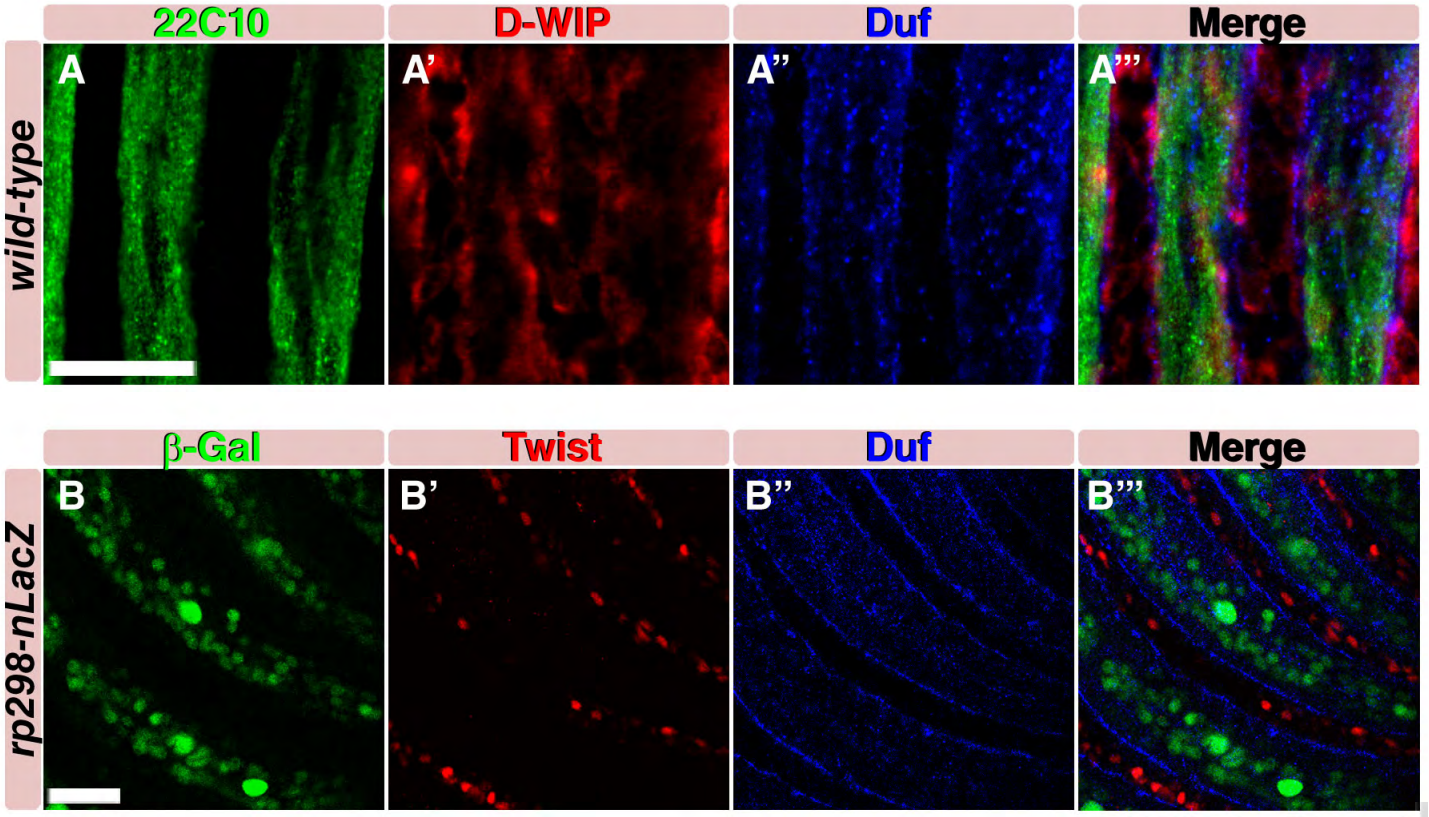

Fig. S1. Duf is expressed only in myotubes during DLM development. Pupal thorax preparations at 18-22 hours APF. All panels display 1 μm confocal sections. (**A-A‴**) Wild-type DLM myofibers [visualized with MAb 22C10 (green)], surrounded by fusion-competent myoblasts, visualized by anti-D-WIP (red). Duf protein (blue) is observed on the membrane of the myofibers and in puncta inside the myotube. (**B-B‴**) Dissected DLM fibers from a *duf* expression reporter line [$duf^{rp298\text{-}lacZ/+}$ (Nose et al., 1998)], double stained for the Duf protein (blue) and β-Gal (green). Myoblasts are marked by anti-Twist antibody (red). Duf protein localization matches the *duf* reporter expression pattern. Scale bars: 20 μm.

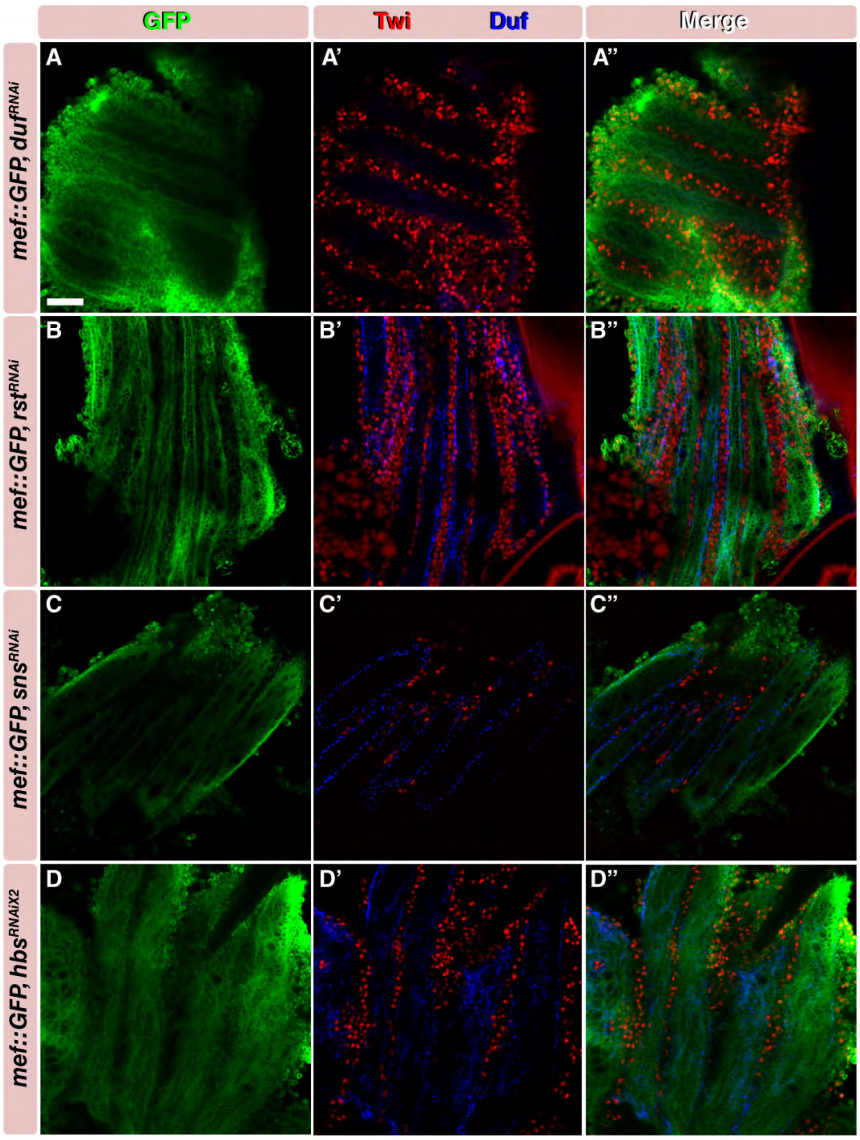

Fig. S2. Knockdown by RNAi of single fusion receptors does not arrest adult myoblast fusion. Dissected pupal muscles 18-24 hours APF. Myoblasts are visualized by Twi (red), myotubes by Duf (blue) and GFP (green) marks both cell types. Pupae expressing dsRNA targeting a single fusion receptor (**A**) Duf [*duf*$^{rp298\text{-}lacZ/Y}$*;UAS-duf*$^{RNAi\ (KK109585)}$*/+; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/+*], (**B**) Rst [*mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/UAS-rst*$^{RNAi\ (JF03087)}$], (**C**) Sns [*UAS-sns*$^{RNAi\ (KK109442)}$*/+; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/+*] and (**D**) Hbs [*UAS-hbs*$^{RNAi\ (KK105913)}$*/+; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/UAS-hbs*$^{RNAi\ (GD27065)}$] display thick, multi-nucleated myofibers that have undergone splitting, and a reduced number of unfused myoblast outside the fibers, implying that single receptor knockdowns do not block the fusion process. Flies expressing a single fusion receptor dsRNA are viable and eclose normally. Scale bar: 20 μm.

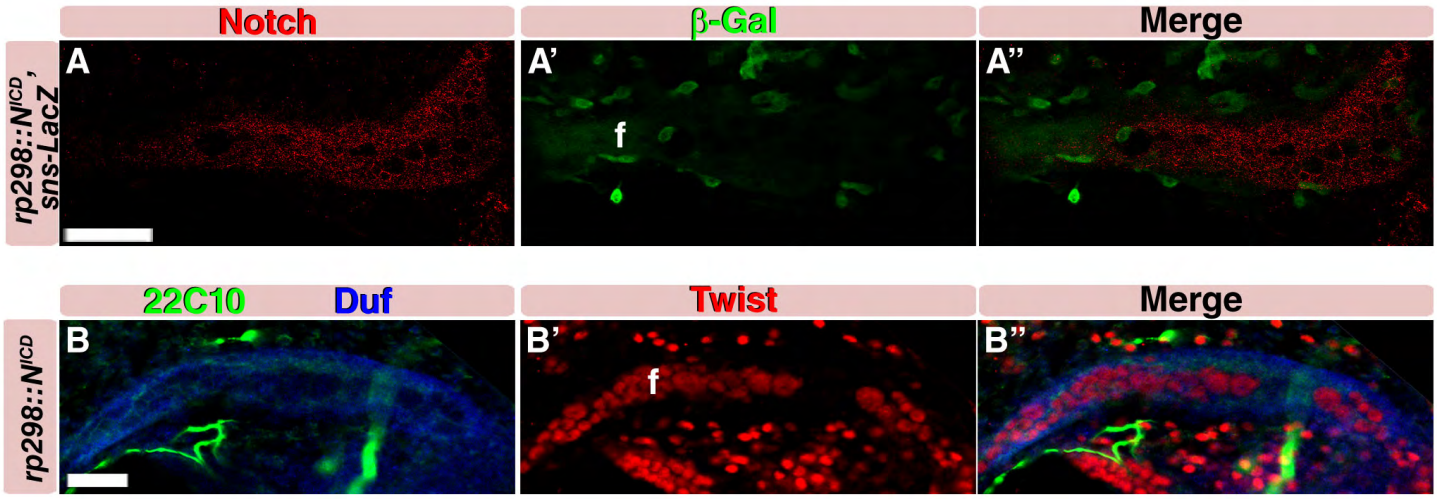

Fig. S3. Ectopic activation of Notch in myotubes does not suppress *sns-lacZ* expression in myoblasts and does not block adult myoblast fusion. (**A-B″**) Dissected pupal muscles 15-19 hours APF, from flies raised at 18°C and shifted to 29°C at the wandering larva stage. GAL80^{ts}, a temperature-sensitive GAL4 suppressor, does not function following this temperature shift, allowing for GAL4-based expression of UAS-bearing transgenes. Under these conditions, activity of the *rp298 (duf)*-GAL4 driver (Menon and Chia, 2001) is confined to the DLM fibers and is absent from myoblasts (Mukherjee et al., 2011). (A) Constitutively active Notch, detected with anti-Notch (red), was expressed in this fashion solely in myotubes, and the effect on *sns-lacZ* expression was monitored (*duf^{rp298GAL4}/+; GAL80^{TS}/UAS-N^{ICD}, sns-lacZ*). *lacZ*-positive myoblasts (green) are still present surrounding the myofiber (f). (B) Myoblast fusion is not blocked by myotube-specific expression of constitutively active Notch (*duf^{rp298GAL4}/+; GAL80^{TS}/UAS-N^{ICD}*). Myotubes fibers (f) are visualized by Duf expression (blue) and MAb 22C10 (green). The myotube is rich in myoblast nuclei after fusion. A large number of nuclei are found inside the fibers, presumably following myoblast fusion with the DLM templates. The nuclei inside the myotube can be visualized owing to an abnormal upregulation of Twist (red), a feature characteristic of Notch overactivation when fusion is not blocked (Anant et al., 1998). Scale bars: 20 μm.

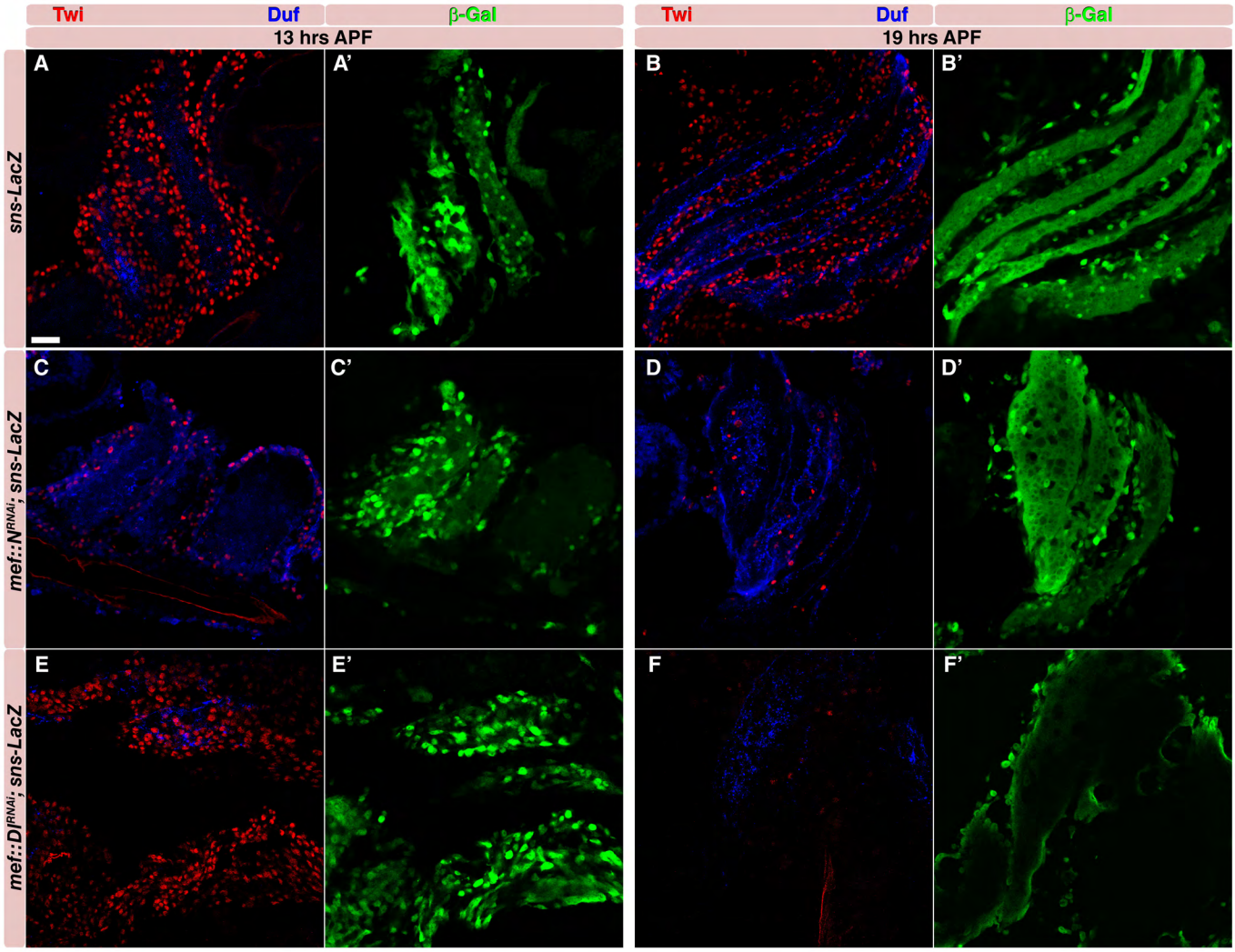

Fig. S4. ***Notch*** **and** ***Delta*** **knockdown inhibits proliferation and expression of Twi in adult myoblasts.** Dissected pupal muscles 13 and 19 hours APF. Myotubes are marked by Duf expression (blue). (**A**,**B**) Wild-type pupae carrying the *sns-lacZ* transgene display a large population of Twi-positive myoblasts (red) and a small population of Sns-positive myoblasts (green), both before (A) and after (B) splitting of the three DLMs. (**C**,**D**) Notch knockdown (*UAS-N$^{RNAi\ (7078)}$/+; GAL80^{TS}/sns-lacZ; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/+*) compromises proliferation and leads to premature differentiation, resulting in a smaller population of myoblasts (C), disappearance of Twi-positive myoblasts by 19 hours APF, and failure of the DLMs to split (D). (**E**,**F**) Delta knockdown (*GAL80^{TS}/ UAS-Delta$^{RNAi\ (GD3720)}$, sns-lacZ; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/+*) in E and *sns-lacZ/+; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/UAS-Delta$^{RNAi\ (JF02867)}$* in F display similar phenotypic features to knockdown of *Notch*. These include premature differentiation, which results in a depletion of the Twi-positive myoblasts, that are present at 13 hours APF (E) but disappear by 19 hours APF (F), and failure of the DLMs to split (F). Scale bar: 20 μm.

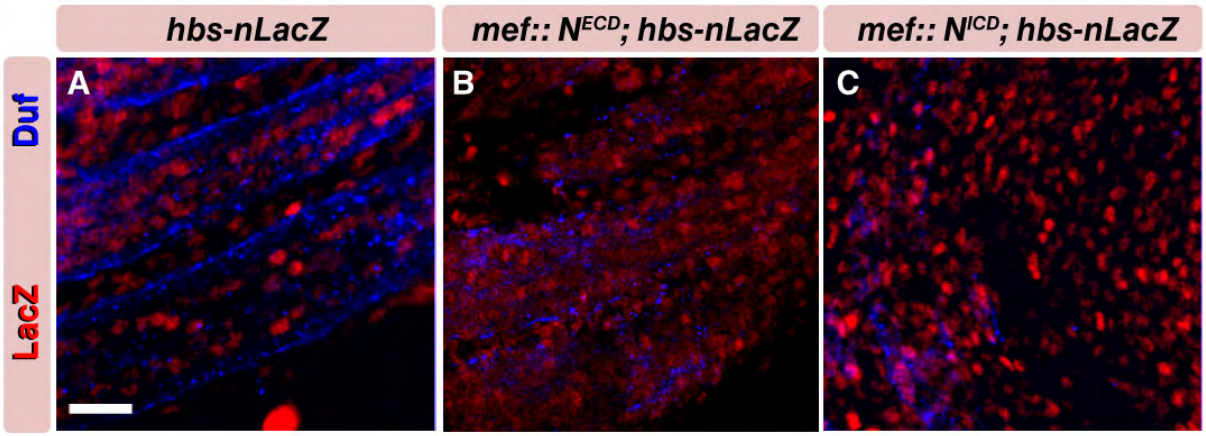

Fig. S5. Influence of Notch signaling on *hibris* expression. Dissected pupal muscles 18-22 hours APF. Myotubes are marked by Duf expression (blue). (**A**) Ubiquitous expression of the enhancer trap allele *hbs-nlacZ/hbs*$^{P36.1}$, a nuclear *hbs* reporter (Artero et al., 2001) in wing disc-derived myoblasts. (**B**) Reducing the levels of Notch signaling by the dominant-negative extracellular domain of Notch (*GAL80*^{TS}*/hbs-lacZ, UAS-N*^{ECD}*; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/+*) leads to a mild reduction in *hbs-lacZ* expression. (**C**) Elevation of the levels of Notch signaling by the intracellular domain of Notch (*GAL80*^{TS}*/hbs-nlacZ, UAS-N*^{ICD}*; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/+*) leads to a mild increase in *hbs-lacZ* levels. Scale bars: 20 μm.

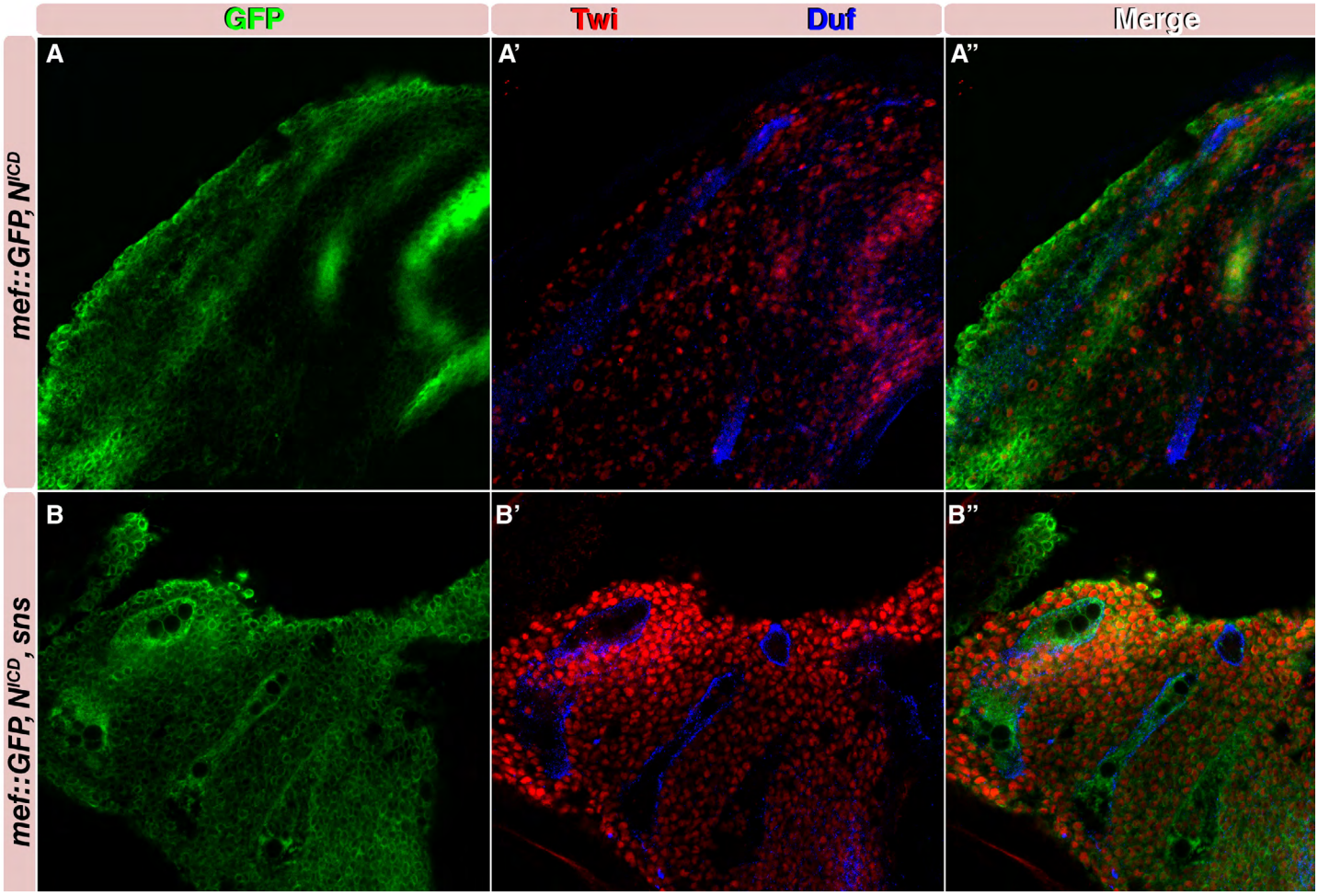

Fig. S6. Ectopic expression of Sns does not rescue fusion following constitutive activation of Notch. Dissected pupal muscles 18-22 hours APF. Myoblasts are visualized by Twi (red), myotubes by Duf (blue) and GFP (green) marks both cell types. (**A-A″**) Constitutive activation of the Notch pathway (*GAL80^{TS}/UAS-N^{ICD}; mef2-GAL4,UAS-CD8GFP,UAS-Dicer2/+*) blocked the myoblast fusion process. (**B-B″**) Ectopic expression of Sns by the GAL4/UAS system in the background of constitutively activated Notch, was not sufficient to rescue the fusion process. Myotubes remained thin, and a large number of unfused myoblasts surrounded the muscle templates. It appears therefore that additional genes, besides *sns*, that are essential for fusion are normally induced following shutdown of Notch signaling. Scale bars: 20 μm.