**Supplementary Materials and Methods**

**Fly husbandry**

Fly stocks were maintained using standard culture conditions. Experimental crosses were grown at 270 C. Muscle-specific expression was achieved with *Mef2*-GAL41. GFP labeling was from *UAS*-CD8::GFP2 or *UAS*-GFP::Gma3. Controls include w1118 or *Mef2*-GAL4 x w1118. *UAS-bru1IR* was previously published as *aret-IR*4. *Salm-/-* is the *salmFRT* allele used in trans to the *salm1* null allele*1*4. *Bru1M2* and *Bru1M3* are CRISPR-generated deletion alleles using the Zhang *et al*. approach5 that delete the C-terminal coding region of Bruno1, including all of RRM3 that is required for RNA binding. More details are available upon request. *weeP26* is a GFP trap line in *Mhc*6.

**Immunofluorescence and microscopy**

Male flies were dissected at the specified timepoints as previously described7. For 24 h APF, 32 h APF and 48 h APF, pupae were dissected via the open-book method7, then fixed for 20 min. in 4% PFA in .05% PBS-Triton-X100. For 72 h APF, 90 h APF and 1 d adult, abdomens were removed, flies were fixed for 15 min. in 4% PFA in .05% PBS-T and then thoraxes were cut longitudinally with a microtome blade. All samples were blocked for at least 1 hour at RT in 5% normal goat serum in PBS-T. Samples were stained with rhodamine-phalloidin and GFP detection reagent at 1:500. After washing 3x in 0.5% PBS-T, samples were mounted in slide mounting buffer containing DAPI. Images were acquired via confocal microscopy (see **Table of Materials**). For whole pupal timecourse and dissection images, GFP fluorescence was imaged live on a fluorescent dissecting microscope (see **Table of Materials**). Additional protocol images were acquired with a cell phone camera (see **Table of Materials**). Images were further processed with Fiji (Image J) and image processing software (see **Table of Materials**). GFP intensity images (Figures 2 & 3) were generated using the “Fire” LUT in Image J.

**RNA isolation**

For timepoints <48 h APF, samples are dissected in black dissecting dishes. The dissections are also possible in transparent glass dissection dishes on a black background, and presumably in any type of concave-shaped dissection dish. With clear glass, there tends to be more diffuse fluorescence. It is also possible to dissect on black silicon dishes (see **Table of Materials**). We prefer a concave surface, however, as tissue does not stay as nicely in place on a flat surface when dissecting under buffer.

IFM dissection was performed in chilled 1x PBS (without Triton-X100 detergent) according to the protocol presented in the main text. We avoid the use of detergent in the dissection buffer to limit the damage to IFM membranes, keep the IFMs alive longer and avoid RNA degradation associated with muscle cell death. Dissection time was limited to 20-30 minutes, because after this time we noticed increasing levels of RNA degradation from RNA integrity traces. IFM samples were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 3-5 min at 2,000 x g. Buffer was completely removed. For standard RNA isolation (Method 1), IFM pellet was resuspended in 50-100 μL of reagent. Samples were immediately frozen on dry ice and stored at -800 C until further processing.

For estimation of the total RNA recovered from the IFMs per 1 d adult fly (**Supplemental** **Figure 1A**), IFM was dissected from 50 1d adult male flies by three different researchers. We additionally performed dissections from 10, 25 and 50 flies, and observed similar yields per fly but increasing total RNA concentrations isolated (as expected). The average yield was calculated plus or minus SEM, as measured by Approach 3. For estimation of the total RNA from IFMs per fly at 30 h APF, 48 h APF, 72 h APF and 1 d adult (**Supplemental** **Figure 1B**), the same researcher dissected 50 flies for each timepoint and performed 2 biological replicates. All samples were isolated with Method 1 (see below), and RNA concentrations measured by Approach 3 (see **Table of Materials**). In the main text (**Figure 4F, G**), we compiled data dissected by 5 researchers over 9 years and isolated using Method 1 (see **Table of Materials**). The data reflect RNA concentration measurements by Approach 1, 2 and 3.

To test the importance of working RNase free, we performed several control experiments. We isolated total RNA from 10 adult male *w1118* flies using Method 1. We compared RNA integrity traces (from Approach 1) and RNA concentrations of an RNA sample freshly isolated using Method 1 to samples that underwent multiple rounds of freeze-thaw (1x, 2x, 3x, 10x, 15x, 25x; freezing on dry ice and thawing on ice), to samples left on the bench (1 h, 4 h, overnight) and RNA samples treated with RNase A (5 min., 15 min., 30 min., 1 hour, .5 μL, .05 μL, .005 μL, .0005 μL). RNase A was kept as a 4 mg/mL stock. With RNase treatment, we generated either flat RNA integrity profiles (indicative of complete degradation) or profiles showing degradation (loss of ribosomal bands and strong signal biased towards the lower nt range). With all other treatments we were not able to discern any differences in RNA integrity profiles. We interpret this result to mean that while working on ice and avoiding freeze-thaw cycles is of course recommended, RNA in a clean prep is relatively stable. However, even miniscule amounts of RNase will rapidly destroy an RNA sample.

*Standard RNA isolation protocol (Method 1)*

Following our published RNA isolation protocol4, IFM pellets were thawed, homogenized in isolation reagent (Method 1, see **Table of Materials**) with a blue pestle, combined and adjusted to a final volume of 500 μL isolation reagent. Samples were mixed well and incubated for 5 min at room temperature. 100 μL of chloroform was added and the samples were shook vigorously for 15 seconds, followed by a 3 minute incubation at room temperature. Samples were centrifuged for 15 min. at 12,000 x g at 40 C. The upper RNA-containing phase was carefully removed (~200 μL total) and placed in a new RNase-free microcentrifuge tube. To increase RNA yield, 1 μL of 20 μg/ μL glycogen was added to all samples and mixed well. Total RNA was precipitated by addition of 250 μL of isopropanol, incubated on dry ice for 10 minutes and then centrifuged at 12,000 x g for 15 minutes at 40 C. It is important to use fresh isopropanol in this step (that is RNase-free and has not otherwise changed concentration), or RNA will not effectively precipitate. The resulting RNA-DNA pellets were washed using 750 μL of 75% Ethanol (in RNase-free water, see **Table of Materials**), centrifuged at 8,000 x g for 10 minutes at 40 C and stored at -800 C as pellets. This allowed us to resuspend the RNA from all samples on the same day for RNA concentration and quality measurements. After removal of 75% Ethanol-RNase-free water, pellets were air dried for approximately 10 minutes and RNA was dissolved in a final volume of 30 μL with RNase-free water preheated to 550 C.

*RNA isolation using commercial kits (Methods 2-6,* see **Table of Materials**)

In **Figure 4G**, we compile data from 3 researchers dissected over 9 years and isolated according to the manufacturers protocol (as described below) for 6 different methods, notably multiple commercial RNA isolation kits. Data represent RNA measurements by Approach 1, 2 and 3. To compare different RNA extraction methods in a more controlled manner (**Supplemental** **Figure 1C**), a single researcher dissected 10 different samples and we show Approach 1 measurements of RNA concentration. Each sample consisted of IFM dissected from 50 1d adult male flies. This allowed us to test 6 extraction methods in duplicate (*i.e.* 2 biological replicates for each method). For all 6 methods, after dissection and removal of PBS, IFM pellets were frozen “dry” (*i.e.* without resuspending in any buffer) on dry ice and stored dry at -800 C until further use. Two of these “dry-frozen” samples were processed as above using Method 1, which we coded Method 1 (dry). After removing aliquots for RNA concentration and integrity measurements, they were further treated with a commercial kit to remove DNA, which we coded Method 2.

The remaining 8 samples were processed according to the relevant manufacturer protocol. For Method 3, dry frozen pellets were re-suspended in 600 μl of RNA isolation buffer from Method 1. The recommended DNase treatment was done using the DNase and reaction buffer from Method 6. For Method 4-6, we used the DNase treatments provided by the respective kits. When using Method 4, IFM pellets were homogenized in 350 μL of buffer as suggested for animal tissues <20mg. For Method 5, we applied the protocol for fibrous tissues. The dry IFM pellets were homogenized in 250 μL of isolation buffer as suggested for RNA samples <5 mg. Using Method 6, we applied the protocol for tissues and homogenized IFM pellets in 300 μL of protection reagent as suggested for samples up to 10 mg. Note that the final volume of all total RNA samples after elution was 30 μL. After elution, all samples were stored at -800C and the RNA quality and quantity was measured on the next day.

**Measurement of total RNA concentrations and quality**

For **Supplemental Figure 1**, all measurements were performed on the same day. Note that multiple devices are commercially available to measure RNA concentrations accurately from small samples, of which we used three different devices (Approach 1, 2 and 3, see **Table of Materials**). Concentrations from Approach 3 are shown in **Supplemental Figure 1A-C**. Total RNA samples were measured 3x 1 μl using Approach 2 to provide higher accuracy. Similarly, 3 μl of each total RNA sample were used for analysis via Approach 3 using Component A and Component B as controls. The total RNA quantity and quality were also assessed by Approach 1 using pico chips. For better resolution, the stock samples were diluted either 1:50 or 1:100 in TE buffer prior to loading on the chips. Data used to generate the plots in **Figure 4 F and G** are a compilation of measurements obtained by Approach 1, 2 or 3. Each dissected sample is represented by a single dot, but given the timeframe and number of researchers involved, it was not possible to use RNA concentration measurements from a single instrument. Data were analyzed with statistical software (see **Table of Materials**).

**Reverse Transcription**

To test the sensitivity of different reverse transcription (RT) kits (**Figure 4K**), we used RNA obtain via Method 6. RT reactions were performed simultaneously using RT Kits 1, 2 and 3 (see **Table of Materials**). In each case, the direct instructions from the respective manufacturer were applied. The resulting cDNA samples were used for the RT-PCR reaction with primers against flight muscle specific splice factor Bruno1 (*bru1*) (F: AGCCTGCCGAATAGTCCC; R: CTTTCAGGGCGGCGTGTC) and Ribosomal protein 49 (*rp49*) (F: GGTATCGACAACAGAGTGCG; R: GAACTTCTTGAATCCGGTGGG) in the same PCR reaction. cDNA was amplified using polymerase for 35 cycles. 2 μL of cDNA was used in each reaction. Band intensity was quantified in image processing software and is presented as total intensity of the *bru1* band divided by the total intensity of the *rp49* band. Intensity of the *rp49* band is comparable between RT Kit #1 and #2. Significance was tested in statistical software using an Unpaired, 2-tailed t-test.

To show the developmental switch in Mhc splicing in wild-type IFM in comparison to leg muscle and IFM from Bruno1 mutant (**Figure 5D**), we used the samples dissected above from *Mef2*-GAL4, *UAS-*mCD8::GFP flies at 30 h APF, 48 h APF, 72 h APF and 1 d adult. We dissected IFMs from 30 1 d adult *bru1M3* mutantsand collected legs from 30 1 day w1118 wild-type flies (see description of dissection below). All RNA samples were isolated with Method 1. The *Mhc* alternative splice events were detected using primer sets previously described for *Mhc* exons 17-198 (here referred to by current annotation as exons 35-38, see below). The cDNA was amplified using a high fidelity polymerase and buffer (see **Table of Materials**) for 30 cycles.

**Mapping of *weeP26***

To verify the insertion location of the *weeP26* GFP trap line previously characterized to be inserted somewhere between exons 18 and 19 of *Mhc*6,8, we performed Splinkerette PCR9. This allowed us to map the insertion point to within 5 nucleotides: **TTCAGGGAGTGCTAGTCGTACTCGTTAT.** The sequences we obtained from the upstream and downstream Splinkerette PCRs are highlighted yellow, implying that the insertion is between any two of the nucleotides in red. Indeed, the P-element does map to the last intron of *Mhc* as reported, but new annotations of the genome attribute many more exons to *Mhc* and former exon 18 consists of two exons based on more detailed splicing knowledge. Thus, we refer to *Mhc* exons by their up-to-date numbering as follows: new [old]; Exon 34 [Exon 17]; Exon 35 & 36 [Exon 18]; Exon 37 [Exon 19]. Even with the updated annotation, there is only a single Mhc isoform (Exon 34-37), where the GFP is spliced into an ORF and actually translated into protein, matching the interpretations of previous studies8.

**Dissection of leg and jump muscle (TDT) samples**

We provide an estimate of total RNA recovered per fly for leg and jump muscle dissections in **Supplemental Figure 1D**. Whole legs were used for RNA isolation for mRNA-Seq (late pupae and adults, 100 flies; young pupae, 150-200 flies) and RT-PCR (30 flies). Legs were cut from the thorax using fine scissors. For pupal timepoints, the pupa was removed from the pupal case as described in Figure 3. The pupal cuticle was slit open ventrally using the tip of a #5 forceps and the legs were cut from the thorax using fine scissors. Legs trapped in the pupal cuticle were pulled free using the forceps. Legs were transferred to 1x PBS in a 1.5 mL microcentrifuge tube and briefly centrifuged at 2,000 x g in a microcentrifuge. Buffer was removed using a 200 µL pipette tip. Note that legs mostly float on top of the buffer, so care must be taken that they stick to the wall of the microcentrifuge tube instead of the pipette tip when removing the PBS. Legs were resuspended in 30 µL RNA isolation reagent (Method 1) and ground with a blue pestle. 70 µL RNA isolation reagent (Method 1) was added to bring the samples to 100 µL. Samples were then processed as above for Method 1.

For mRNA-Seq experiments, jump muscle was dissected from 150-180 1 d adults. Adult flies were dissected according to the protocol in the text through Step 3.7, resulting in a pile of thorax hemisections. To isolate the jump muscle, make a single cut with fine scissors in the dorsal-ventral orientation in the middle of the dorsal longitudinal IFMs. This cut allows access to the jump muscle behind the IFMs. The jump muscle attaches just above the second leg in the ventral part of the thorax, and along the dorsal part of the thorax behind the IFMs, so is orientated like the dorsal-ventral IFMs (by contrast to the dorsal-longitudinal IFMs that run the length of the thorax from anterior to posterior). Then cut in the anterior-posterior plane (with the tips of the fine scissors inserted nearly to the cuticle) once at the top (dorsal part) of the thorax and once at the bottom (ventral part) above the second leg. The jump muscle can then be removed from the thorax using a #5 forceps. This dissection is difficult, as it is performed without seeing the jump muscle but knowing its location, and requires extensive training. An alternative (but slower) approach is to proceed through Step 3.9 in the protocol, in other words to completely remove the IFMs to gain access to the jump muscle below. Then use a cut in the anterior-posterior plane at the top (dorsal) of the thorax and a cut at the bottom (ventral) of the thorax above the second leg to release the now-visible jump muscle from its attachments. The jump muscle can then be removed from the thorax using a #5 forceps. Practicing with a fluorescent label, for example *Act79B*-GAL4 or *Mef2*-GAL4 driving a GFP reporter, can help visualize the jump muscle to learn its structure and attachment sites. Dissected jump muscles were transferred to a 1.5 mL microcentrifuge tube and then processed as above for Method 1.

**mRNA-Seq**

mRNA-sequencing data used in this manuscript have been published previously4,10 and are publicly available in NCBI’s Gene Expression Omnibus (GEO) under accession numbers GSE107247 and GSE63707. Data were mapped to the *Drosophila* genome (BDGP6.80 from ENSEMBL). The raw data processing pipeline is available from Spletter *et al*.10.

Here we performed a differential expression analysis in R either at the gene unit level (DESeq2) or at the exon level (DEXSeq) to detect differences in splicing between *bru-IR* and wildtype IFM at 72 h APF. Data were plotted in R using ggplot211. Data for sarcomere proteins at 72 h APF are available in **Supplementary Table 1**. Adult data analysis and tables are available from Spletter *et al.*4Read counts were visualized using the UCSC Genome Browser. Splice junction counts were obtained from .bed files generated in the mapping pipeline. For our analysis of the indicated genotypes and timepoints, we first determined the total number of splice junctions using the *Mhc* Exon 34 donor. We then determined the percent of the total events corresponding to Exon 34-35, Exon 34-36 and Exon 34-37 junctions. Our data supports the Flybase annotation of three distinct splice events using the Exon 34 donor.

**Mass spectrometry**

We performed whole proteome mass spectrometry on 4 biological replicates for IFMs dissected from w1118 and *bru1M2* 1 d adults. Samples consisted of 30 flies per replicate and were prepared using a peptide preparation kit (see **Table of Materials**) following the manufacturer protocol. Label-free mass spectrometry was performed on a mass spectrometer (see **Table of Materials**). Peptides were identified using Max Quant and then analyzed using Perseus and R. Data were filtered requiring 3 of 4 replicates in one sample to have a value, and missing intensities were replaced by a single low value (intensity=20), as in multiple cases peptides are completely missing in one sample and present in the other, and are thus biologically relevant. For volcano plots we used an FDR=.05. Sarcomere proteins were identified using a previously compiled list10. Data are available in **Supplementary Table 2**.

**References**

1. Ranganayakulu, G., Schulz, R. A. & Olson, E. N. Wingless signaling induces nautilus expression in the ventral mesoderm of the Drosophila embryo. *Developmental biology* **176** (1), 143–148, doi:10.1006/dbio.1996.9987 (1996).

2. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. *Trends in neurosciences* **24** (5), 251–254 (2001).

3. Dutta, D., Bloor, J. W., Ruiz-Gómez, M., VijayRaghavan, K. & Kiehart, D. P. Real-time imaging of morphogenetic movements in Drosophila using Gal4-UAS-driven expression of GFP fused to the actin-binding domain of moesin. *Genesis (New York, NY : 2000)* **34** (1-2), 146–151, doi:10.1002/gene.10113 (2002).

4. Spletter, M. L., Barz, C., *et al.* The RNA-binding protein Arrest (Bruno) regulates alternative splicing to enable myofibril maturation in Drosophila flight muscle. *EMBO reports* **16** (2), 178–191, doi:10.15252/embr.201439791 (2015).

5. Zhang, X., Koolhaas, W. H. & Schnorrer, F. A versatile two-step CRISPR- and RMCE-based strategy for efficient genome engineering in Drosophila. *G3 (Bethesda, Md.)* **4** (12), 2409–2418, doi:10.1534/g3.114.013979 (2014).

6. Clyne, P. J., Brotman, J. S., Sweeney, S. T. & Davis, G. Green fluorescent protein tagging Drosophila proteins at their native genomic loci with small P elements. *Genetics* **165** (3), 1433–1441 (2003).

7. Weitkunat, M. & Schnorrer, F. A guide to study Drosophila muscle biology. *Methods (San Diego, Calif.)* **68** (1), 2–14, doi:10.1016/j.ymeth.2014.02.037 (2014).

8. Orfanos, Z. & Sparrow, J. C. Myosin isoform switching during assembly of the Drosophila flight muscle thick filament lattice. *Journal of cell science* **126** (1), 139–148, doi:10.1242/jcs.110361 (2013).

9. Potter, C. J. & Luo, L. Splinkerette PCR for mapping transposable elements in Drosophila. *PloS one* **5** (4), e10168, doi:10.1371/journal.pone.0010168 (2010).

10. Spletter, M. L., Barz, C., *et al.* A transcriptomics resource reveals a transcriptional transition during ordered sarcomere morphogenesis in flight muscle. *eLife* **7**, 1361, doi:10.7554/eLife.34058 (2018).

11. Wickham, H. ggplot2. *Wiley Interdisciplinary Reviews: Computational Statistics* **3** (2), 180–185, doi:10.1002/wics.147 (2011).