

ARTICLE

Postsynaptic BMP signaling regulates myonuclear properties in *Drosophila* larval muscles

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The syncytial mammalian muscle fiber contains a heterogeneous population of (myo)nuclei. At the neuromuscular junction (NMJ), myonuclei have specialized positioning and gene expression. However, it remains unclear how myonuclei are recruited and what regulates myonuclear output at the NMJ. Here, we identify specific properties of myonuclei located near the *Drosophila* larval NMJ. These synaptic myonuclei have increased size in relation to their surrounding cytoplasmic domain (size scaling), increased DNA content (ploidy), and increased levels of transcription factor pMad, a readout for BMP signaling activity. Our genetic manipulations show that local BMP signaling affects muscle size, nuclear size, ploidy, and NMJ size and function. In support, RNA sequencing analysis reveals that pMad regulates genes involved in muscle growth, ploidy (i.e., *E2f1*), and neurotransmission. Our data suggest that muscle BMP signaling instructs synaptic myonuclear output that positively shapes the NMJ synapse. This study deepens our understanding of how myonuclear heterogeneity supports local signaling demands to fine tune cellular function and NMJ activity.

Introduction

Skeletal muscle cells (myofibers) contain hundreds of post-mitotic (myo)nuclei that exhibit heterogeneity despite sharing one cytoplasm. Single nuclear sequencing in mammalian muscle revealed that myonuclei have distinct transcriptional profiles based on their intracellular position (Dos Santos et al., 2020; Kim et al., 2020; Petrany et al., 2020). For example, myonuclei adjacent to the neuromuscular junction (NMJ) express genes specifically required for the myofiber's postsynaptic structures. These intracellular differences are consistent with the myonuclear domain hypothesis that considers the limited synthetic capacity of individual myonuclei and the physical constraints on cellular transport and diffusion (Hall and Ralston, 1989; Pavlath et al., 1989). It proposes that each nucleus within the syncytial myofiber supplies gene products only to its immediately surrounding cytoplasm (myonuclear domain). Thus, myonuclei are spaced throughout the myofiber to allow for the efficient distribution of gene products for muscle homeostasis and function. In contrast, myonuclei adjacent to the NMJ are clustered, which locally increases nuclear size scaling (relationship between myonuclear size and surrounding cytoplasmic domain size) and DNA content (Sanes and Lichtman, 2001), resulting in local enrichment of NMJ components at the postsynaptic membrane (Duclert and Changeux, 1995; Schaeffer et al., 2001). An open question is

how synaptic myonuclei are specified and their activity programmed to provide gene products for effective synaptic transmission.

The NMJ is a specialized synapse composed of the myofiber's postsynaptic structures in opposition to the motor neuron's presynaptic axonal endings (boutons). The *Drosophila melanogaster* larva provides an easily accessible and highly conserved NMJ, ideal for studying synaptic development and function through genetic manipulations and visualization (Broadie and Bate, 1995; Deng et al., 2017). While presynaptic bouton formation and maturation have been thoroughly examined (reviewed in Chou et al. [2020]), the postsynapse is far less studied. The *Drosophila* postsynapse consists of complex membrane folds (subs synaptic reticulum, SSR) that increase muscle surface area. Membrane receptors within the SSR, including glutamatergic neurotransmitter receptors, participate in synaptic signaling, which leads to muscle contraction (DiAntonio et al., 1999; Nguyen and Stewart, 2016; Zou and Pan, 2022). The SSR develops following new bouton formation (Vasin et al., 2019), and critical regulators of SSR development include scaffolding proteins (Lahey et al., 1994), adhesion proteins (Banovic et al., 2010; Xing et al., 2018), and actin modulators (Blunk et al., 2014; Christophers et al., 2024). Like at the vertebrate postsynapse,

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Drosophila myonuclei can support SSR development. For example, work on synapse-to-myonucleus signaling revealed that the Frizzled nuclear import pathway promotes SSR maturation, but the mechanism is not fully understood (Restrepo et al., 2022; Speese et al., 2012). However, it is unknown whether all *Drosophila* myonuclei are equally affected by NMJ-derived signals and/or establish transcriptional heterogeneity. Previous work from our lab and others has identified differences in myonuclear position, size scaling, and DNA ploidy in the center of the muscle cell near the larval NMJ (Perillo and Folker, 2018; Windner et al., 2019). Nevertheless, *Drosophila* synaptic myonuclei remain uncharacterized, providing an opportunity to advance our understanding of postsynaptic adaptations and investigate whether local myonuclear changes are a conserved muscle feature.

Bone morphogenetic protein (BMP) ligands are critical signaling proteins at both the mammalian and *Drosophila* NMJ (Bayat et al., 2011; Fish et al., 2023, Preprint; Yilmaz et al., 2016). In *Drosophila*, a retrograde muscle-to-motor neuron BMP pathway that promotes NMJ growth has been extensively studied (Aberle et al., 2002; Marqués et al., 2002; McCabe et al., 2003). The BMP ligand, Glass bottom boat (Gbb), is secreted by the postsynaptic muscle and activates the presynaptic neuronal BMP receptors. This leads to phosphorylation and nuclear import of the transcriptional effector Mad (pMad), which regulates the expression of key presynaptic NMJ genes (Ball et al., 2010; Kim and Marqués, 2010; Smith et al., 2012). Studies in BMP signaling mutants revealed that loss of BMP signaling results in reduced bouton number and impaired NMJ synaptic transmission, specifically reduced release of neurotransmitters, and a diminished postsynaptic excitatory response (Aberle et al., 2002; Marqués et al., 2002; McCabe et al., 2003). These effects are attributed to disrupting the retrograde pathway because transgene expression of BMP effectors specifically in the motor neuron, but not in the muscle cell, rescued these mutant NMJ defects (Aberle et al., 2002; McCabe et al., 2004).

However, some data suggest that postsynaptic BMP signaling contributes to NMJ development and muscle growth. BMP signaling mutants have smaller muscles, which are rescued to normal size by muscle-specific transgene expression and only partially rescued by neuronal expression (McCabe et al., 2004). The muscle expresses BMP receptors and Mad, which localize to the postsynaptic membrane, and the active form of Mad (pMad) is reduced at the NMJ following muscle-specific Mad knock-down (KD) (Dudu et al., 2006; Fuentes-Medel et al., 2012). Like the retrograde pathway, manipulations of postsynaptic BMP signaling affect NMJ structure and neurotransmission (Fuentes-Medel et al., 2012; Rawson et al., 2003; Sulkowski et al., 2016). However, it remains to be investigated whether postsynaptic BMP signaling affects the morphology, position, and transcriptional output of myonuclei.

In this study, we defined synaptic myonuclei in *Drosophila* larval muscles and found that they have increased size and DNA content, and are enriched in pMad, the active BMP transcription factor. We investigate the effects of postsynaptic BMP signaling on synaptic myonuclei by muscle-specific KD of the core BMP signaling components: the type II receptor Punt (*put*), the type I receptor thickveins, and the transcription factor Mad. We show

that postsynaptic BMP signaling perturbations affect muscle size, nuclear size, and DNA ploidy. RNA sequencing confirms that Mad KD reduces mRNA levels of the critical endoreplication G1/S regulator *E2f1*, suggesting that postsynaptic BMP signaling promotes endoreplication. Mad KD also disrupts the expression of glutamate receptor subunits, NMJ structure and function, and larval locomotion. We propose that local BMP signaling at the postsynapse exposes synaptic myonuclei to higher levels of pMad, which locally increases nuclear DNA copy numbers and expression of genes that promote synaptic function. This study provides novel insights to the mechanisms regulating myonuclear heterogeneity and postsynaptic development and is important for our understanding of processes underlying NMJ functionality in the contexts of development and disease.

Results

Defining synaptic myonuclei in *Drosophila* larval muscles

To characterize synaptic myonuclei at the *Drosophila* NMJ, we used the well-studied ventral longitudinal muscles VL3 and VL4 (muscles 6 and 7) from abdominal segments 2 and 3 (Fig. 1 A). VL3 and VL4 muscles differ in size, nuclear number, and nuclear arrangement with two rows versus one row, respectively (Fig. S1, A and B; Manhart et al., 2018; Windner et al., 2019). While the innervating motor neuron is shared between VL3 and VL4, each muscle develops its own NMJ (Kohsaka et al., 2012). We previously identified a size-scaling relationship between myonuclei and their surrounding cytoplasmic domain. Both nuclear size scaling and DNA content were found to be intracellularly patterned: myonuclei with the largest size and highest DNA ploidy occur in the middle of the muscle cell where the NMJ is located (Windner et al., 2019). Here, we asked whether myonuclei closer to the NMJ are significantly different from those positioned more distant to the NMJ.

To identify the NMJ, we immunolabeled the scaffolding protein discs large (Dlg) that closely associate with the SSR and is a widely used postsynaptic marker (Chen and Featherstone, 2005; Zhang et al., 2017). As synaptic myonuclei cannot be identified visually, we used the average distance (*d*) between myonuclei and the SSR to separate them into two populations, synaptic nuclei ($\leq d$), and non-synaptic nuclei ($> d$) (Fig. 1 A and Fig. S1 C). The average nuclear-NMJ distance was $56.25 \pm 9.98 \mu\text{m}$ for the larger VL3 muscle and $49.23 \pm 8.87 \mu\text{m}$ for the smaller VL4 muscle (mean $\pm$ SD), with ~ 8 synaptic nuclei (48% of myonuclei) in VL3 and ~ 6 synaptic nuclei (58% of myonuclei) in VL4 (Fig. S1, D and E). Given these differences, we initially examined myonuclear attributes separately for VL3 and VL4 muscles.

Increased nuclear size scaling at the mammalian NMJ is established by locally decreasing myonuclear domain sizes and indicates an increased potential of these myonuclei to contribute gene products to the NMJ. To assess nuclear size scaling, we measured the size of nuclei and their cytoplasmic domains as previously described (Windner et al., 2019). We found that nuclear size scaling is significantly increased in myonuclei closer to the NMJ, dropping below average to $41\text{--}60 \mu\text{m}$ (diameter of ~ 3 nuclei) distance (Fig. 1 B). This size scaling increase resulted

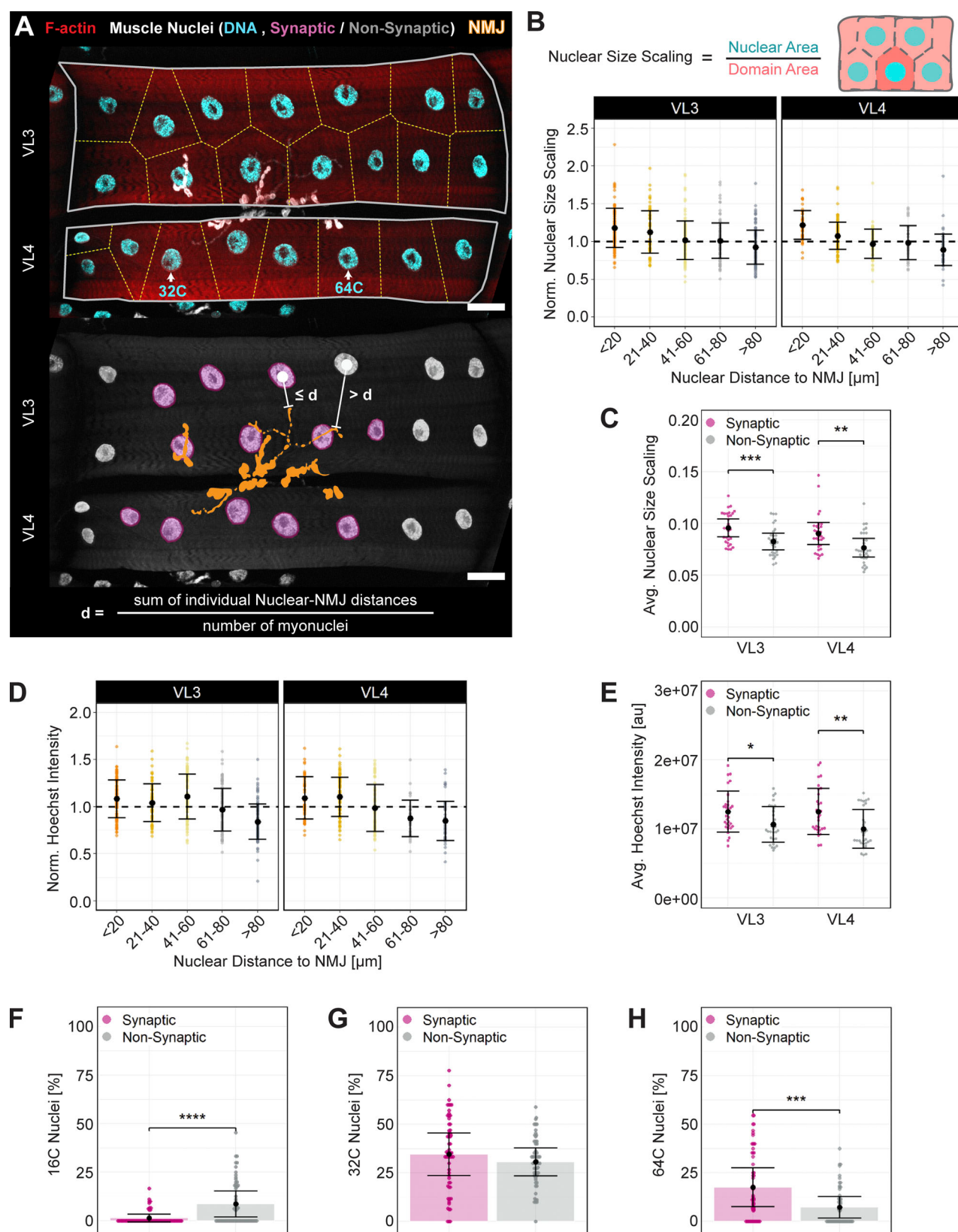


Figure 1. **Synaptic myonuclei in *Drosophila* larval muscles are characterized by position, size, and DNA content.** (A) Confocal images (sum slice projection) of ventral longitudinal muscles VL3 and VL4 (or muscles 6 and 7) from wandering *Dmef2 > mCherry-RNAi* control larvae. Top, fluorescent labeling of muscle cells (phalloidin, red), nuclear DNA (Hoechst, cyan), and NMJ (anti-Dlg, white). Myonuclear domains (yellow dashed lines) and example myonuclei with a ploidy of 64C and 32C are indicated. Bottom, same cells highlighting the NMJ (orange), NMJ-proximal synaptic myonuclei (magenta), and distal non-synaptic myonuclei (dark gray). Synaptic myonuclei are defined per cell as nuclei positioned less than or equal to the average nuclear distance to the NMJ (d). Scale bars

= 30 μ m. **(B)** Nuclear size scaling, equal to nuclear size divided by cytoplasmic domain size, normalized by the cell's average and plotted against distance from the NMJ (20 μ m bins). One-way ANOVA was performed (VL3: $P = 5.9\text{e-}13$; VL4: $P = 1.2\text{e-}15$). **(C)** Average nuclear size scaling for synaptic and non-synaptic myonuclei. **(D)** Hoechst fluorescence intensity, normalized by the cell's average and plotted against distance from the NMJ (20 μ m bins). One-way ANOVA was performed (VL3: $P < 2.2\text{e-}16$; VL4: $P = 3.6\text{e-}13$). **(E)** Average Hoechst fluorescence intensity for synaptic and non-synaptic myonuclei. **(F–H)** Ploidy distribution based on Hoechst fluorescence intensities (refer to Fig. S1 H) in synaptic and non-synaptic myonuclei from VL3 and VL4 muscles for (F) 16C, (G) 32C, and (H) 64C nuclei. Data are shown as mean $\pm$ SEM, except for in B and D, where mean $\pm$ SD is plotted ($n = 19$ larvae, 30 VL3 muscles and 30 VL4 muscles). Statistical comparisons were made for all groups with significant differences noted (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

from an increase in nuclear sizes, while cytoplasmic domain sizes remained unchanged (Fig. S1, F and G). When calculating average nuclear size scaling for synaptic and non-synaptic myonuclei, we found similar values in both VL muscles (Fig. 1 C). Therefore, in both VL3 and VL4, synaptic myonuclei have significantly increased nuclear size scaling compared with non-synaptic myonuclei.

Each larval muscle contains multiple nuclei, and each myonucleus increases its DNA content by endoreplication to support cell growth and metabolic demands, resulting in DNA copy numbers of 16C, 32C, and 64C (Windner et al., 2019). To assess myonuclear DNA content, we used the fluorescence intensity of the DNA stain Hoechst. We found that like nuclear size scaling, myonuclei with increased Hoechst intensity were positioned ≤ 60 μ m from the NMJ, supporting the use of average nuclear-NMJ distance for identifying synaptic myonuclei (Fig. 1 D). We calculated the average Hoechst intensity for synaptic and non-synaptic myonuclei, revealing that synaptic myonuclei have significantly increased DNA content in both VL muscles (Fig. 1 E). To further evaluate nuclear DNA content, we assigned ploidy numbers to Hoechst intensity measurements (Fig. S1 H). This analysis revealed that synaptic myonuclei have higher ploidy numbers (more 64C nuclei and fewer 16C nuclei) (Fig. 1, F–H), indicating that they undergo additional rounds of endoreplication compared with non-synaptic myonuclei.

At the mammalian NMJ, increased DNA content and nuclear size scaling were achieved by clustering myonuclei at the postsynapse (Bruusgaard et al., 2003; Hansson et al., 2020). We observed no change in cytoplasmic domain sizes for myonuclei near the *Drosophila* NMJ (Fig. S1 G). To directly test whether clustering occurs, we measured internuclear distance and observed similar average distances for synaptic and non-synaptic myonuclei in VL3 and VL4 muscles (Fig. S1 I). Thus, at the *Drosophila* NMJ, a local increase in myonuclear DNA content and nuclear size scaling is established by providing more DNA per synaptic nucleus and increasing nuclear size, respectively, in the absence of nuclear clustering. These synaptic nuclear properties are observed in both VL muscles, indicating that it is a general feature of larval VL muscles. Based on these data, we define populations of synaptic and non-synaptic myonuclei in *Drosophila* muscles by their distance to the NMJ, nuclear size scaling, and DNA content.

BMP signaling is increased in synaptic myonuclei

A key signaling pathway at the NMJ is the BMP pathway. While postsynaptic pathway activation and nuclear import have been described (Dudu et al., 2006), how this affects the muscle, particularly the myonuclei, is unknown. To confirm the expression of the core BMP pathway components (Fig. 2 A), we initially

screened our previous RNA sequencing dataset from larval muscle-enriched preps (Christophers et al., 2024). As expected, known muscle-enriched genes showed moderate to high expression levels. NMJ-related genes and BMP pathway components also showed expression but at lower levels. This analysis confirmed the muscle expression of many BMP pathway components and informed our choice of targets for RNAi KD. At the receptor level, Put can interact with either Tkv- or Saxophone (*sax*) to activate BMP signaling. In larval muscles, Tkv protein was previously shown to localize specifically to the SSR membrane (Dudu et al., 2006), indicating that muscle BMP signaling activation occurs at the postsynapse. In addition, in the *Drosophila* wing disc, receptor complexes composed of Tkv-Sax heterodimers promote BMP signaling, whereas Sax-Sax homodimers antagonize BMP signaling by sequestering Gbb (Bang and Wharton, 2006; Bauer et al., 2023). Given the complex signaling effects of Sax, we focused on receptors Tkv and Punt, in combination with transcription factor Mothers against dpp (*Mad*) for subsequent KD experiments.

To analyze BMP signaling activity in *Drosophila* larval muscles, we performed immunolabeling for pMad. We observed strong, punctate pMad signal directly at the NMJ as well as diffuse and punctate pMad signal in myonuclei (Fig. 2, C–E). These patterns are consistent with previous studies (Dudu et al., 2006; O'Connor-Giles et al., 2008; Smith et al., 2012; Sulkowski et al., 2014). We quantified the nuclear pMad signal and found significantly higher levels in myonuclei closer to the NMJ, dropping below average at a distance of 60 μ m (Fig. 2 F). We calculated average nuclear pMad intensities, revealing that synaptic myonuclei contain significantly more pMad signal than non-synaptic myonuclei (Fig. 2 G) in both VL muscles (Fig. S2 A). This increase remained when nuclear pMad intensity was normalized to the nuclear area, indicating that synaptic myonuclei have higher pMad levels independent of size (Fig. S2 B).

pMad functions as part of a transcription complex to promote target gene expression; however, it can also associate with evolutionarily conserved zinc-finger protein Schnurri (*Shn*) to repress gene transcription (Marty et al., 2000). To test if BMP target gene repression is also affected by the NMJ, we performed *Shn* immunolabeling. We found that *Shn* is present in all myonuclei (Fig. 2 H), and that, unlike pMad, there was no difference in *Shn* levels between synaptic and non-synaptic myonuclei (Fig. 2 I). This suggests that *Shn*-pMad complexes have equal potential to inhibit gene expression in all myonuclei, while high pMad levels in synaptic myonuclei may signify increased expression of pMad target genes. Altogether, these data suggest that BMP pathway activation originating from the NMJ establishes a pMad gradient across the myonuclei, and high levels of pMad are characteristic of synaptic nuclei.

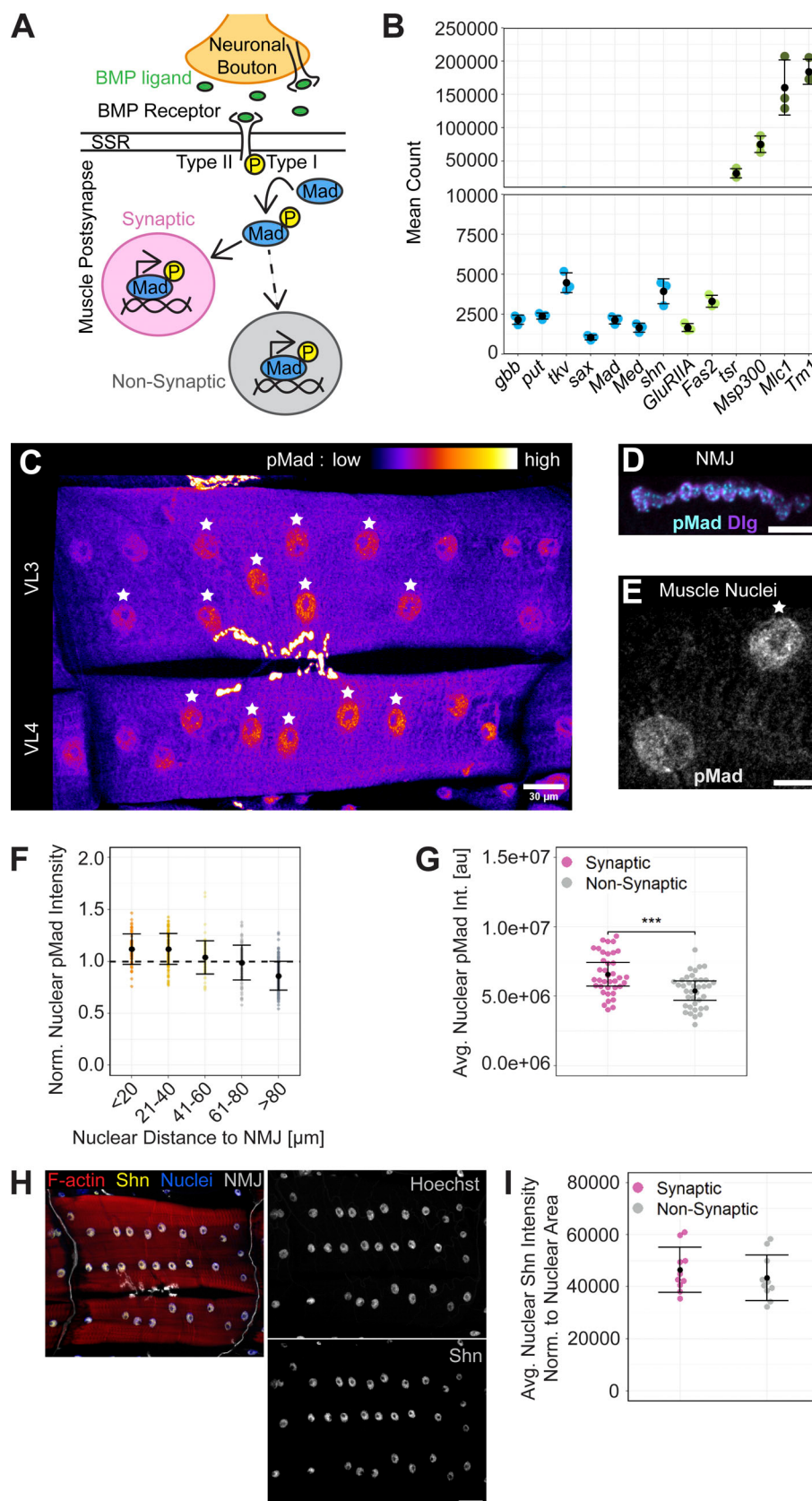


Figure 2. BMP signaling effector pMad is increased within synaptic myonuclei. (A) Diagram of the postsynaptic BMP signaling pathway in *Drosophila* larval muscle. BMP ligand (Gbb, green) binds to receptor hetero-tetramers composed of two Type I (Tkv or Sax) and two Type II (Punt) receptors in the postsynaptic muscle membrane (SSR) opposed to the neuronal boutons (orange). Activation of the receptor complex leads to phosphorylation of Mad (pMad,

blue), its nuclear translocation, and subsequent regulation of target gene transcription. We hypothesize that synaptic myonuclei (magenta) are exposed to higher pMad levels than non-synaptic myonuclei (gray). **(B)** Normalized mRNA counts for *Drosophila* BMP pathway genes (blue, ligand: Glass bottom boat [*gbb*]; receptors: Punt [*put*], thickveins [*tkv*], saxophone [*sax*]; transcription factor: Mad [*Mad*]; co-factors: Medea [*Med*], Schnurri [*shn*]) and known muscle-expressed genes (green, NMJ related: glutamate receptor IIA [*GluRIIA*], Fasciclin 2 [*Fas2*]; muscle function related: twinstar [*tsr*], muscle-specific protein 300 kDa [*Msp300*], myosin alkali light chain 1 [*Mlc1*], tropomyosin 1 [*Tm1*]) in third instar control *Mhc* > *mCherry-RNAi* larvae from a published bulk RNA sequencing dataset (Christophers et al., 2024). **(C)** Confocal image (sum slice projection) of VL3–VL4 muscle pair from a *Dmef2* > *mCherry-RNAi* control larva immunolabeled for pMad. Colors indicate pixel intensities, with the highest levels at the NMJ (white-yellow) and within synaptic myonuclei (white stars). **(D)** Confocal image of a VL3/4 NMJ branch (sum slice projection) showing pMad (cyan) and SSR (Dlg, magenta). **(E)** Confocal image of nuclear pMad (sum slice projection) in a synaptic nucleus (white star) next to a non-synaptic nucleus. **(F)** Nuclear pMad intensity, normalized by the cell's average and plotted against distance from the NMJ (20 μ m bins) (n = 11 larvae, 40 muscle pairs of VL3 and VL4). One-way ANOVA was performed (P < 2.2e–16). **(G)** Average nuclear pMad intensity in synaptic and non-synaptic myonuclei from control muscles (n = 11 larvae, 40 muscle pairs of VL3 and VL4). **(H)** Confocal images (sum slice projections) of VL muscles from a *Dmef2* > *mCherry-RNAi* larva labeled for anti-Schnurri (Shn, yellow and shown individually in gray), F-actin (phalloidin, red), muscle nuclei (Hoechst, blue and shown individually in gray), and the NMJ (anti-Dlg, gray). **(I)** Average nuclear Shn intensity normalized to the nuclear area in synaptic and non-synaptic control myonuclei (n = 4 larvae, 10 muscles). Data are shown as mean $\pm$ SEM in plot G and as mean $\pm$ SD in plots B, F, and I. Statistical comparisons were made for all groups with significant differences noted (** P < 0.001). Scale bars = 30 μ m (C and H), 10 μ m (D and E).

Postsynaptic BMP signaling promotes muscle size and myonuclear size

To investigate the effects of postsynaptic BMP signaling on synaptic myonuclei, we performed muscle-specific, RNAi-mediated KD of the core pathway components: the two BMP receptors, Punt (type II) and Tkv (type I), and the downstream transcription factor Mad (Fig. 2, A and B; and Fig. 3 A). While Tkv exclusively acts in BMP signaling, Punt can also dimerize with the type I receptor Baboon to activate Activin signaling mediated by the transcriptional effector Smox. This pathway was previously shown to regulate larval muscle growth (Kim and O'Connor, 2021), which may affect our pathway analysis. We first determined KD efficiencies by qPCR on larval muscle-enriched preps, revealing a 20–30% KD of *put*, *tkv*, and *Mad* RNA levels using the muscle-specific driver *Dmef2* (Fig. S2 C). Given the similar nuclear patterns in VL3 and VL4 control muscles, we combined data from both muscles for the analysis of KD phenotypes. Muscle KD of Punt, Tkv, or Mad reduced the size of muscle cells and myonuclei (Fig. 3, B and C; and Fig. S2 D), indicating a role for postsynaptic BMP signaling in promoting muscle growth. Among the KD groups, Punt KD exhibited the strongest reduction in nuclear size, consistent with Punt acting as a type II receptor for both BMP and Activin pathways. Tkv KD and Mad KD, both specifically targeting BMP signaling, had a similar reduction in nuclear size (Fig. 3 C). We used a second Mad RNAi line to confirm these results (Fig. S2 E) and chose the line that produced a stronger phenotype for subsequent analyses. Muscle KD of Punt, Tkv, or Mad did not alter the average distance between nuclei and the NMJ, despite having smaller muscles (Fig. S2 F). We attribute this to a decrease in relative NMJ length, not a difference in nuclear distribution (Fig. S2 G). Interestingly, VL3 muscles with Tkv KD or Mad KD had a ~9–14% increase in the number of synaptic myonuclei compared with control VL3 muscles (Fig. S2 H), suggesting that *Drosophila* muscles can adjust nuclear positioning in relation to the NMJ to compensate for limited growth.

When we assessed nuclear size scaling, we found that in Tkv KD and Punt KD muscles, the reduction of both cell and nuclear sizes resulted in similar nuclear size scaling for synaptic and non-synaptic myonuclei compared with control. However, in Mad KD muscles, only non-synaptic myonuclei had control-like size scaling, whereas synaptic myonuclei showed significantly

reduced scaling (Fig. 3 D). These data suggest that receptor level KD affects all myonuclei equally, while Mad KD more strongly affects synaptic myonuclei. Nevertheless, muscles of all genotypes maintain increased nuclear size scaling for synaptic relative to non-synaptic myonuclei.

Next, we analyzed nuclear pMad levels in Punt KD, Tkv KD, and Mad KD muscles. We found that average protein levels are reduced in all groups compared with control (Fig. 3, E and F). In Punt KD and Tkv KD muscles, nuclear pMad was reduced by ~17%, while Mad KD muscles had ~50% nuclear pMad reduction, indicating that Mad KD exerts a stronger effect on postsynaptic BMP signaling. When we examined the intracellular distribution of nuclear pMad, we found that Mad KD muscles maintained a gradient as observed in control muscles, as well as similar differences (~20%) between synaptic and non-synaptic nuclei (Fig. 3, G and H). Punt KD and Tkv KD muscles also showed a difference in nuclear pMad levels between synaptic and non-synaptic nuclei; however, Punt KD reduced the difference to ~10%, while Tkv KD increased it to ~30% (Fig. 3 H). These data indicate that the pMad gradient is regulated at the receptor level, with opposing roles for type I and type II receptors. Since type II receptors phosphorylate type I receptors, which then phosphorylate Mad (Fig. 2 A), and different BMP receptor dimers can have distinct effects on pathway activation (Bang and Wharton, 2006; Bauer et al., 2023), it is possible that the composition of the BMP receptor complex establishes the gradient by regulating the availability and dispersion of pMad in the muscle.

Together, our results show that postsynaptic BMP pathway perturbations reduce muscle growth and the intracellular distribution of pMad, yet, maintain differences between synaptic and non-synaptic myonuclei. Mad KD resulted in the lowest nuclear pMad levels but with a control-like gradient. Reduced size scaling of synaptic myonuclei in Mad KD muscles suggests a reduced potential of these myonuclei to contribute to postsynaptic processes.

Postsynaptic BMP signaling promotes myonuclear DNA content via the regulation of E2f1

We next used Hoechst labeling to determine whether postsynaptic BMP signaling affects myonuclear DNA content (Fig. 4 A). We observed a strong positive correlation between total Hoechst

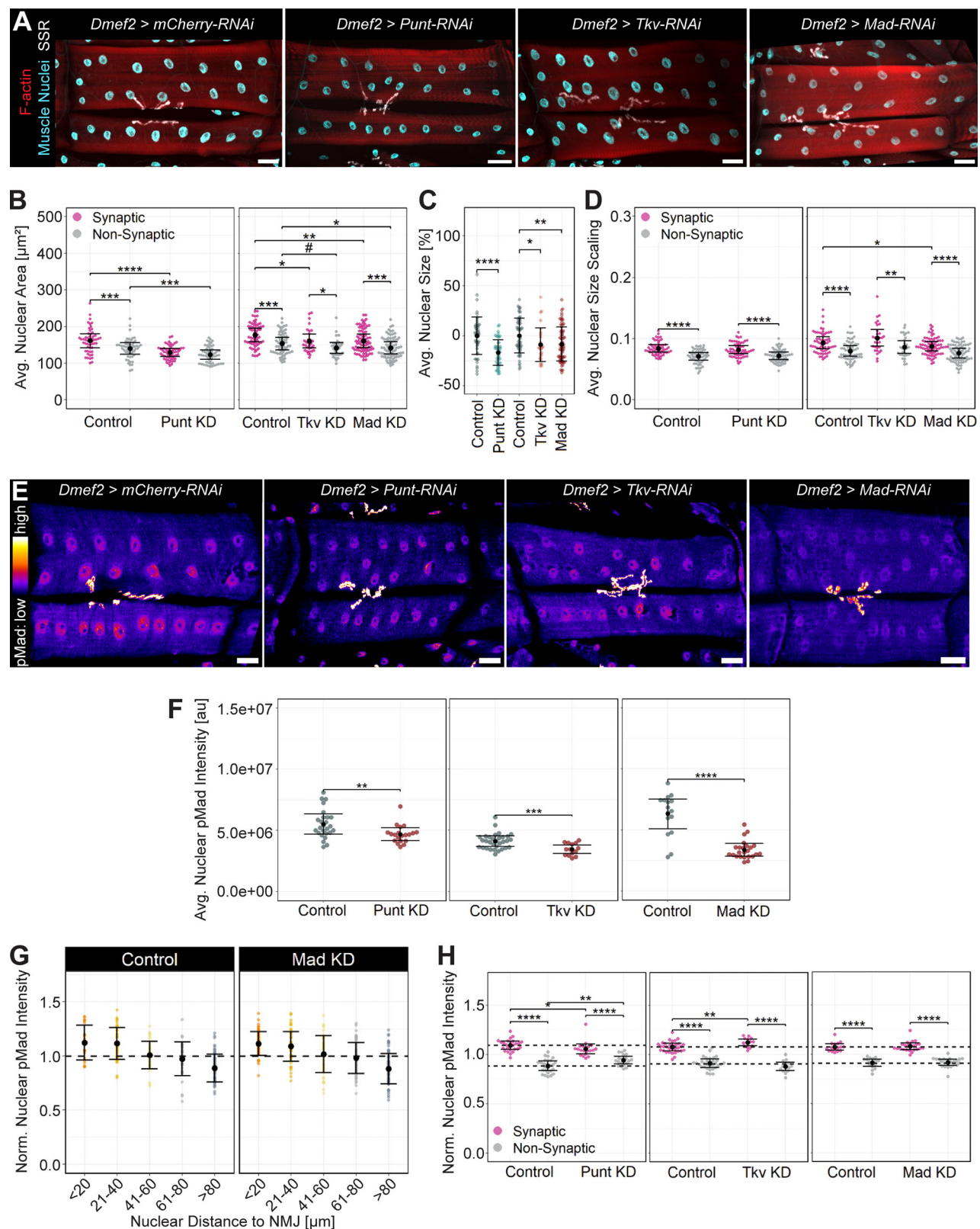


Figure 3. Muscle-specific KD of BMP pathway components reduces nuclear size and pMad levels but maintains a nuclear pMad gradient. (A) Confocal images of VL muscles (sum slice projection) labeled with phalloidin (F-actin, red), Hoechst (DNA, cyan), and anti-Dlg (SSR, white) in control and Punt, thickveins (Tkv), and Mad KD (KD) muscles. Scale bars = 30 μm . **(B)** Average size of synaptic and non-synaptic myonuclei in control and Punt-deficient VL muscles ($n = 17$ larvae, 56 muscles in both groups), and control, Tkv, and Mad KD muscles (control: $n = 19$ larvae, 60 muscles; Tkv KD $n = 12$ larvae, 30 muscles; Mad KD $n = 20$ larvae, 76 muscles). **(C)** Average nuclear size, plotted as percent difference from the control average for each respective experiment (Punt KD versus Tkv KD and Mad KD) for control and KD muscles. **(D)** Average nuclear size scaling for synaptic and non-synaptic myonuclei. Genotypes and sample numbers as in

B. (E) Confocal images of control, Punt KD, Tkv KD, and Mad KD muscles (sum slice projection pseudo-colored to indicate pixel intensity) labeled for pMad. Scale bars = 30 μ m. (F) Average nuclear pMad intensity in Punt KD, Tkv KD, and Mad KD muscles and respective control muscles (control: $n = 6$ –12 larvae, 16–32 muscles; Punt KD: $n = 8$ larvae, 20 muscles; Tkv KD: $n = 5$ larvae, 14 muscles; Mad KD: $n = 9$ larvae, 24 muscles). (G and H) Nuclear pMad intensity, normalized by the cell's average, (G) plotted against distance from the NMJ (20 μ m bins) for control and Mad KD muscles, and (H) shown for synaptic and non-synaptic myonuclei in control and KD muscles. One-way ANOVA was performed in G (control: $P = 7.1e-15$; Mad KD: $P < 2.2e-16$). Data are shown in all plots as mean $\pm$ SEM, except in C and G where mean $\pm$ SD is plotted. Distinct plots with different controls are included for Punt KD, Tkv KD, and Mad KD due to the experiments being conducted separately. Statistical comparisons were made for all groups with significant differences noted ($\#P = 0.054$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

intensity and total nuclear pMad intensity in control muscles and in Mad KD muscles, supporting a relationship between postsynaptic BMP signaling and myonuclear DNA content (Fig. 4 B). We found that average nuclear Hoechst intensity was reduced in Mad KD muscles (two different RNAi lines) and Punt KD muscles but was similar to controls in Tkv KD muscles (Fig. 4, C and D; and Fig. S2 I). In accordance with a reduced nuclear pMad gradient, Hoechst intensity differences between synaptic and non-synaptic nuclei were strongly reduced in Punt KD muscles (Fig. 4 C). These data are consistent with Punt supporting Activin signaling in promoting overall muscle growth (Kim and O'Connor, 2021), which involves an increase in DNA content. In Tkv KD and Mad KD muscles, average nuclear Hoechst intensity was still increased in synaptic compared with non-synaptic nuclei, altogether supporting a role for postsynaptic BMP signaling in regulating nuclear DNA content.

To achieve a more detailed analysis of DNA content, we used Hoechst intensity to designate nuclear ploidy (Fig. S3 A). In control muscles, synaptic nuclei exhibit increased ploidy (more 64C and less 16C) compared with non-synaptic nuclei. Consistent with average Hoechst intensity, overall 64C ploidy was reduced in muscles with KD of Punt and Mad, but not Tkv (Fig. 4, E–G). Muscles across all groups had significantly higher ploidy (greater 32C and/or 64C) in the synaptic myonuclei, confirming that a local DNA increase is maintained at the postsynapse. A reduction of 64C nuclei in Punt KD and Mad KD muscles correlated with a significant increase of 32C ploidy in the synaptic nuclei compared with the non-synaptic nuclei (Fig. 4, E and G). These data reveal that perturbations to BMP signaling affect nuclear DNA content, specifically altering the frequencies for 64C overall and 32C locally in the synaptic myonuclei.

To further explore the role of BMP signaling, we performed bulk RNA sequencing on control and Mad KD muscle-enriched larval preparations. As expected, Mad KD muscles had reduced expression of *Mad* RNA ($\log_2FC = -0.56$; $P = 0.00018$), consistent with a ~30% reduction as found by qPCR. Expression of *brinker* (*brk*), a gene that is downregulated by Mad normally (Jaźwińska et al., 1999; Müller et al., 2003; Pyrowolakis et al., 2004), was increased in Mad KD muscles ($\log_2FC = 1.04$; $P = 4.79e-07$) (Fig. S3 B). Consistent with reduced sizes of Mad KD muscles, GO analysis for biological processes showed downregulation of metabolic pathways (e.g., carbohydrate catabolic process, glucose metabolic process, and ATP generation from ADP) involved in nutrient and cellular energy production that contribute to muscle growth (Demontis and Perrimon, 2009; Piccirillo et al., 2014) (Fig. S3 C). Conversely, muscle Mad KD led to an upregulation in genes related to developmental processes (e.g.,

metamorphosis and postembryonic organ morphogenesis) and transcription/translation (e.g., cellular response to unfolded protein and polytene chromosome puffing) (Fig. S3 D). These data support a growth-promoting role for postsynaptic BMP signaling and suggest that changes in muscle size and myonuclear function may induce a cell stress response. In addition, we examined the expression of muscle-specific structural genes (e.g., *Tm1*, *Tm2*, *Mlc2*, *Mhc*) that contribute to muscle mass and observed these genes were significantly downregulated in Mad KD muscles (Fig. S3 E), further supporting that BMP signaling affects muscle growth.

To address changes in myonuclear DNA content following Mad KD, we examined regulators of endoreplication. *E2f1* is a key transcriptional regulator of endoreplication and is required to drive the expression of cell cycle genes like *CycE* to trigger S phase initiation (Duronio et al., 1995; Royzman et al., 1997; Sekar et al., 2023; Weng et al., 2003). Conversely, *E2f1* degradation and reduction of *CycE* activity resets the cell cycle to the G phase (Zielke et al., 2011). In control muscles, *E2f1* was highly expressed, whereas *CycE* was expressed at a lower level (Fig. 4 H). By RNA sequencing, muscle-specific Mad KD significantly reduced *E2f1* expression ($\log_2FC = -0.34$; $P = 0.0157$), whereas *CycE* expression was not affected ($P = 0.9566$) (Fig. 4 I). Validating the sequencing data, qPCR showed reduced levels of *E2f1* and increased *brk* levels with Mad KD (Fig. S3 F). Further support for a Mad-*E2f1* relationship was obtained by analysis of open access databases by JASPAR (2018 CORE collection; Khan et al., 2018) and the Swiss Institute of Bioinformatics (Eukaryotic Promoter Database, EPDnew; Dreos et al., 2015) for *D. melanogaster* that maps transcription factor binding motifs to the upstream promoter and intron regions of genes. Using these databases, we cross-referenced *E2f1* hits for the consensus Mad binding motif and found several potential Mad binding sites within *E2f1* regulatory regions (Fig. S4, A and B). To validate this approach, we also assessed the known Mad target gene *brk* and, as expected, found multiple Mad binding sites in *brk* regulatory regions (Fig. S4 C). Thus, it is possible that BMP signaling directly promotes endoreplication by regulating *E2f1* transcription. A reduction in myonuclear DNA content could also indirectly contribute to reduced muscle growth by affecting the expression of genes required for NMJ growth and function. As such, we next evaluated the expression of NMJ-related genes and the synapse itself.

Postsynaptic BMP signaling shapes NMJ size

Retrograde BMP signaling has been viewed as the main BMP pathway at the NMJ, contributing to NMJ growth and differentiation. Only one study concluded that postsynaptic BMP signaling affects bouton number and thereby NMJ size (Fuentes-

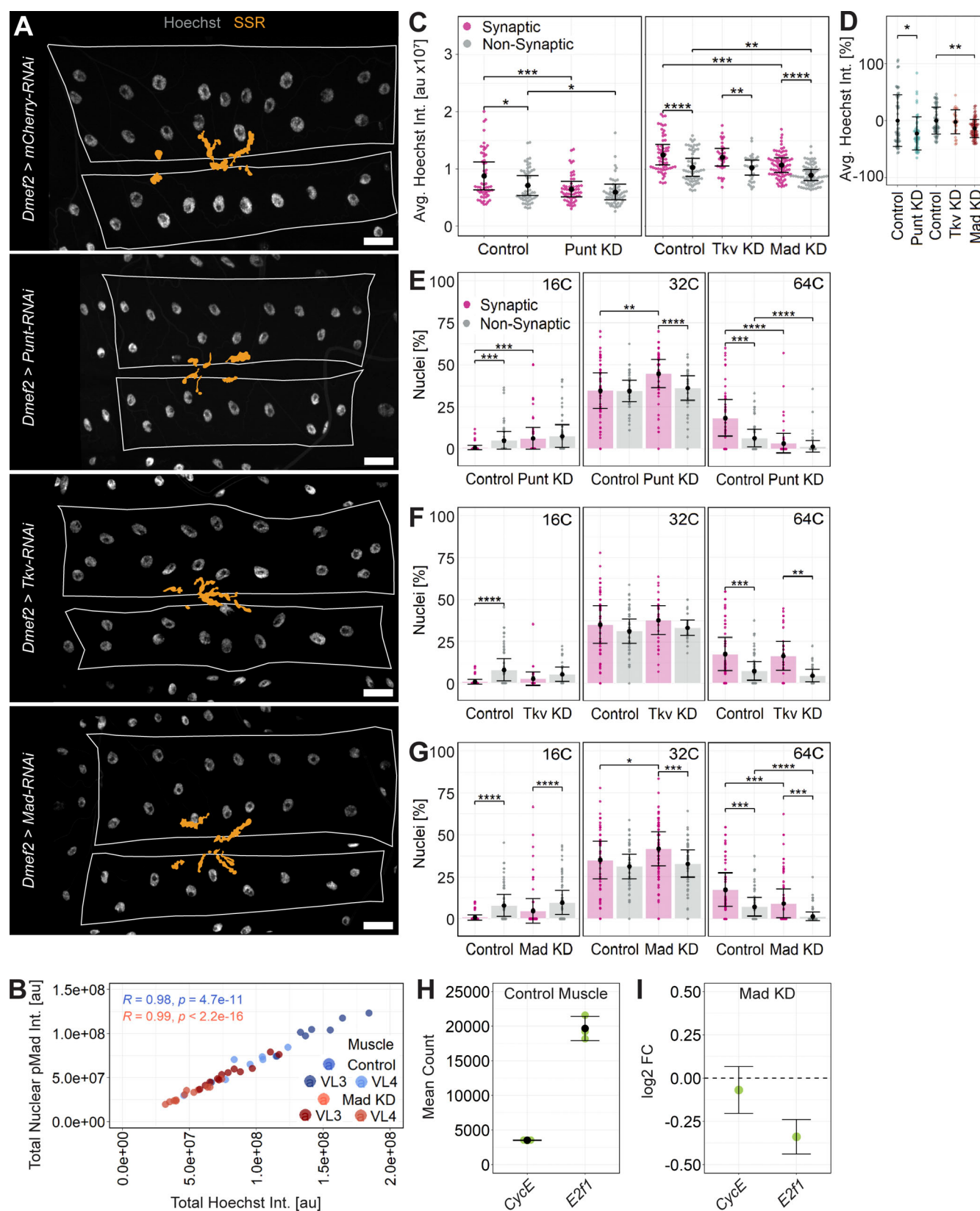


Figure 4. **Postsynaptic BMP signaling regulates myonuclear DNA content and endoreplication.** (A) Confocal images (sum slice projection) of control and Punt, Tkv, and Mad KD (KD) muscles labeled with Hoechst (DNA, white) and anti-Dlg (NM), orange mask). Scale bars = 30 μ m. (B) Total/cumulative nuclear pMad intensity plotted against total/cumulative Hoechst intensity for control and Mad KD VL muscles. Pearson's correlation coefficient (R) and statistical significance (p) are indicated and reveal a strong linear correlation between nuclear pMad and DNA content for both genotypes (control: $n = 6$ larvae, 16 muscles; Mad KD: $n = 9$ larvae; 24 muscles). (C) Average Hoechst intensity per nucleus from control and Punt KD VL3/4 muscles ($n = 17$ larvae, 56 muscles in

both groups), and control, Tkv, and Mad KD muscles (control: $n = 19$ larvae, 60 muscles; Tkv KD $n = 12$ larvae, 30 muscles; Mad KD $n = 20$ larvae, 76 muscles). **(D)** Average Hoechst intensity, plotted as percent difference from the control average for each respective experiment (Punt KD versus Tkv KD and Mad KD), for control and KD muscles. **(E–G)** Frequency of 16C, 32C, and 64C ploidy in synaptic and non-synaptic myonuclei of (E) Punt KD, (F) Tkv KD, and (G) Mad KD VL muscles and corresponding controls. **(H)** Normalized mRNA counts of endoreplication regulators *CycE* and *E2f1* in muscle-enriched preps of control larvae ($N = 3$ replicates, $n = 6–10$ larvae). **(I)** Fold change of *CycE* and *E2f1* expression in Mad KD preps ($N = 3$ replicates, $n = 6–10$ larvae per genotype). Data are shown as mean $\pm$ SEM, except in plots D, H, and I where mean $\pm$ SD is shown. Distinct plots with different controls are included for Punt KD compared to Tkv KD and Mad KD due to the experiments being conducted separately. Statistical comparisons were made for all groups with significant differences noted (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Medel et al., 2012). To expand upon this finding, we examined the effects of postsynaptic BMP pathway manipulations on NMJ size. To first test whether our manipulations affect the retrograde BMP pathway, we performed immunostaining for BMP ligand Gbb. While Gbb is expressed in both muscles and motor neurons, muscle Gbb is seen as the critical ligand for retrograde pathway activation (McCabe et al., 2003) (Fig. 5 A). We found a similar number of Gbb-positive puncta in Tkv and Mad KD muscles compared with control (Fig. 5 B). Moreover, by RNA sequencing, we observed no change in *gbb* expression in Mad KD samples (Fig. S5 A). These data suggest that Gbb production is unaffected in Tkv and Mad KD muscles and that Gbb is available to activate retrograde BMP signaling to direct presynaptic bouton growth independent of postsynaptic BMP pathway inhibition.

To assess postsynaptic changes in BMP signaling deficient muscles, we measured the total SSR area for VL3 and VL4 using Dlg immunostaining (Fig. 5 C). Since NMJ size correlates with muscle size (Ho and Treisman, 2020), and KD of BMP signaling components reduces muscle size (Fig. S2 D), we normalized total SSR area by cell area (SSR size scaling). We found proportionally increased SSR area in Tkv KD muscles yet similar proportions in Punt KD and Mad KD muscles (Fig. 5 D).

We next counted the number of large 1b boutons at the VL3/VL4 NMJ. Normalized by muscle area, we observed an increased bouton number with Tkv KD but not Punt KD nor Mad KD (Fig. 5 C inserts, Fig. 5 E). We divided total SSR area by bouton number to approximate the average SSR area per bouton and found that in Punt KD and Tkv KD muscles, SSR area per bouton was unchanged, while Mad KD muscles had increased SSR area per bouton compared with control (Fig. 5 F).

Together, these results suggested that postsynaptic BMP signaling acts independently of the retrograde pathway to coordinate bouton numbers and SSR size with muscle size. KD of the *put* did not affect NMJ size or morphology, consistent with Activin signaling not regulating bouton number as previously published (Kim and O'Connor, 2014). Tkv KD increased bouton number and SSR area, overall leading to a proportionally larger NMJ. In contrast, KD of the downstream effector Mad had no effect on the bouton number but increased SSR area per bouton. Thus, both Tkv KD and Mad KD show proportionally increased NMJ structures, indicating that NMJ size and the proper coordination of NMJ size to muscle size depend on postsynaptic BMP signaling.

Postsynaptic BMP signaling impacts NMJ function

To test whether Mad KD also affects NMJ function, we first examined the expression of NMJ-related genes in our RNA sequencing dataset. At the glutamatergic *Drosophila* NMJ, ionotropic glutamate receptors (GluRII) in the SSR allow for

postsynaptic calcium (Ca^{2+}) influx and muscle membrane depolarization following neurotransmitter binding (reviewed in Menon et al. [2013]). An individual GluRII consists of subunits C, D, and E, in addition to either A or B that together are required for proper receptor expression and function at the NMJ (Qin et al., 2005). Strikingly, we found changes in the expression of several GluRII subunits with muscle Mad KD (Fig. 6 A): the expression of *GluRIIE* ($\log_2\text{FC} = -0.6710$; $P = 3.68\text{e-}11$) and *GluRIIC* ($\log_2\text{FC} = -0.5763$; $P = 0.0012$) were reduced, *GluRIID* ($\log_2\text{FC} = 0.5040$; $P = 0.0001$) was increased, and *GluRIIA* and *GluRIIB* were unchanged (Fig. 6 A). These RNA sequencing results were tested and confirmed by qPCR (Fig. S5 B).

GluRIIA was previously reported to be reduced at the protein level in Mad KD muscles (Sulkowski et al., 2016). We confirmed these data, finding significantly reduced GluRIIA protein at the NMJs of Mad KD muscles (Fig. 6, B and C). Regulation of *GluRIIA* expression has been suggested to involve *GluRIIA* mRNA localization to the SSR and local postsynaptic translation driven by the activity of eIF4E, a family of essential translation initiation factors (Menon et al., 2004; Sigrist et al., 2000). In accordance with previous studies, we found a member of the eIF4E family, eIF4E3, which was significantly downregulated ($\log_2\text{FC} = -6.4810$, $P = 2.95\text{e-}15$) in Mad KD muscles by RNA sequencing and qPCR (Fig. S5, C and D). Furthermore, we observed a statistical trend for the upregulation of Pumilio (*pum*) ($\log_2\text{FC} = 0.4295$, $P = 0.0976$), a translational repressor known to target eIF4E and *GluRIIA* mRNA to regulate their postsynaptic protein levels (Fig. S5 C) (Menon et al., 2004; Menon et al., 2009). Together, our data strongly support the role of BMP signaling in promoting GluRIIA protein levels at the postsynapse by modulating the expression of translation machinery regulators required for proper local translation of *GluRIIA* mRNA.

The absence or impaired function of GluRIIA inhibits the muscle's response to spontaneous glutamate release by the motor neuron, resulting in decreased amplitude (quantal size) and frequency of spontaneous miniature excitatory junctional potentials (mEJPs) in the postsynaptic muscle (DiAntonio et al., 1999; Petersen et al., 1997). Single-action potentials as well as the spontaneous release of neurotransmitters from the motor neuron result in Ca^{2+} influx at the postsynaptic membrane and can be measured using Ca^{2+} indicator GCaMP6f localized to the SSR (*Mhc-CD8-GCaMP6f-Sh*) (Desai and Lnenicka, 2011; Newman et al., 2017). To determine if neurotransmission is affected in Mad KD muscles, we performed live imaging of postsynaptic Ca^{2+} influx during spontaneous mEJPs (Fig. 6 D). We found a statistical trend for reduced frequency of quantal events in Mad KD muscles compared with control (Fig. 6 E), whereas the amplitude was unchanged (Fig. 6 F). This mild functional phenotype

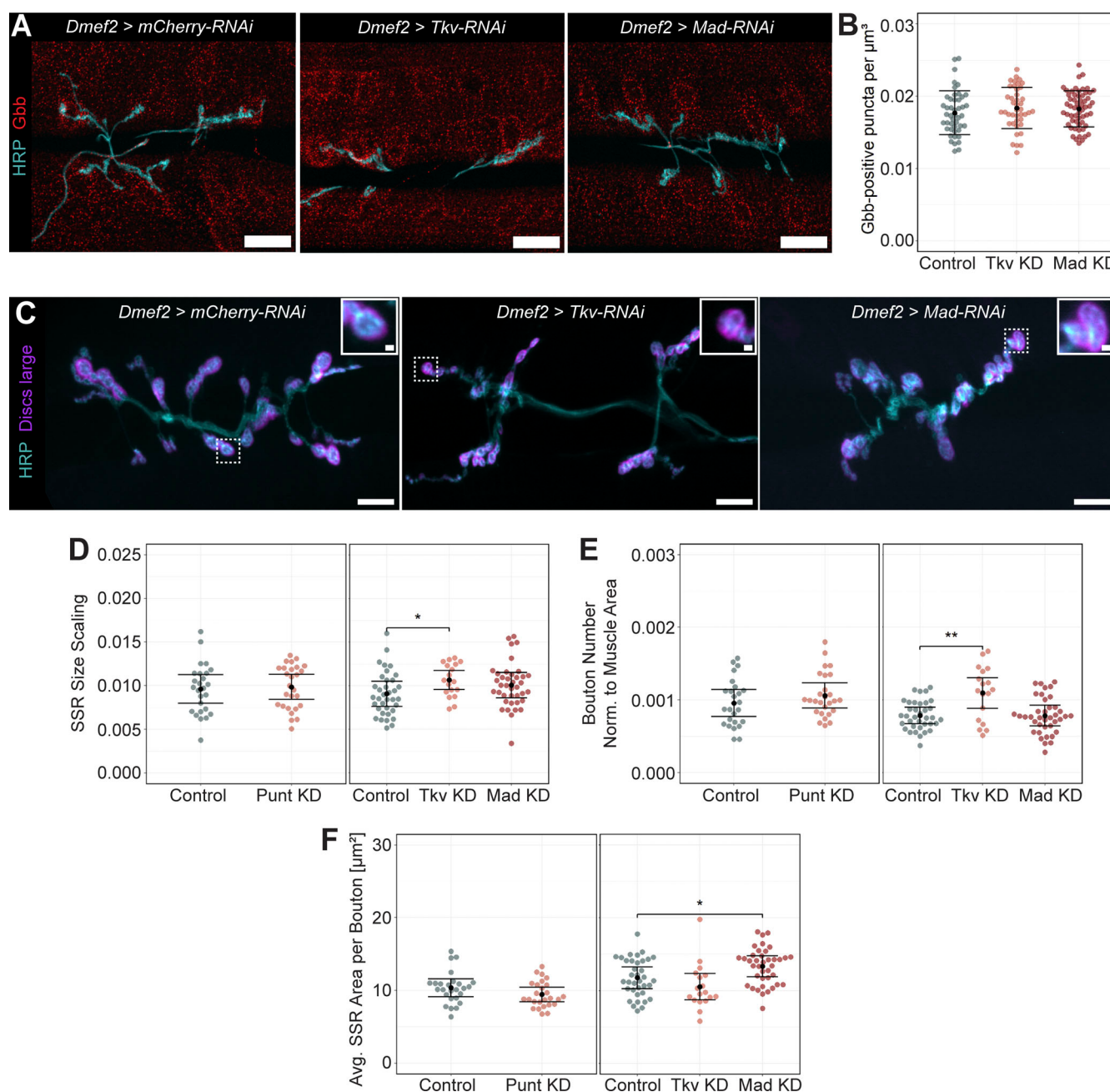


Figure 5. Postsynaptic BMP signaling acts independently of the retrograde BMP pathway to influence NMJ structure. (A) Confocal images (maximum intensity projection) showing muscles of the indicated genotypes labeled for presynaptic NMJ marker HRP (cyan) and BMP ligand Gbb (red). Scale bars = 30 μm . (B) Quantification of Gbb density (puncta per μm^3) in VL3 and VL4 muscles of the indicated genotypes (control: $n = 10$ larvae, 46 muscles; Tkv KD: $n = 12$ larvae, 42 muscles; Mad KD: $n = 13$ larvae, 58 muscles). Data are shown as mean $\pm$ SD. (C) Confocal images (sum slice projection) of VL3/VL4 NMJs showing postsynaptic (anti-Dlg, magenta) and presynaptic (anti-HRP, cyan) structures in control, Tkv, and Mad KD muscles. For each genotype, an individual 1b bouton and surrounding SSR (dashed line boxes) is magnified in the top right corner. Scale bars = 10 and 1 μm in magnified insert. (D–F) Quantification of (D) SSR size scaling (SSR area based on anti-Dlg labeling normalized to total VL3/VL4 muscle cell area), (E) 1b bouton number normalized to the muscle cell area, and (F) SSR area per bouton (SSR area divided by 1b bouton number). Plots show mean $\pm$ SEM ($n = 26$ –36 control, 27 Punt KD, 18 Tkv KD, and 39 Mad KD NMJs). Statistical comparisons were made for all groups with significant differences noted (* $P < 0.05$, ** $P < 0.01$). Distinct plots with different controls are included for Punt KD compared to Tkv KD and Mad KD due to the experiments being conducted separately.

is consistent with a reduction but not a loss in GluRIIA protein at the postsynapse and suggests that BMP signaling promotes the postsynaptic response to NMJ neurotransmission.

Last, we performed a locomotion assay to measure neuromuscular function on a larger scale. We quantified larval

locomotion to calculate average velocity. Larval crawling velocity was reduced by ~ 19 –22% for larvae with Punt KD, Tkv KD, or Mad KD muscles compared with control (Fig. 6 G). These data are consistent with our previous work showing that an increase or decrease of nuclear DNA content in larval muscles

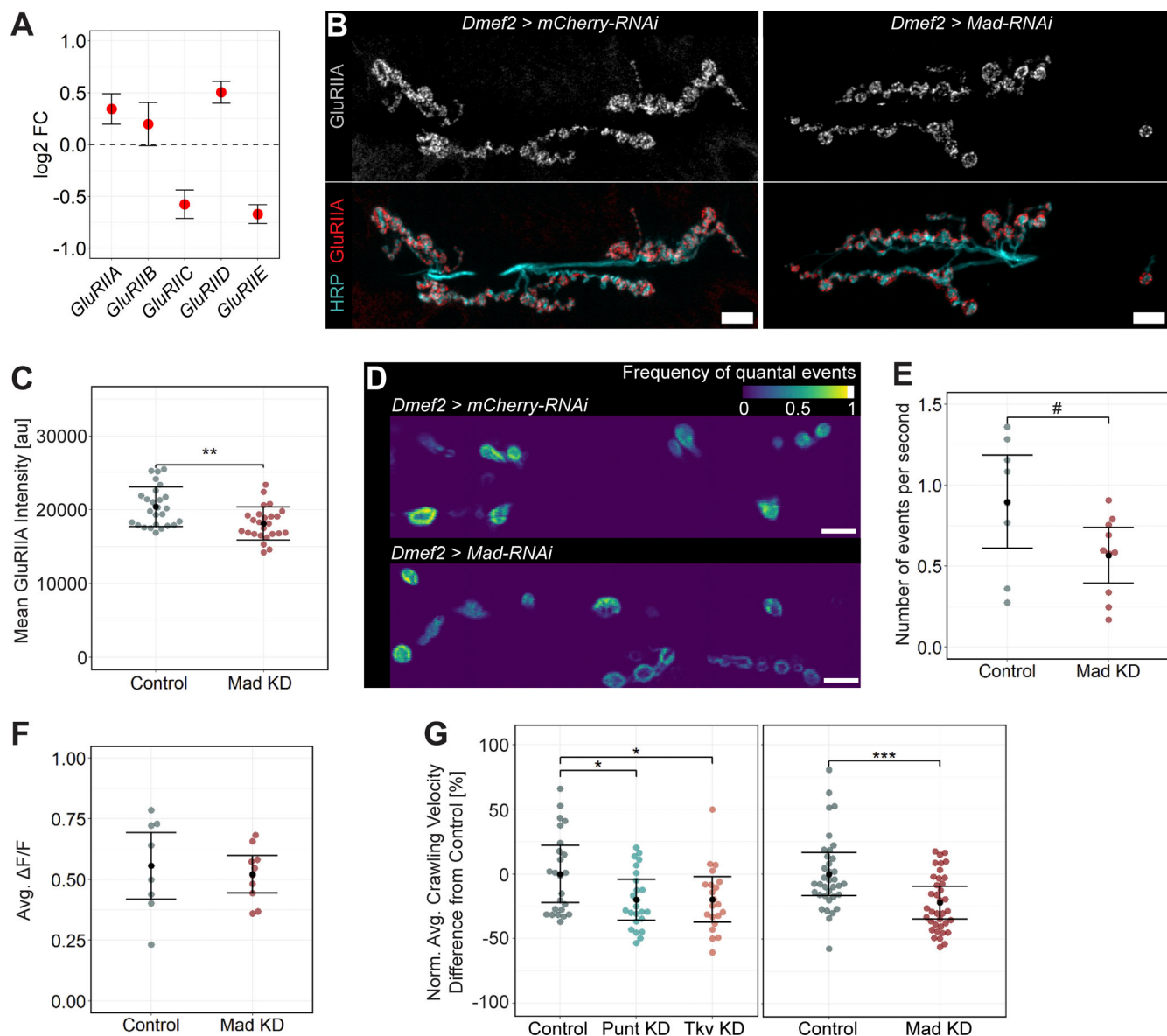


Figure 6. Postsynaptic BMP signaling influences NMJ gene expression and NMJ function. (A) Fold change of glutamate receptor (GluRII) subunits in Mad KD compared with control muscles from RNA sequencing analysis ($N = 3$ replicates, $n = 6$ –10 larvae per genotype). Data are shown as mean $\pm$ SD. (B) Confocal images (sum slice projection) of the VL3/VL4 NMJ labeled for HRP (cyan) and GluRII subunit A protein (GluRIIA, red and shown individually in gray) in control and Mad KD muscles. Scale bars = 10 μ m. (C) Mean GluRIIA intensity at the control and Mad KD NMJs ($n = 26$ control and 25 Mad KD NMJs). Data are shown as mean $\pm$ SD (** $P < 0.01$). (D) Confocal images (sum slice projection) of control and Mad KD NMJs expressing Ca^{2+} indicator GCaMP6f with SSR localization (*Mhc-CD8-GCaMP6f-Sh*). Pseudocoloring indicates the frequency of quantal events, defined as the change in GCaMP6f fluorescence from baseline ($\Delta F/F$) above a threshold value, at the NMJs of control and Mad KD muscles over time (20–40 s). Scale bars = 10 μ m. (E and F) Quantification of the quantal event (E) frequency and (F) amplitude at the NMJs of control and Mad KD muscles ($n = 8$ control and 10 Mad KD NMJs). (G) Mean crawling velocity, normalized to larval length and plotted as percent difference from the control average, for larvae with control, Punt KD, Tkiv KD, and Mad KD muscles (Control: $n = 24$ –37 larvae; Punt KD: $n = 24$ larvae; Tkiv KD: $n = 21$ larvae; Mad KD: $n = 36$ larvae). Data are shown as mean $\pm$ SEM (# $P = 0.067$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Distinct plots with different controls are included for Punt KD and Tkiv KD compared with Mad KD due to the experiments being conducted separately.

results in reduced larval velocity (Windner et al., 2019) and confirm that postsynaptic BMP signaling is required for optimal muscle function.

Discussion

Each skeletal myofiber in mammals contains hundreds of myonuclei, all sharing one cytoplasm and contributing to

optimal muscle function. Several myonuclei are physically clustered at the NMJ and have a distinct transcriptional profile. Whether these myonuclear specializations are conserved in *Drosophila* and how synaptic myonuclei coordinate their gene expression with NMJ requirements are unclear. In this study, we identified synaptic myonuclei in *Drosophila* larval muscles. Postsynaptic BMP signaling results in an intracellular gradient of nuclear pMad with the highest signal in synaptic myonuclei,

which regulates endoreplication, via *E2f1*, and NMJ activity, via NMJ-related genes (i.e., GluRII subunits). Our data suggest that local activation of BMP signaling affects muscle cell size, nuclear size, DNA content, and synaptic properties. Altogether, this study reveals how local signaling in a syncytial cell orchestrates nuclear activity for optimal cell function.

Synaptic myonuclei in *Drosophila* larval muscles

The skeletal muscle's postsynapse is characterized by the accumulation of the membrane and proteins necessary for synaptic integrity and signal transmission. Data from mammals suggest that growth and maintenance of the postsynaptic protein milieu requires local production of specific mRNAs that are provided by (sub)synaptic myonuclei (Belotti and Schaeffer, 2020). As such, synaptic myonuclei are important contributors to NMJ activity. Here, we identify synaptic myonuclei at the *Drosophila* NMJ by their distance to the NMJ (Figs. 1 and S1), like previous studies in mammalian myofibers (Bai et al., 2022; Grady et al., 2005). We uncover several specific adaptations in *Drosophila* synaptic myonuclei: (1) increased nuclear size scaling, signifying that each myonucleus has ample potential to meet local signaling and metabolic demands; (2) greater DNA content, which increases transcriptional capacity; and (3) higher levels of BMP signaling (pMad) indicating differential transcription in synaptic compared with non-synaptic myonuclei.

In contrast to mammals, *Drosophila* synaptic and non-synaptic myonuclei have similar internuclear distances and do not cluster under control conditions. However, a previous study reported specific nuclear spacing for NMJ-adjacent myonuclei in VL3 muscles, which is regulated by microtubules and associated motor proteins (Perillo and Folker, 2018). We observed a VL3-specific increase in the percent of synaptic nuclei for Tkv KD and Mad KD muscles, suggesting that *Drosophila* muscles use nuclear positioning to compensate for defective synaptic nuclear properties. Altogether, our data strongly suggest that myonuclear changes at the NMJ are a conserved muscle feature. While mammalian synaptic myonuclei are visibly clustered (Hansson et al., 2020), *Drosophila* larval muscles establish a gradient of nuclear size scaling and DNA content to achieve local nuclear adaptations.

A recent study identified a reliable protein marker for mammalian synaptic myonuclei, the nuclear anchoring protein Nesprin-1, which when used in combination with proximity to the NMJ can better distinguish synaptic from non-synaptic myonuclei than NMJ proximity alone (Ruiz et al., 2023). In *Drosophila*, no protein has yet been uncovered to distinguish synaptic myonuclei. While our study indicates pMad as a promising marker, a nuclear structural protein would be better than a diffusible signaling effector to identify these nuclei. In the absence of such a protein marker, it is possible that we are overestimating the number of synaptic myonuclei per NMJ or masking subpopulations within the synaptic myonuclei. Nuclei that spatially overlap with the SSR could experience shorter-range NMJ signals that may have additional effects on gene expression. Future work identifying markers specific to synaptic myonuclei in *Drosophila* would be highly useful to corroborate our distance-based identification approach. Importantly, our work indicates several measures (distance, size, and DNA

ploidy) to identify and study synaptic myonuclei in *Drosophila*, thereby providing an important platform for future studies.

BMP signaling creates an intracellular pMad gradient

In mammals, synaptic myonuclei transcribe genes encoding NMJ components in response to local signals that relay information on synaptic activity (DeChiara et al., 1996; Ruegg and Bixby, 1998; Sanes and Lichtman, 2001). Of the many local signals, we investigated the BMP pathway as it plays an important role in the mammalian NMJ. In mammals, BMP receptor complexes associate with the postsynaptic regulator MuSK to promote BMP target gene expression (Jaime et al., 2024; Yilmaz et al., 2016) and to regulate overall NMJ structure and postsynaptic localization of voltage-gated sodium channels (Nav1.4) required for muscle fiber excitability (Fish et al., 2023, Preprint). As in mammals, BMP receptors are also found in the postsynaptic membrane of *Drosophila* muscles (Chou et al., 2013; Dudu et al., 2006). We measured BMP signaling activity by quantifying nuclear pMad, the active BMP transcriptional effector, and observed a striking intracellular gradient, with the highest levels within the synaptic myonuclei and the lowest levels within the non-synaptic myonuclei (Fig. 2). Whether BMP signaling in mammals also has specific effects on synaptic myonuclei remains to be determined.

Signaling gradients are usually established by the distribution of ligands to transmit positional information across the extracellular space. In contrast, syncytial cells that contain multiple nuclei in one large cytoplasmic space can use downstream effectors to convey positional information within the cell. In *Drosophila*, a nuclear transcription factor gradient has been described specifically for Bicoid in the syncytial blastoderm (Ali-Murthy and Kornberg, 2016). In muscle, the robustness of the pMad gradient is remarkable given that muscle cells contract, which likely results in continuous cytoplasm mixing (Koslover et al., 2017). In mammalian myofibers, the transcription factor GABP is globally expressed but specifically phosphorylated by local signaling pathways to activate NMJ gene transcription in synaptic myonuclei (Belotti and Schaeffer, 2020). We also find localized transcription factor phosphorylation in *Drosophila* muscles, revealing that similar mechanisms could regulate gene expression in synaptic myonuclei in mammals and *Drosophila*. By showing different pMad gradients downstream of BMP type I and type II receptor manipulations, our data further indicate that phosphorylation may also affect the distribution of the activated transcription factor. Synaptic and non-synaptic myonuclei contain similar levels of transcriptional repressor Shn, thereby, inhibition of gene expression likely occurs equally across myonuclei by repressive pMad-Shn transcriptional complexes. While the mechanisms that regulate the expression of pMad target genes in synaptic compared with non-synaptic myonuclei remain to be deciphered, localized transcription factor activation (i.e., phosphorylation) seems to be a conserved feature of muscle cells that establishes positional information and generates localized adaptations to promote post-synaptic development and function.

BMP signaling affects myonuclear size and DNA content

As synaptic myonuclei show enrichment for pMad, we dissected the postsynaptic BMP signaling pathway to understand the role

of BMP signaling in synaptic myonuclear adaptations. Muscle KD of each BMP pathway component (Punt, Tkv, and Mad) resulted in smaller muscles (i.e., smaller cytoplasmic domain sizes) and smaller nuclei, but maintained most differences between synaptic and non-synaptic nuclei (Figs. 3, 4, and S2). In addition, we found genotype-specific phenotypes that further highlight the complexity of BMP signaling. Overall, KD of Tkv had mild or no effects on nuclear size and DNA content/ploidy and did not alter differences between synaptic and non-synaptic nuclei. This is attributed to a smaller reduction in BMP signaling as evidenced by pMad labeling due to several factors: (1) achieving only ~20% KD of highly expressed Tkv in larval muscles and/or (2) larval muscles expressing two type I BMP receptors, Tkv and Sax, thereby Sax-mediated signaling may provide some compensation.

Punt KD in muscles produced a similar reduction in nuclear pMad levels (~17%) as Tkv KD, yet resulting in the most robust reduction in size and DNA content (less 64C, more 32C ploidy) with the effects on DNA content being stronger in synaptic than non-synaptic nuclei. Punt is believed to be the only BMP type II receptor expressed in larval muscle (Akiyama et al., 2024), where it functions to phosphorylate the type I receptor for activation. However, Punt also associates with Activin receptors (Babo), and like BMP, the Activin pathway has been shown to influence NMJ activity along with muscle size (Ellis et al., 2010; Fuentes-Medel et al., 2012; Kim and O'Connor, 2014; Kim and O'Connor, 2021). It will be of interest in future studies to assess whether Activin signaling through Punt-Babo receptor activity regulates intracellular heterogeneity by specifically affecting synaptic myonuclear adaptations.

Muscle KD of Mad, the transcriptional effector of BMP signaling, reduced nuclear pMad levels the most (50%) and showed robust effects on size and DNA content with significant DNA reduction in synaptic and non-synaptic nuclei. In addition to the overall reduction in muscle size and nuclear size, Mad KD results in reduced nuclear size scaling specifically in the synaptic myonuclei. To provide sufficient gene products to the cytoplasmic domains as well as the NMJ, transcription and/or translation would need to be upregulated. Indeed, we observed that pathways related to transcription and translation were upregulated (e.g., cellular response to unfolded protein, polytene chromosome puffing) in Mad KD muscles. However, expression of *elF4E3*, a component of the postsynaptic translational machinery localized to the NMJ, and a locally translated protein, GluRIIA subunit, were significantly reduced in Mad KD muscles. Therefore, these data suggest insufficient compensation of synaptic nuclei in Mad KD muscles to meet the demand for critical postsynaptic proteins.

Our data indicate that BMP signaling directly regulates DNA endoreplication and thus nuclear DNA content. In support, we found reduced expression of the endoreplication regulator *E2f1* with muscle Mad KD and potential Mad binding sites in *E2f1* regulatory regions. Furthermore, pMad was previously shown by ChIP sequencing to bind to *E2f1* in the *Drosophila* embryonic mesoderm (Junion et al., 2012) and intersect with *E2f1* activity to promote endoreplication in *Drosophila* prostate-like secondary cells (Sekar et al., 2023). *E2f1* promotes the G1-S phase transition

and is required for DNA replication in cells undergoing mitosis and endoreplication (Duronio et al., 1995). *E2f1* transcription is not under cell cycle control, but levels of *E2f1* protein and its regulated transcripts (e.g., *CycE*) do oscillate with the cell cycle due to posttranscriptional regulation of *E2f1* (Øvrebø et al., 2022; Zielke et al., 2011). This specific translational control of the *E2f1* function may explain why our BMP manipulations only mildly reduced DNA content. Like in flies, myonuclei in mammals are recently understood to undergo endoreplication to increase DNA content (Borowik et al., 2022), but the regulation of this process is unknown. We propose that in larval muscle, endoreplication is regulated by a nuclear pMad gradient and that this intracellular pattern is involved in establishing myonuclear heterogeneity and a postsynaptic increase in DNA content.

A deficit in endoreplication of nuclear DNA content could also indirectly affect nuclear size and muscle growth. *E2f1* was shown in *Drosophila* adult skeletal muscle to regulate muscle mass by controlling the expression of several structural genes (e.g., *Tm1*) (Zappia and Frolov, 2016). We also observed the downregulation of multiple structural genes in Mad KD muscles that encode components of the myofiber's contractile apparatus (Fig. S3). Furthermore, we observed a downregulation of genes involved in metabolic pathways in Mad KD muscles that are known to promote muscle growth (Demontis and Perrimon, 2009; Piccirillo et al., 2014). Thus, BMP signaling could affect muscle cell size directly by promoting the expression of structural and metabolic genes and/or indirectly via *E2f1* and regulation of DNA content.

Postsynaptic BMP signaling contributes to NMJ size, synaptic activity, and muscle function

BMP signaling at the *Drosophila* NMJ is largely studied as a retrograde pathway that activates presynaptic BMP receptors and drives neuronal gene expression to promote NMJ growth through modulating presynaptic actin dynamics and bouton budding (Aberle et al., 2002; Ball et al., 2010; Marqués et al., 2002; McCabe et al., 2003; Piccioli and Littleton, 2014). Our findings suggest that postsynaptic BMP signaling independently plays a role in NMJ size regulation. We find that KD of Tkv disrupts the size relationship between the muscle and the NMJ, including both the presynaptic and postsynaptic sides. This scaling disruption does not occur in Mad KD nor Punt KD muscles and may be driven by non-canonical Tkv activity. Non-canonical BMP signaling has previously been reported for the neuronal type II receptor Wit that can associate with actin regulator Limk to regulate the NMJ (Piccioli and Littleton, 2014); therefore, a non-canonical BMP pathway could also exist in the postsynaptic muscle.

Mad KD increased the SSR size per bouton (Fig. 5). An increase in the size of the SSR, due to greater membrane folds or less compaction between folds, has previously been observed following muscle overexpression of SSR-associated proteins and motor neurons with increased activity (Budnik et al., 1996; Teodoro et al., 2013). SSR size can affect synaptic activity; for instance, boutons with normally large SSRs (e.g., type Ib) have slower mEJP rise times and produce reduced postsynaptic voltage changes in the muscle compared to boutons with smaller

surrounding SSRs (e.g., type Is) (Nguyen and Stewart, 2016). In addition to SSR size, our data indicate that postsynaptic BMP signaling also affects the expression of glutamate receptor (GluRII) subunits at the mRNA and protein levels (Fig. 6). Reduced GluRII protein and increased SSR size likely both restrict synaptic function, as evidenced by reduced frequency of spontaneous quantal events in Mad KD muscles, signifying an impaired postsynaptic response to neurotransmitter release. However, our NMJ structural results are based on confocal microscopy and further ultrastructural studies will be needed to confirm that SSR size is affected with no corresponding presynaptic bouton changes in Mad KD muscles. In accordance with previous studies, we suggest that postsynaptic BMP signaling supports proper NMJ activity and ultimately muscle function by regulating the transcription of GluRII subunits and through promoting the expression of postsynaptic machinery (i.e., eIF4E3) involved in local GluRII translation (Rawson et al., 2003; Sulkowski et al., 2016).

Altogether, our study introduces postsynaptic adaptations in *Drosophila* that include local changes in DNA content and size scaling of adjacent myonuclei and works toward a more comprehensive understanding of BMP pathway activation and its role at the NMJ. Postsynaptic signaling and the role of synaptic myonuclei have been of great interest in the context of aging and muscle diseases where the loss of synaptic myonuclei corresponds with NMJ structural and functional defects. A recent study revealed that activation of 4EBP1, a mRNA translation regulator, led to increased acetylcholine receptor subunit expression and neurotransmission in aged mice (Ang et al., 2022). Based on mammalian studies and our data, postsynaptic BMP signaling could also be a good target for intervening in conditions that implicate NMJ activity in their initiation and/or progression.

Materials and methods

Drosophila husbandry, stocks, and crosses

Experimental crosses of *Drosophila melanogaster* were kept on a standard cornmeal medium at 25°C in 12-h light/12-h dark conditions under humidity control. RNA interference and overexpression studies relied on the Gal4-UAS system (Brand and Perrimon, 1993). For muscle-specific expression, we used the *Dmef2-Gal4* driver line (Ranganayakulu et al., 1998) crossed with the following UAS lines that target core components of the BMP signaling pathway: *UAS-Mad-RNAi* (12635; Vienna *Drosophila* Resource Center [VDRC]; 31316; Bloomington *Drosophila* Stock Center [BDSC]), *UAS-tnv-RNAi* (35653; BDSC), and *UAS-put-RNAi* (848; VDRC). The driver lines were crossed to *UAS-mCherry-RNAi* (35785; BDSC) for the control RNAi. We recombined the *Dmef2-Gal4* driver line with *Mhc-CD8-GCaMP6f-Sh* line (Newman et al., 2017) (67739; BDSC) to create *Dmef2-Gal4, Mhc-CD8-GCaMP6f-Sh* flies for quantal live imaging.

Larval staging and muscle selection

For all experiments, both male and female larvae were used at the wandering third instar stage. Staging was confirmed using the developmental landmarks of mouth hook and spiracle

morphologies (Bodenstein, 1950). Larval body wall muscles ventral longitudinal 3 (VL3) and 4 (VL4) were studied in anterior abdominal hemisegments 2 and 3 due to consistent muscle size and NMJ span and size.

RNA isolation and qRT-PCR

Six to fifteen larvae at the third instar wandering stage were dissected and rinsed in ice-cold HL3.1 medium (Feng et al., 2004) with all internal organs removed and the larval body wall kept intact as previously described (Brent et al., 2009). The head and tail segments of the larva were also removed. Total RNA was isolated from muscle-enriched larval fillets using TRIzol reagent (15596026; Thermo Fisher Scientific) and chloroform extraction, followed by purification with the TURBO DNA-free Kit (AM1907; Thermo Fisher Scientific). cDNA was synthesized from 1 µg of RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (18080-051; Thermo Fisher Scientific), and qRT-PCR reactions performed using the CFX96 Real-Time PCR system (BioRad) with SYBR Select Master Mix for CFX (4472942; Applied Biosystems). Three independent mRNA preparations per genotype were made and analyzed in triplicate. Relative gene expression was calculated relative to *Rpl32* by the delta-delta Ct method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Data are shown as fold change in gene expression and ΔCt values were used for statistical comparison. Table S1 contains information on the oligonucleotides used.

RNA sequencing and analysis

Six to ten wandering third instar larvae per genotype were dissected per replicate as described above for a total of three replicates. Total RNA was isolated using TRIzol and chloroform extraction from the muscle-enriched larval fillets. The lysates were submitted to the Memorial Sloan Kettering Cancer Center (MSKCC) Integrated Genomics Operations (IGO) core for RNA quality assessment and Illumina next-generation sequencing. The sequencing libraries were constructed using Poly A enrichment following oligo-dT-mediated purification. The libraries were then sequenced on the Illumina HiSeq 4000 in a PE50 run. Over 40 million reads were generated per library. FASTQ data was processed in R with the ShortRead package (Morgan et al., 2009) and quality control was conducted with the Rfastp package (Wang and Carroll, 2023). Reads were mapped and aligned to the *Drosophila* genome (release dm6) using Rsubread (Liao et al., 2019) and counted with the GenomicAlignments package (Lawrence et al., 2013). Gene differential expression analysis was performed using DESeq2 (Love et al., 2014). Last, enrichment analysis was performed with Goseq (Young et al., 2010) using the GO database.

Immunolabeling and confocal imaging

Larvae were dissected in silica plates in ice-cold HL3.1 buffer as described in Windner et al. (2019). Muscles were fixed with 4% paraformaldehyde in HL3.1 for 20 min for all antibodies except GluRIIA for which samples were fixed in Bouin's fixative (15990; Electron Microscopy Sciences) for 5 min. Larval fillets were washed in PBT (0.3% Triton X-100 in PBS pH 7.4) and transferred into tubes for immunolabeling. Samples were blocked in

PBT-BSA (0.1% BSA, 0.3% Triton X-100 in PBS pH 7.4) for 30 min and incubated with the following primary antibodies overnight at 4°C: mouse anti-Lamin C (LC28.26; 1:50; DSHB), mouse anti-Dlg (4F3; 1:200–1:300; DSHB), guinea pig anti-phosphorylated Smad 1/5/8 (a gift from E. Laufer; 1:500; previously shown to be specific to *Drosophila* phosphorylated Mad in Guo et al. [2013]), mouse anti-GluRIIA (DSHB, 8B4D2 [MH2B]; 1:50), rabbit anti-Schnurri3 (a gift from F.M. Hoffmann and G. Boekhoff-Falk; 1:100; previously published in Staehling-Hampton et al. [1995]), or mouse anti-Gbb (3D6-24; 1:50; DSHB). Then larval fillets were washed in PBT-BSA and incubated with Alexa Fluor-conjugated secondary antibodies, F-actin probe 488-conjugated Phalloidin, and 647-conjugated anti-Horseradish Peroxidase (HRP) that labels insect neuronal membranes, and/or Hoechst 33342 for DNA at a 1:400 concentration for 1 h at room temperature. Last, larval fillets were washed in PBT, mounted on glass slides, and cured over 24 h in ProLong Gold (P36930; Invitrogen). All antibodies used are described in Table S1. Confocal z-stacks of VL3 and VL4 muscles were acquired using an A1R laser-scanning microscope (Nikon) with an oil-immersion objective (CFI Plan APO Lambda S 40× Sil/1.25). The same confocal laser and system settings were used for all samples under direct comparison. Approximately, seven larvae of each genotype were dissected, stained, and imaged for each experiment that was repeated for a total of three independent experiments unless otherwise noted.

Muscle nuclear size, DNA content, nuclear pMad, nuclear Shn, and position analysis

Sum slice projections of confocal z-stacks were made in ImageJ (Fiji) for 2D quantification of VL3 and VL4 muscles. Muscle areas for VL3 and VL4 were traced by hand as determined by phalloidin labeling using the polygon selection tool. These cell outlines were used to measure muscle cell area and the x- and y-coordinates of the anterior and posterior ends of individual cells. Automated thresholding of fluorescence intensities using the triangle mode of Lamin C and/or Hoechst labeling was used to create binary images of VL nuclei. Nuclear number, size (areas), and position (centroids) within each muscle cell were recorded. The binary images served as masks to measure Hoechst, nuclear pMad, and nuclear Shn fluorescence intensities (sum of pixel values in a nuclear area). Hoechst values were used to estimate DNA content and ploidy as previously done (Dej and Spradling, 1999; Losick et al., 2016; Sher et al., 2013; Unhavaithaya and Orr-Weaver, 2012; Windner et al., 2019). In addition, nuclear centroids were used to determine nearest neighbor distances and make Voronoi tessellations to measure cytoplasmic domain areas (Du et al., 2010). Average nuclear values for all nuclei in an individual muscle cell from 6 to 13 muscles per experiment across three replicates were graphed and used for statistical analyses.

Nuclear distance to the NMJ measurement and identification of synaptic nuclei

Sum slice projections used for muscle nuclear analyses were also used to generate binary images of the NMJ for the VL3 and VL4 muscles. This was done by automated thresholding of Dlg labeling using Triangle mode in Fiji that included both 1b and 1s

synapse elements. The shortest distance from multiple objects (nuclear centroids) to one feature (the NMJ) was determined using a Fiji macro (Michael Cammer and others at NYU Langone Medical Center; available at https://microscopynotes.com/imagej/shortest_distance_to_line/index.html). The NMJ was divided for VL3 and VL4 based on Dlg and phalloidin labeling, and then the shortest nuclear distance to the NMJ was measured for nuclei of an individual muscle cell, calculated separately for VL3 and VL4. An average nuclear distance to the NMJ (d) was calculated for each muscle cell using the individual nuclear distances to the NMJ's SSR (nearest Dlg positive element). Synaptic nuclei were classified as nuclei positioned near ($\leq d$) the SSR and non-synaptic nuclei as those positioned farther away ($> d$) from the SSR.

NMJ size analyses

Sum slice projections of confocal z-stacks of Dlg immunostaining were made in ImageJ (Fiji) for 2D quantification of the NMJ for the VL3/VL4 muscle pair. We performed automated thresholding of Dlg fluorescence intensity using Yen mode to create binary images of the VL3/4 NMJ. This thresholding approach produced a binary of 1b synapse components only, purposefully excluding 1s synapse components due to the difficulty of reliably measuring 1s synapse area and number for the VL3/4 NMJ. The size (area) of the Dlg-positive SSR, the NMJ's post-synaptic element, was recorded. The number of 1b boutons was quantified by using the counter tool and counting the number of Dlg-positive rings that surround 1b boutons, and optical slice by optical slice in the confocal z-stack. The average SSR area per bouton was calculated by dividing the SSR area by 1b bouton number. The SSR scaling was determined by dividing SSR area by total muscle area for VL3 and VL4, with area determined for the individual muscles as described above (see "Muscle nuclear size, DNA content, nuclear pMad, nuclear Shn, and position analysis"). Values for individual VL3/VL4 NMJs (7–13 NMJs per genotype from one experiment) across three replicates were graphed and used for statistical analyses.

Quantification of muscle Gbb

In Imaris 10.0 (Bitplane), confocal z-stacks of phalloidin, Gbb, and HRP immunostainings were processed. Surfaces were created manually for VL3 and VL4 muscles separately based on phalloidin labeling. The spots function was used to detect Gbb-positive puncta (diameter equals 1 μm) in 3D under automated thresholding within the VL3 and VL4 surfaces. The number of Gbb-positive puncta and the surface volumes (μm^3) were recorded for each muscle. The number of Gbb-positive puncta per μm^3 was calculated by dividing the number of puncta by the surface volume of an individual muscle cell. Muscles were excluded from analysis if there were HRP-positive motor axons overlayed on the muscle, an artifact of dissection, since Gbb is expressed both in the muscle and motor neuron and would impact Gbb count. This analysis was conducted in duplicate with 42–58 muscles from 10 to 13 larvae per genotype.

Glutamate receptor intensity analyses

In Imaris 10.0 (Bitplane), confocal z-stacks of GluRIIA and HRP immunostainings were processed as previously done

(Christophers et al., 2024). A three-dimensional surface for HRP was generated with a surface detail grain level of 0.355 μm , smoothing enabled, and auto-thresholding. Small surfaces were removed that were outside of the NMJ. A mask was created from the HRP surface using the distance transform setting and then this mask was used to create a second surface, with a surface detail level of 0.355 μm and a manual threshold of 0–0.5 μm to limit the final surface to a shell extending from the edge of the HRP surface to 0.5 μm away. The sum of the sum intensity of the GluRIIA channel within this 0.5 μm -wide surface was recorded and normalized to the expanded HRP volume. This analysis was conducted in duplicate with 25–26 NMJs from nine larvae per genotype.

Quantal live imaging and analysis

Seven to nine wandering third instar larvae were dissected and pinned to visualize the body wall muscles in ice-cold HL3.1 buffer on 5.5-cm wide, sylgard plates. Live VL3/VL4 NMJs in abdominal hemisegment 3 were imaged at ambient temperature using a Stellaris 5 laser-scanning confocal microscope (Leica) with a water-immersion objective (HC FLUOTAR L VISIR 25x/0.95) and HyD S detector in counting mode. Confocal images of SSR-localized GCaMP6f signal were acquired continuously for 2–20 s sessions by scanning bidirectionally at 1,000 Hz, with a pixel size per voxel size of 0.728 μm and an area size of 512 $\times$ 100 pixels to provide a frame rate of 18.18 frames per second with 2.25x zoom applied in Leica LASX software. The pinhole size was 65.1 μm , calculated at 1.5 A.U. for 451 nm emission. Images were analyzed in FIJI based on a published protocol (Chen et al., 2024) followed by manual setting of the threshold ($\Delta F/F = 0.25$) to separate signal to noise for peak event identification in Python using the sci-kit image package. Quantal event frequency was calculated as the number of peak events over time, and quantal event amplitude was the average fluorescence ($\Delta F/F$) of the peak events. Due to larval movement, only positionally stable NMJs were included for analysis that were collected over a minimum of 20 s, ranging from a total of 20–40 s. This analysis was conducted in duplicate with 8–10 NMJs from 7 to 9 larvae per genotype.

Larval locomotion assay and tracking

Wandering third instar larvae were extracted from food vials and placed, one at a time, in the center of a 15-cm apple juice agar plate (stained blue with food dye for improved contrast). Each larva was recorded for 3 min or until the larva reached the plate's edge, using an iPhone 14 Pro Max. 9–12 larvae per genotype were recorded for a total of two or three replicates. Movie files were then converted into image sequences (one frame per second) in Adobe Photoshop. The image sequences were uploaded to ImageJ (FIJI) and calibrated using a ruler captured alongside the plate. Each larva was tracked for the first 53 s of recording by tracing the posterior end of the larva using the Manual Tracking plug-in. Larval length was measured by taking the distance of a straight line from the anterior to posterior ends of the larva during an initial frame when the larva was straight and not actively crawling. Average velocity was calculated per larva with the exclusion of larval stops (frames with no forward movement) and normalized to larval length.

Statistical analysis

Correlation coefficients (R), pairwise comparisons between two groups determined by two-tailed student's *t* test (alpha of 0.05), and three or more group comparisons determined by one-way ANOVA were computed using R statistical software (version 1.4.1717). Data are shown as mean $\pm$ SEM, unless otherwise noted, with asterisks used to denote significance level ($P = * < 0.05$, $** < 0.01$, $*** < 0.001$, $**** < 0.0001$) and the sample size reported in the figure legend. All plots were made using R with the ggplot2 package (Wickman, 2016).

Online supplemental material

Further details about nuclear and RNA analyses in *Drosophila* body wall muscles in control and following muscle-specific BMP manipulations are available in the supplemental material. Fig. S1 shows the characterization of synaptic myonuclei in *Drosophila* larval VL3 and VL4 muscles. Fig. S2 shows muscle cell size and myonuclear proximity to the NMJ following KD of BMP signaling components. Fig. S3 shows genes and pathways associated with muscle growth are affected by KD of postsynaptic BMP signaling components. Fig. S4 shows that predicted Mad binding sites are identified at the *E2f1* locus. Fig. S5 shows that the expression of GluRII subunits and postsynaptic mRNA translation regulator eIF4E3, but not BMP ligand Gbb is dysregulated in Mad KD muscles. Table S1 lists antibodies and oligonucleotides used in the study.

Data availability

The data underlying Figs. 1, 2, 3, 4, 5, and 6; and Figs. S1, S2, S3, S4, and S5 are available in the published article and its online supplemental material. The RNA sequencing data underlying Figs. 4 and 6; and Figs. S3 and S5 are openly available in Gene Expression Omnibus (GEO) (GSE263700).

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Visualization, Writing - original draft, Writing - review & editing, S.E. Windner: Methodology, Writing - review & editing, G. Armetta: Formal analysis, Writing - review & editing, M.K. Baylies: Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

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Supplemental material

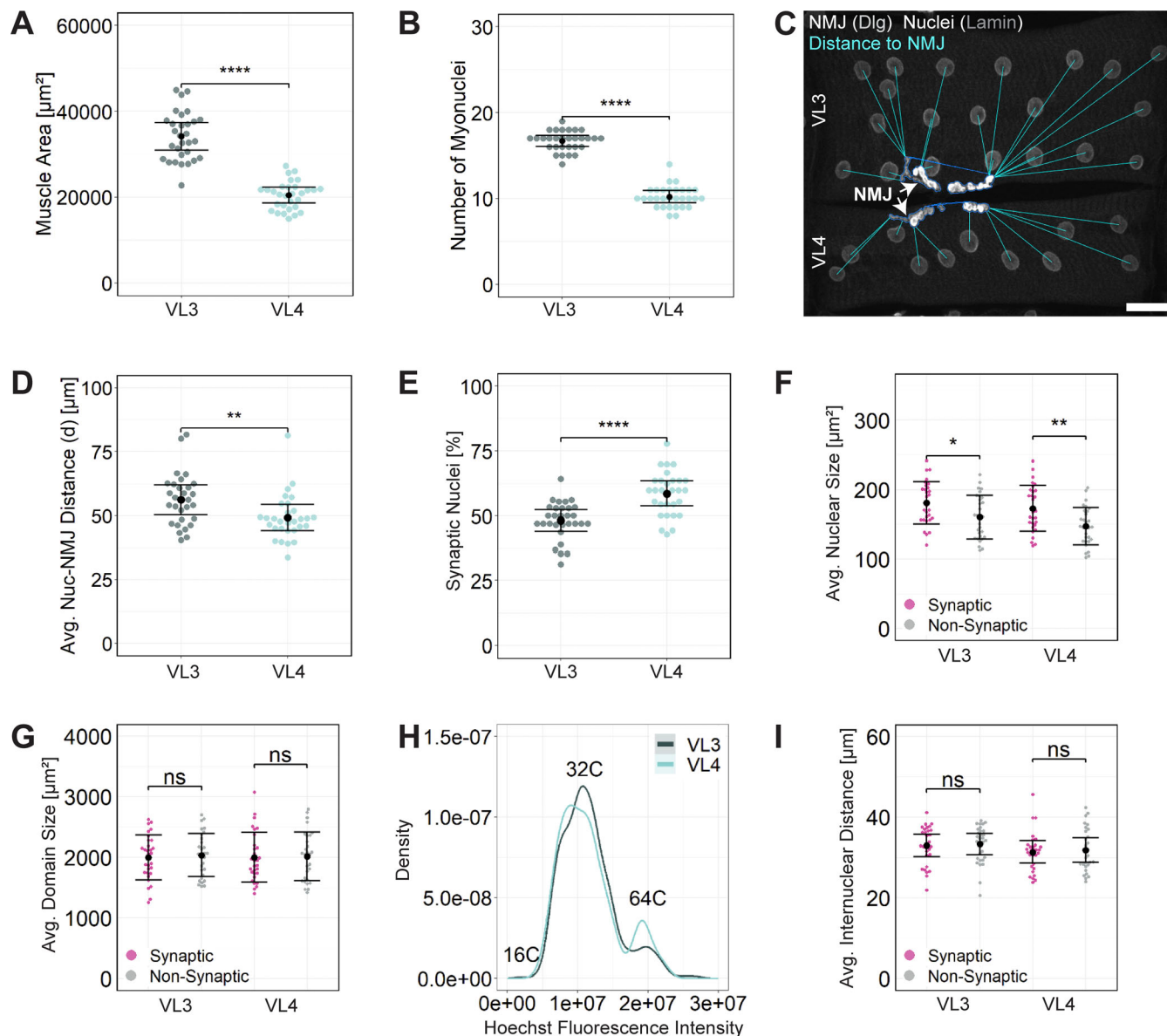


Figure S1. **Characterization of synaptic myonuclei in *Drosophila* larval VL3 and VL4 muscles.** (A) Average VL3 and VL4 muscle area based on actin (phalloidin). (B) Myonuclear number for individual VL3 and VL4 muscles. (C) Confocal image (sum slice projection) of a VL3/VL4 muscle pair labeled for NMJ marker Dlg (white, arrows) and nuclear envelope protein Lamin C (gray). Cyan lines indicate the direct distance between individual myonuclear centers and the nearest NMJ component in VL3 and VL4 muscles. Scale bar = 30 μm . (D) Average nuclear distance to the NMJ (d) for VL3 and VL4 muscles. (E) Average percent of synaptic myonuclei in VL3 and VL4 muscles. (F) Average nuclear size for synaptic and non-synaptic myonuclei in VL3 and VL4 muscles. (G) Average cytoplasmic domain size determined by Voronoi tessellations for synaptic and non-synaptic myonuclei from VL3 and VL4 muscles. (H) A smooth density estimate plot of Hoechst intensities for VL3 and VL4 myonuclei where corresponding ploidy levels are denoted (16C, 32C, and 64C). (I) Average internuclear distance, determined by measuring the nearest neighbor distance for each myonucleus, for synaptic and non-synaptic myonuclei from VL3 and VL4 muscles. Data are shown as mean $\pm$ SEM ($n = 19$ larvae, 30 muscle pairs of VL3 and VL4 from abdominal hemi-segments 2 and 3; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

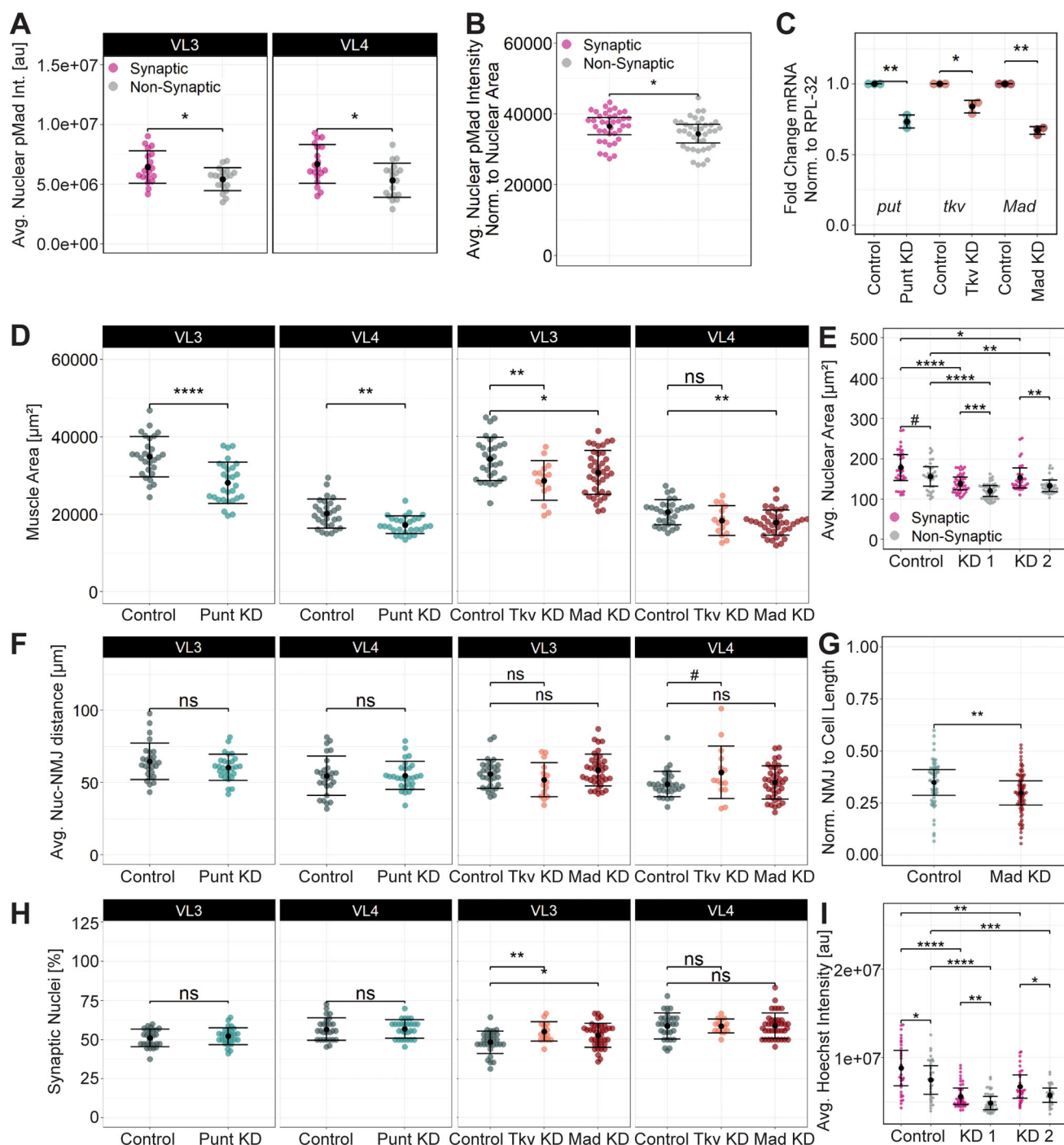


Figure S2. Muscle cell size and myonuclear proximity to the NMJ following KD of BMP signaling components. (A) Average nuclear pMad intensity in synaptic and non-synaptic myonuclei of VL3 and VL4 control muscles ($n = 11$ larvae, 40 VL3/4 muscle pairs). (B) Average nuclear pMad intensity normalized to the nuclear area in synaptic and non-synaptic control myonuclei. (C) qPCR data showing Punt, Tkv, and Mad KD (KD) efficiencies. Fold change in expression of *put*, *tkv*, and *Mad* mRNA normalized to housekeeping gene *rpl-32* (three replicates; *put* and *tkv*: $n = 10$ – 15 larvae per genotype; *Mad*: 6–10 larvae per genotype). (D) VL3 and VL4 muscle area based on phalloidin labeling for control and Punt KD muscles (Control: $n = 17$ larvae, 28 VL3/VL4 muscle pairs; Punt KD: $n = 17$ larvae, 28 VL3/VL4 muscle pairs), and control, thickveins, and Mad KD muscles (Control: $n = 19$ larvae, 30 VL3/VL4 muscle pairs; Tkv KD: $n = 12$ larvae, 15 VL3/VL4 muscle pairs; Mad KD: $n = 20$ larvae, 38 VL3/VL4 muscle pairs). (E) Average size of synaptic and non-synaptic myonuclei in control and Mad KD muscles from two different Mad RNAi lines (control: $n = 10$ larvae, 32 muscles; Mad KD 1: $n = 11$ larvae, 42 muscles; Mad KD 2: $n = 10$ larvae, 30 muscles). Of note, Mad KD 1 is also used in the main figures. (F) Average nuclear distance to the NMJ in VL muscles from Punt, Tkv, and Mad KD muscles and corresponding controls. (G) NMJ length normalized to muscle cell length in control and Mad KD muscles (Control: $n = 19$ larvae, 60 muscles; Mad KD: $n = 20$ larvae, 76 muscles). (H) Average percent of synaptic myonuclei in VL muscles from Punt, Tkv, and Mad KD muscles and corresponding controls. (I) Average Hoechst intensity per nucleus for synaptic and non-synaptic myonuclei from control and Mad KD muscles using two different Mad RNAi lines. Data are shown as mean $\pm$ SD for all plots, except in B, E, G, and I, where mean $\pm$ SEM is plotted (# $P = 0.069$ or 0.067 , * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$), and distinct plots with different controls are included for Punt KD compared with Tkv KD and Mad KD due to being conducted separately.

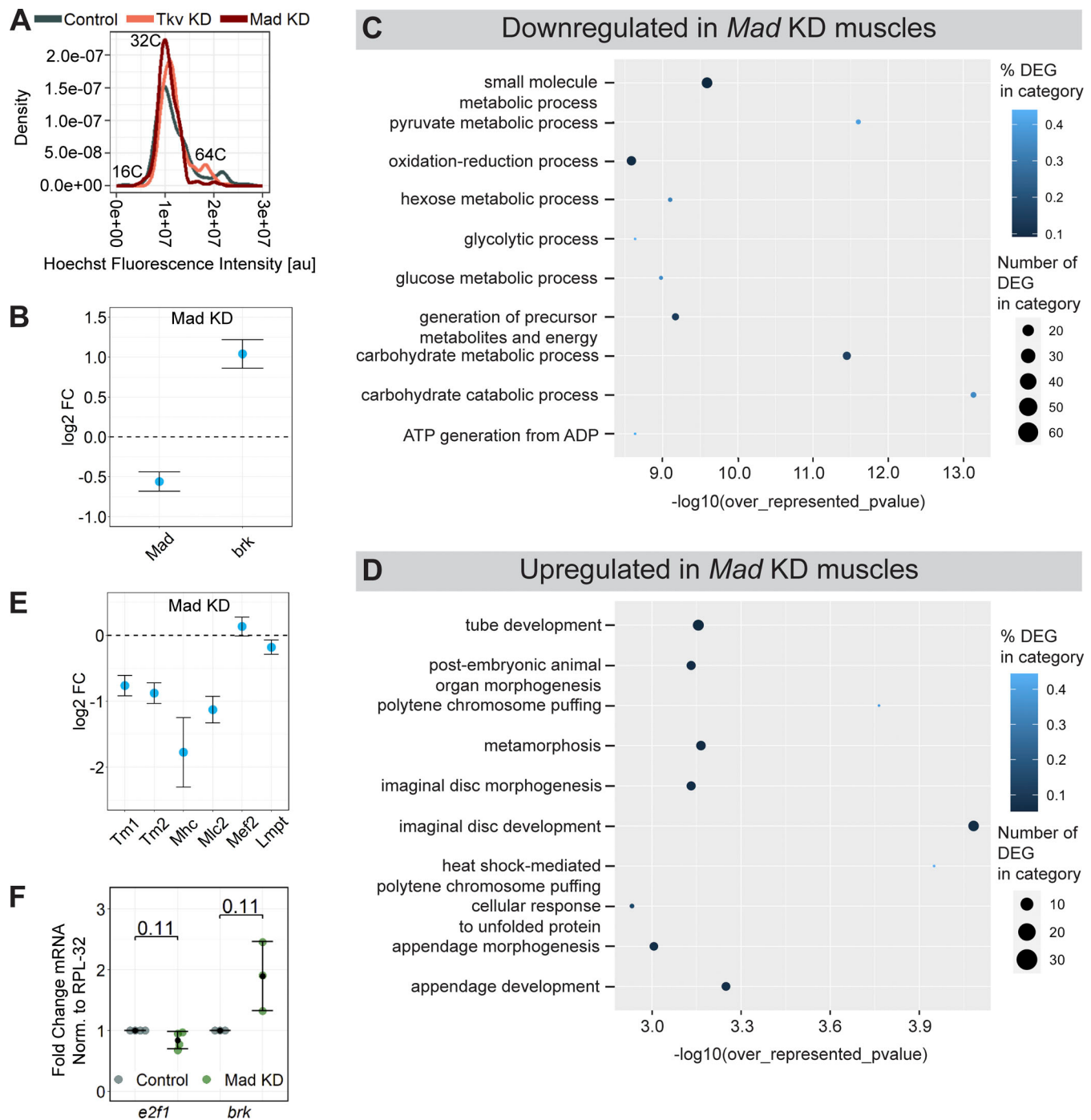


Figure S3. **Genes and pathways associated with muscle growth are affected by KD of postsynaptic BMP signaling components.** (A) A smooth density estimate plot of myonuclear Hoechst intensities in control, Tkv KD, and Mad KD muscles. Peaks indicate 16C, 32C, and 64C nuclei. (B) Fold change of *Mad* and *brk* mRNA in Mad KD compared to control muscle preps. (C and D) Top 10 (C) downregulated and (D) upregulated Gene Ontology (GO) gene sets for biological processes in Mad KD compared to control muscles. (E) Fold change of structural genes in Mad KD muscles. (F) qPCR data showing downregulation of *E2f1* and upregulation of *brk* in Mad KD muscles ($N = 3$ –4 replicates; $n = 6$ –12 larvae per genotype). RNA sequencing data was used for plots B–D and E ($N = 3$ replicates, $n = 6$ –10 larvae per genotype). Data are shown as mean $\pm$ SD.

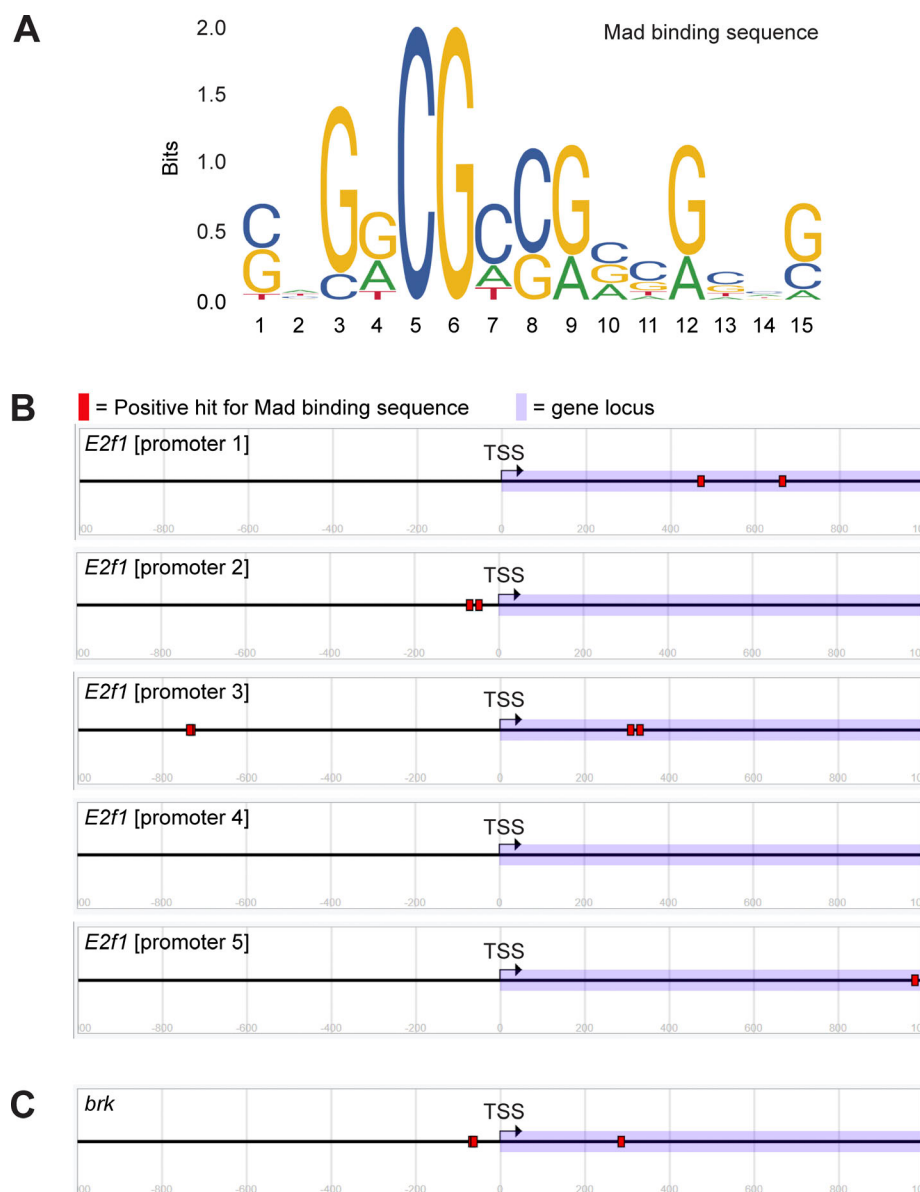


Figure S4. **Predicted Mad binding sites are identified at *E2f1* locus.** (A–C) Mad binding motif shown as a position-specific scoring matrix (PSSM) and was determined by CHIP-sequencing/-exo methods in *Drosophila* and made available by the JASPAR database (Khan et al., 2018). The relative rate of each nucleotide at each DNA position of Mad binding is indicated. Using the Mad-specific PSSM, potential Mad binding sites (red) were determined in the regulatory promoter and intron regions for (B) *E2f1* and (C) known Mad target gene *brk*. The transcription start site (TSS, arrow) of each unique promoter for each gene (purple) was accessed on and validated by the Eukaryotic Promoter Database (Dreos et al., 2015). As shown, *E2f1* has five unique promoters corresponding to distinct *E2f1* transcripts, whereas *brk* has one promoter.

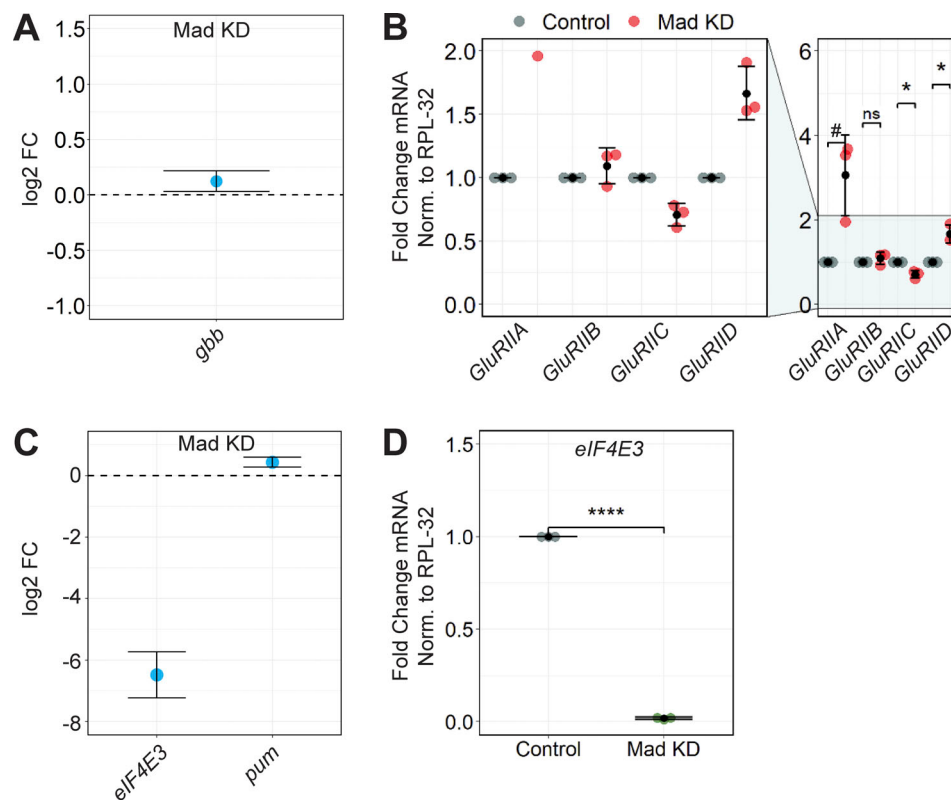


Figure S5. **Expression of GluRII subunits and postsynaptic mRNA translation regulator eIF4E3, but not BMP ligand Gbb are dysregulated in Mad KD muscles.** (A) Fold change of *gbb* in Mad KD compared to control muscle preps. (B) qPCR was conducted on GluRII subunits in control and Mad KD larval muscle preps to validate RNA sequencing results ($N = 3$ replicates; $n = 10$ – 14 larvae per genotype). (C) Fold change of *eIF4E3* and *pum* in Mad KD compared to control muscles. (D) qPCR results of *eIF4E3* expression in control and Mad KD larval muscle preps ($N = 3$ replicates; $n = 6$ – 10 larvae per genotype). RNA sequencing data was used for plots A and C ($N = 3$ replicates, $n = 6$ – 10 larvae per genotype). Data shown as mean $\pm$ SD (# $P = 0.065$, * $P < 0.05$, **** $P < 0.0001$).

Provided online is Table S1. Table S1 lists antibodies and oligonucleotides used in the study.