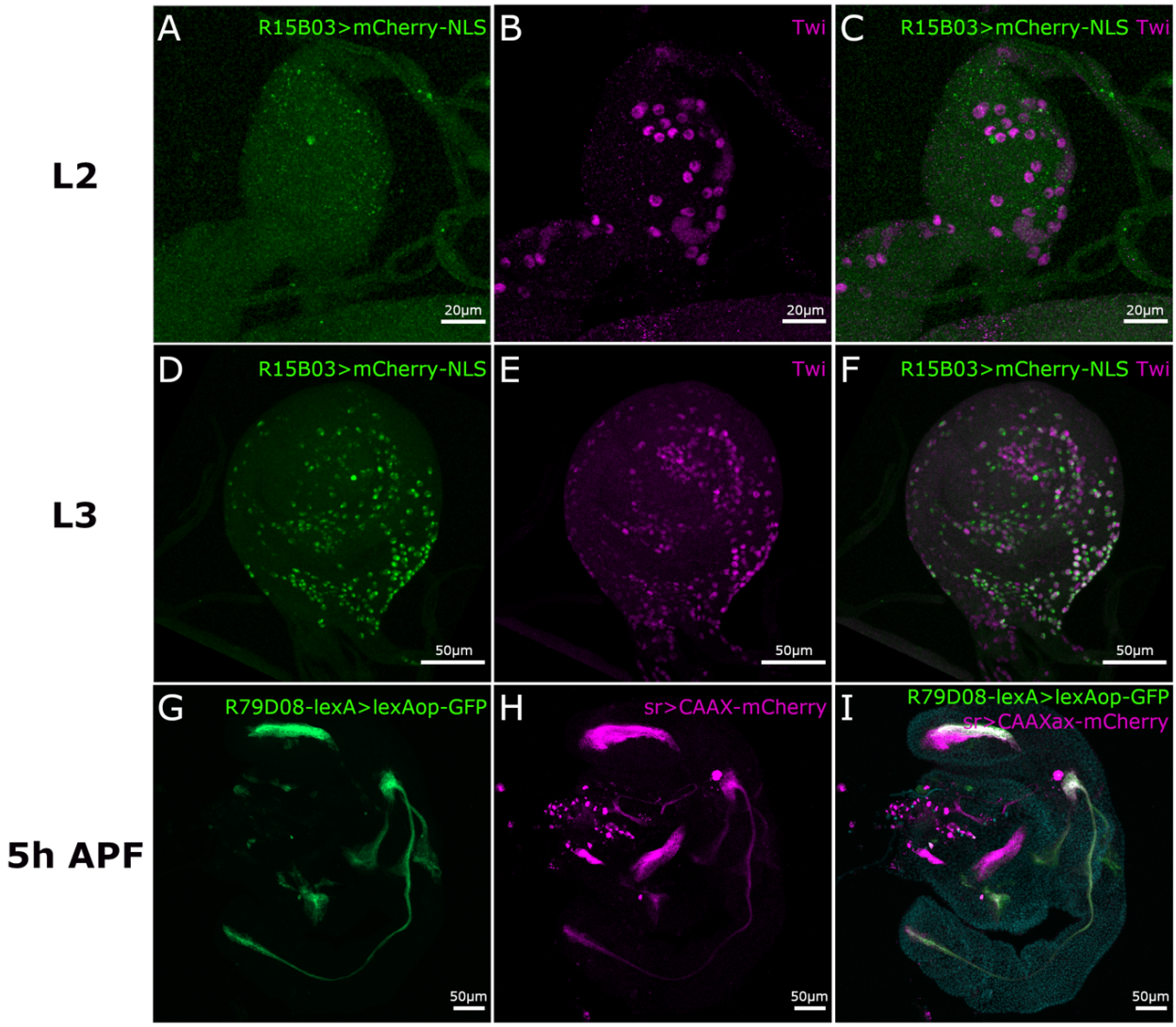
(A-D) L3 leg discs immunostained for Twi (magenta). **(A-B)** Expression of *UAS-AmaRNAi* in myoblasts using R32D05-Gal4 driver leads to strong depletion of myoblasts when compared to R32D05-Gal4>UAS-mCherryRNAi control. **(C-D)** Comparison between leg discs of W1118 control and Nrt^1/Nrt^2 transheterozygous show no difference in myoblast number. **(E)** Dot-plot graph showing the mean number of Twi-positive myoblasts per disc, in R32D05-Gal4>UAS-mCherryRNAi (control) leg disc versus R32D05-Gal4>UAS-AmaRNAi (Ama KD) leg disc; and between W1118 control leg discs *versus* Nrt^1/Nrt^+ and Nrt^2/Nrt^+ heterozygous leg discs and Nrt^1/Nrt^2 transheterozygous leg discs. **(F-G)** R32D05-Gal4>UAS-GFP (green) leg discs immunostained for pH3 (magenta); a small portion of myoblasts are proliferating in control **(F),** while only few cells remained after *UAS-AmaRNAi* expression in the myoblasts **(G)**; none

of them are pH3-positive. **(I-J)** R32D05-Gal4>UAS-GFP (green) leg discs immunostained for dcp1 (magenta); several apoptotic cells can be found amongst the GFP-positive cells remaining after *UAS-AmaRNAi* expression (arrows in J). **(H)** Graphs showing the percentage of mitotic myoblasts (on left) and the percentage of apoptotic myoblasts (on right). Compared to UAS-mCherryRNAi (control), the percentage of mitotic myoblasts is significantly reduced when *UAS-AmaRNAi* is expressed in the myoblasts from early larval stages (Ama KD) and when it is expressed from the beginning of L2 stage using Gal80^{ts} (Ama KD from L2). Compared to UAS-mCherryRNAi (control), the percentage of apoptotic myoblasts is significantly higher when *UAS-AmaRNAi* is expressed in the myoblasts from early larval stages (Ama KD) and when it is expressed from the beginning of L2 stage using Gal80^{ts} (Ama KD from L2). **(K)** Dot-plot graph showing the mean number of Twi-positive myoblasts per disc, in R32D05-Gal4 leg discs crossed with different UAS-transgenic lines affecting the FGFR pathway. Statistical analysis reveals an increase myoblast total number when overexpressing an activated ERK (rl OE), a constitutive active FGFR (FGFR OE) or when downregulating *sty* expression (Sty KD), compared to control RNAi. Inversely, overexpressing *sty* (Sty OE) or a dominant negative FGFR (FGFR DN) reduce the number of myoblasts. Note that overexpressing *Ama* (Ama OE) is not sufficient to induce an increase of myoblast number. **(L)** Dot-plot graph showing the mean number of Twi-positive myoblasts per disc in rescue experiments using R32D05-Gal4 driver. Overexpressing *UAS-Ama* together with *UAS-Sty* (Ama OE ; Sty OE) show no significant difference in myoblast number when compared to *UAS-LacZ, UAS-Sty* overexpression (Sty OE). Co-expressing *UAS-StyRNAi* with *UAS-AmaRNAi* (Sty KD ; Ama KD) is not sufficient to rescue the loss of myoblasts after *UAS-AmaRNAi* ; UAS-LacZ (Ama KD) expression. Co-expressing UAS-Ama with UAS-HtlDN (FGFR DN; Ama OE) partially rescues the decrease of myoblast number of *UAS-HtlDN ;* UAS-mCherryRNAi (FGFR DN) expression. Co-expression of *UAS-Ama* and *UAS-StyRNAi* (Sty KD ; Ama OE) together enhances the phenotype of UAS-StyRNAi (Sty KD).
In all graphs, error bars represent s.d.; *$p < 0.05$, **$p<0.01$, ***$p < 0.001$ and ****$p < 0.0001$, ns: not significant, (Mann-Whitney test). n=number of discs. Scale bars= 50µm



Suppl. Figure 3: Ama downregulation leads to myoblast depletion in second larval instar before any *Nrt* expression is observed

(A-I) Confocal optical sections of R32D05-Gal4>UAS-GFP (green) leg discs at mid L2 stage immunostained for Twi **(A-F)** and for Nrt **(G-I)**. **(A-C)** In control leg discs, R32D05-Gal4 induces *GFP* expression (Green) in all Twi-positive myoblasts (magenta). **(D-F)** *AmaRNAi* expression using R32D05-Gal4 driver leads to the loss of most of the myoblasts. **(G-H)** Nrt protein (magenta) is not detected in midL2 stage.



Suppl. Figure 4: Expression patterns of R15B03-Gal4 and R79D08-lexA drivers
(A-F) R15B03-Gal4>UAS-mCherryNLS (green) leg disc immunostained for Twi (magenta). **(A-C)** L2 leg disc shows no *mCherryNLS* expression in Twi-positive myoblasts, indicating that R15B03-Gal4 driver is not expressed in myoblasts at this stage. **(D-F)** During L3 stage, *mCherryNLS* starts to be expressed in Twi-positive myoblasts as well as in few other cells. **(G-H)** 5h APF R79D08-lexA>lexAop-mCD8::GFP; Sr-Gal4>UAS-CAAXmCherry leg discs, R79D08--lexA driving lexAop-GFP allows the visualization of developing tendons identified through the expression of the well characterized *Sr-Gal4* driver expression (magenta).

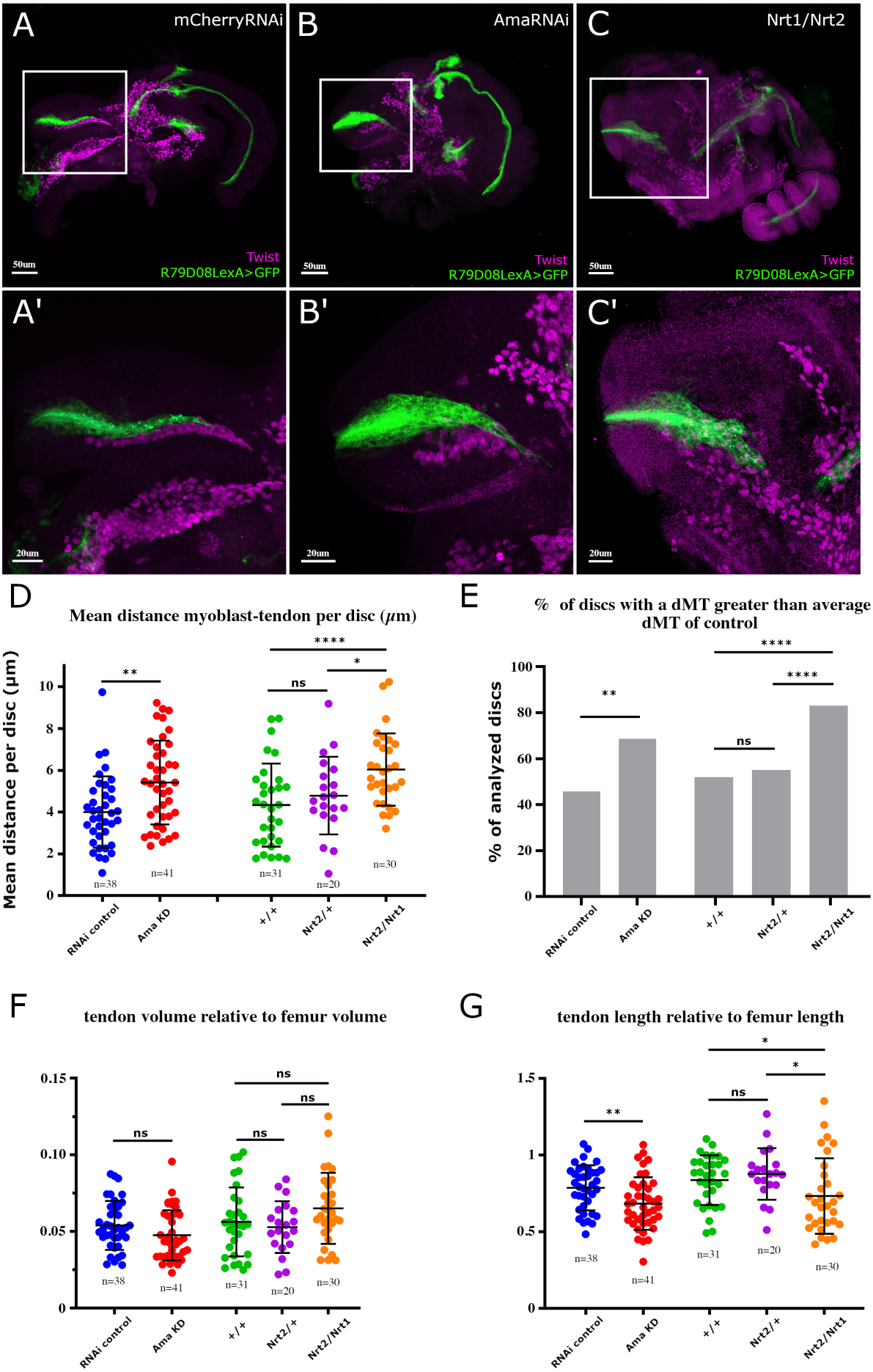

A
mCherryRNAi
Twist
R79D08LexA>GFP
50um
B
AmaRNAi
Twist
R79D08LexA>GFP
50um
C
Nrt1/Nrt2
Twist
R79D08LexA>GFP
50um
A'
20um
B'
20um
C'
20um
D
Mean distance myoblast-tendon per disc (μm)
Mean distance per disc (μm)
**
ns
****
*
n=38
n=41
n=31
n=20
n=30
RNAi control
Ama KD
+/+
Nrt2/+
Nrt2/Nrt1
E
% of discs with a dMT greater than average dMT of control
% of analyzed discs
**
ns
****
****
F
tendon volume relative to femur volume
ns
ns
ns
ns
G
tendon length relative to femur length
**
ns
*
*

Figure 5: Myoblast positioning and tendon morphogenesis require Ama and Nrt

(A-C) Confocal optical sections of 5 h APF leg disc immunostained for Twi from **(A)** R79D08-lexA>lexAop-mCD8::GFP; R15B04-Gal4>UAS-mCherryRNAi, from **(B)** R79D08-lexA>lexAop-mCD8::GFP; R15B04-Gal4>UAS-AmaRNAi and from **(C)** R79D08-lexA>lexAop-mCD8::GFP; Nrt^1/Nrt^2 transheterozygous. **(A'-C')** Higher magnifications of the dorsal femur regions from (A), (B) and (C), respectively. **(A, A')** In control leg discs expressing *UAS-mCherryRNAi* using R15B03-Gal4 late myoblast driver, the tilt (green) has elongated within the dorsal femur cavity to form a long internal structure along which the myoblasts (magenta) are aligned. **(B, B')** When *UAS-AmaRNAi* is expressed in the myoblasts, they lose their adhesion with the tilt; the tilt itself appears wider and shorter compared to control. **(C, C')** The same observations can be made in Nrt^1/Nrt^2 transheterozygous leg discs, with a misdistribution of the myoblasts along the tilt and an elongation default of the tilt. **(D)** Dot-plot graph showing the mean distance between myoblasts and the tilt surface; each dot corresponds to the mean distance between the tilt and all the myoblasts for one disc. The average of mean distance for R79D08-lexA>lexAop-mCD8::GFP; R15B04-Gal4>UAS-AmaRNAi (Ama KD) leg discs is statistically higher compared to R79D08-lexA>lexAop-mCD8::GFP; R15B04-Gal4>UAS-mCherryRNAi (control RNAi) leg disc ($p<0.0016$). This average is also higher when comparing R79D08-lexA>lexAop-mCD8::GFP; Nrt^1/Nrt^2 transheterozygous leg discs with both R79D08-lexA>lexAop-mCD8::GFP (+/+) ($p<0.0008$) and R79D08-lexA>lexAop-mCD8::GFP; Nrt^2/Nrt^+ ($p<0.0201$) control leg discs. **(E)** Graphs showing the percentage of discs for which the mean distance between the myoblasts and the tilt is higher than the average mean distance of the controls UAS-mCherryRNAi and +/+ respectively. **(F)** Dot-plot graph showing the volume of the tilt, each dot corresponds to one disc. No statistically significant difference is observed between the control lines and the lines expressing *AmaRNAi*, as well as the mutant lines for Nrt **(G)** Dot-plot graph showing the lengths of the tilt, each dot corresponds to one disc. *UAS-AmaRNAi* (Ama KD) myoblast late expression leads to shortening of the tilt length compared to UAS-mCherryRNAi control ($p<0,0042$). This shortening is also evident in transheterozygous mutants Nrt^1/Nrt^2 compared to wild-type Nrt homozygous ($p<0,025$) and Nrt^2/ Nrt^+ ($p<0,011$).

In all graphs, error bars represent s.d.; $*p < 0.05$, $**p<0.01$, $***p < 0.001$ and $****p < 0.0001$ ns: not significant, Mann-Whitney test (D) and (G), Fisher's exact test (E). n=number of discs.