SUPPLEMENTARY MATERIAL

Integration of Insulin Receptor/FOXO signaling and dMyc activity during muscle growth regulates body size in *Drosophila*.

Fabio Demontis and Norbert Perrimon

SUPPLEMENTARY FIGURES

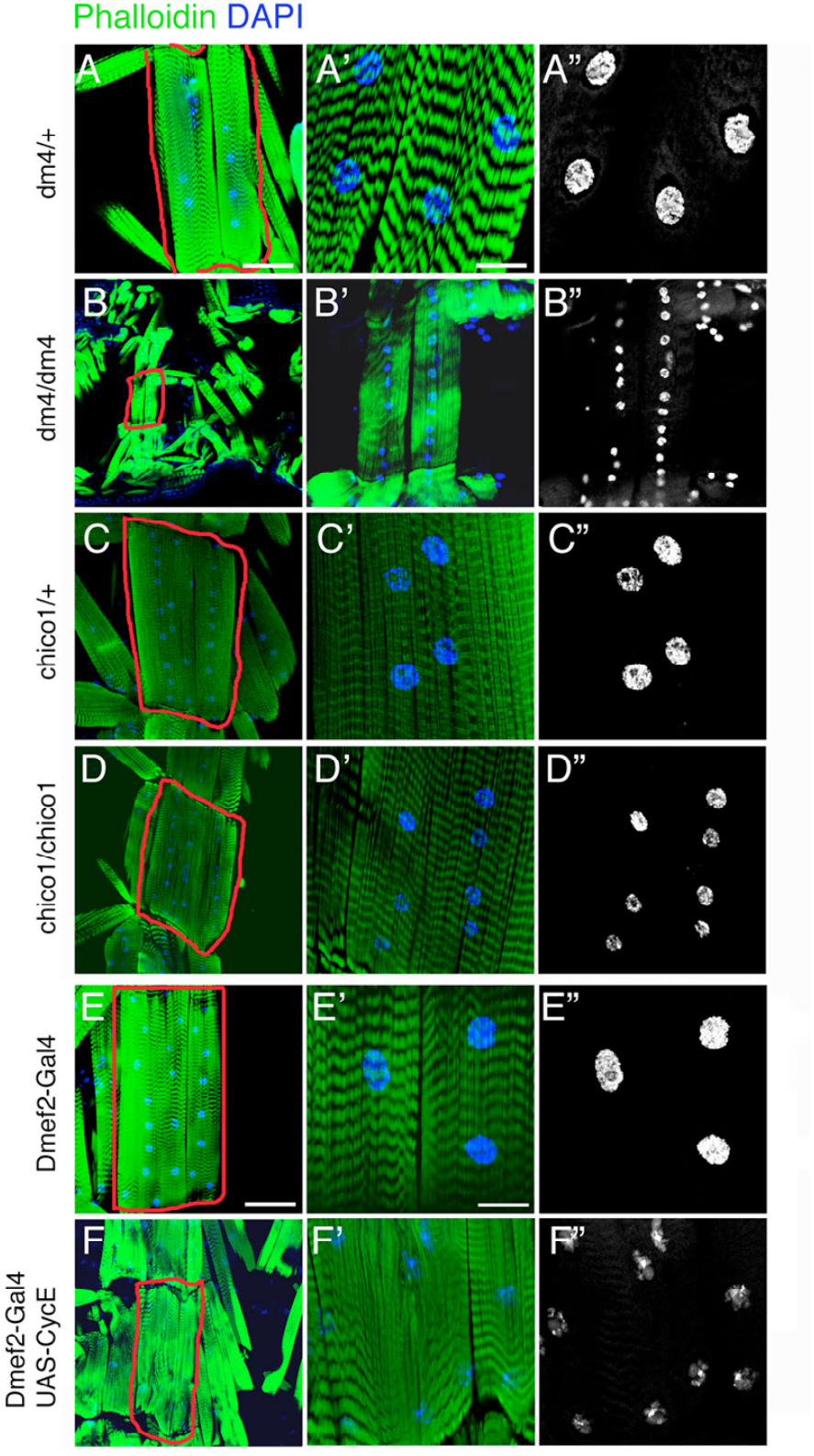

Figure S1 Demontis and Perrimon

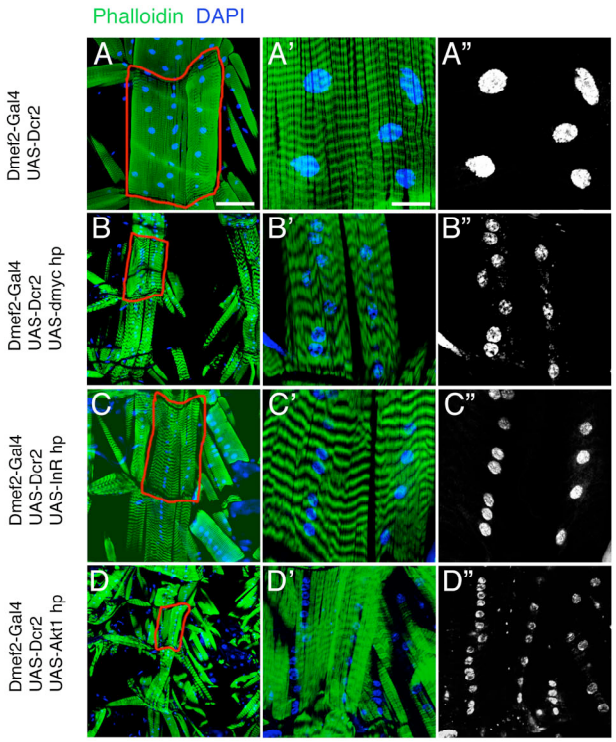

Figure S2 Demontis and Perrimon

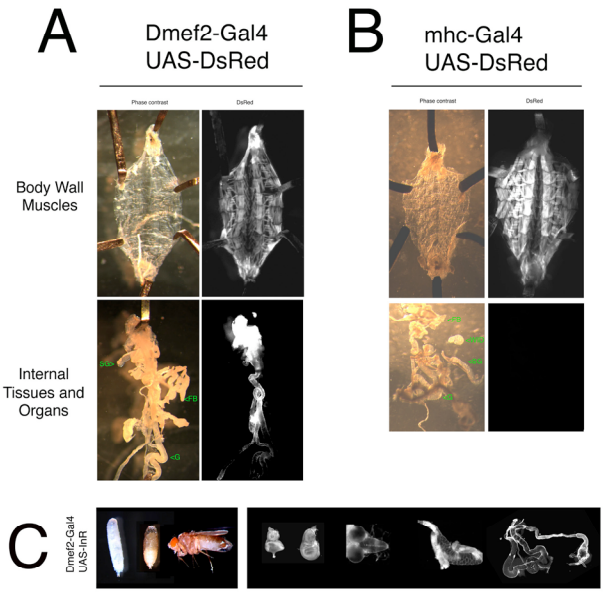

Figure S3 Demontis and Perrimon

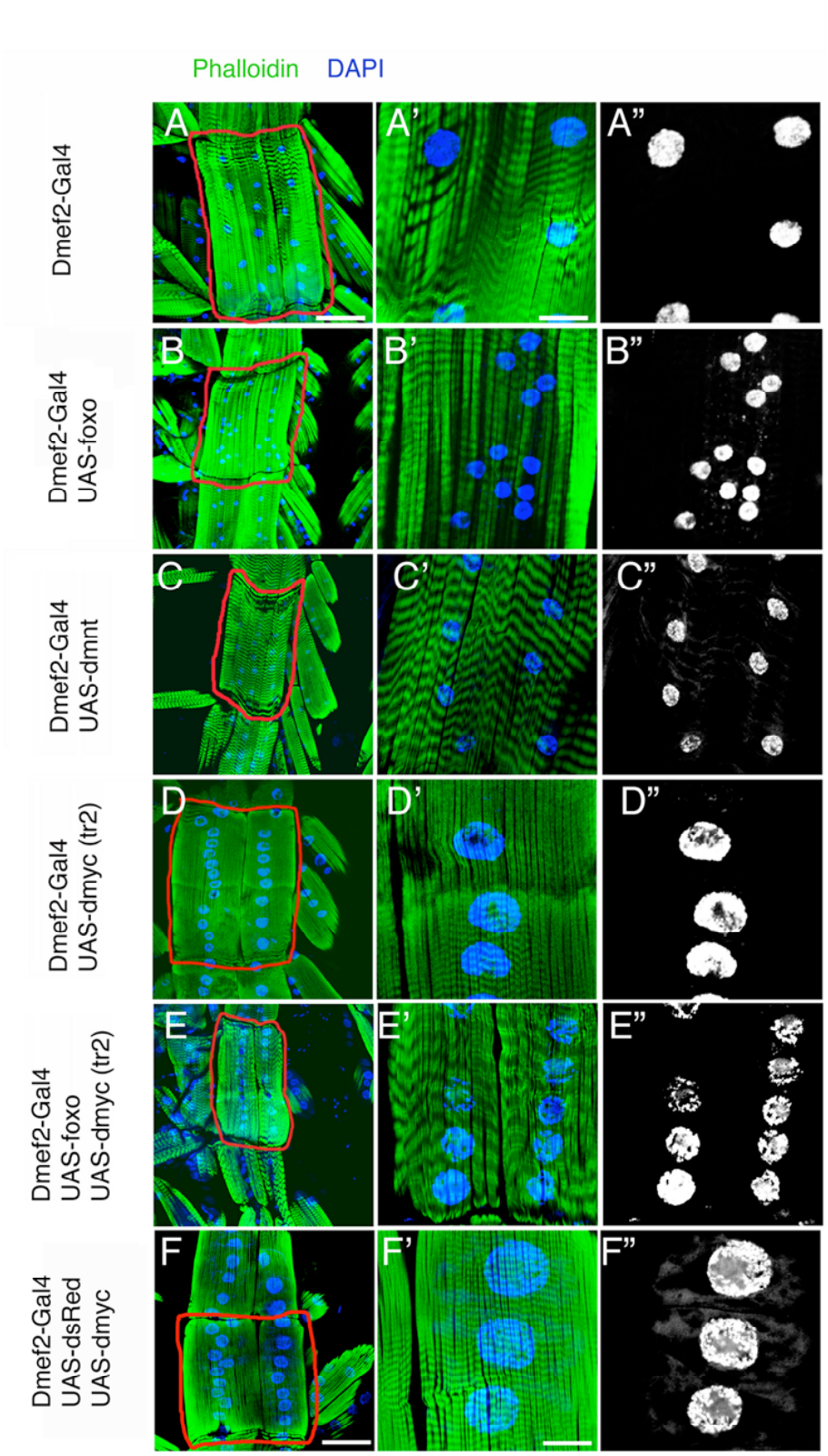

Figure S4 Demontis and Perrimon

PG157-Gal4 UAS-GFP

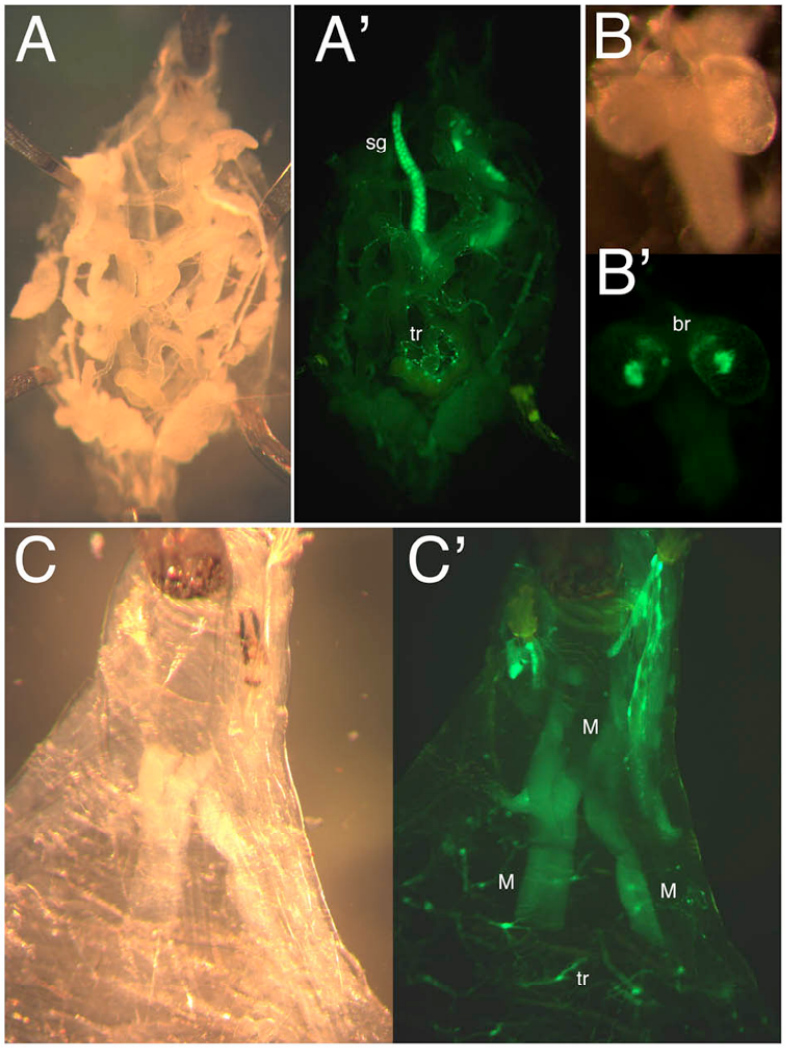

Figure S5 Demontis and Perrimon

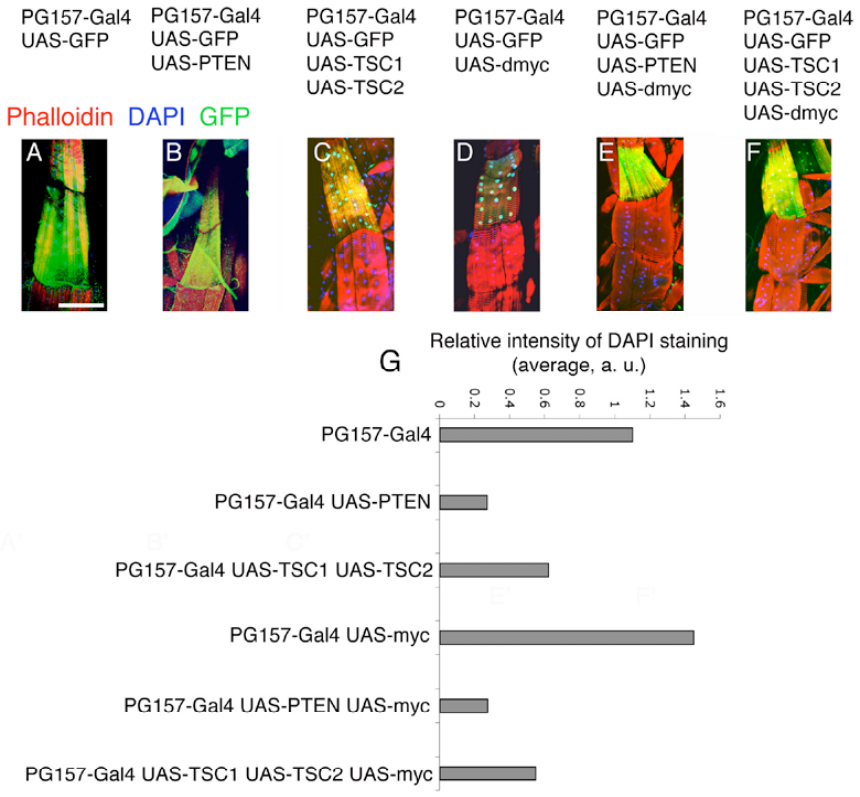

Figure S6 Demontis and Perrimon

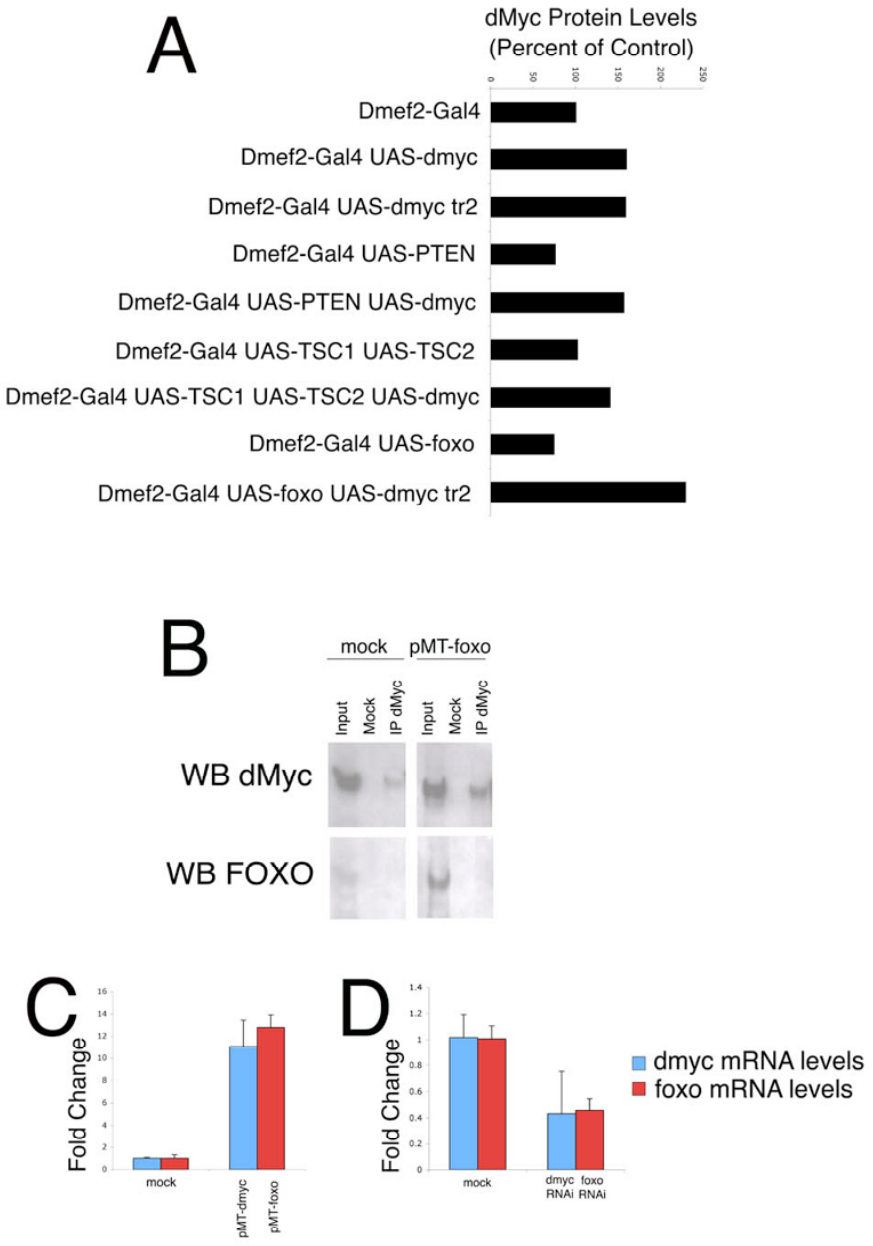

Figure S7 Demontis and Perrimon

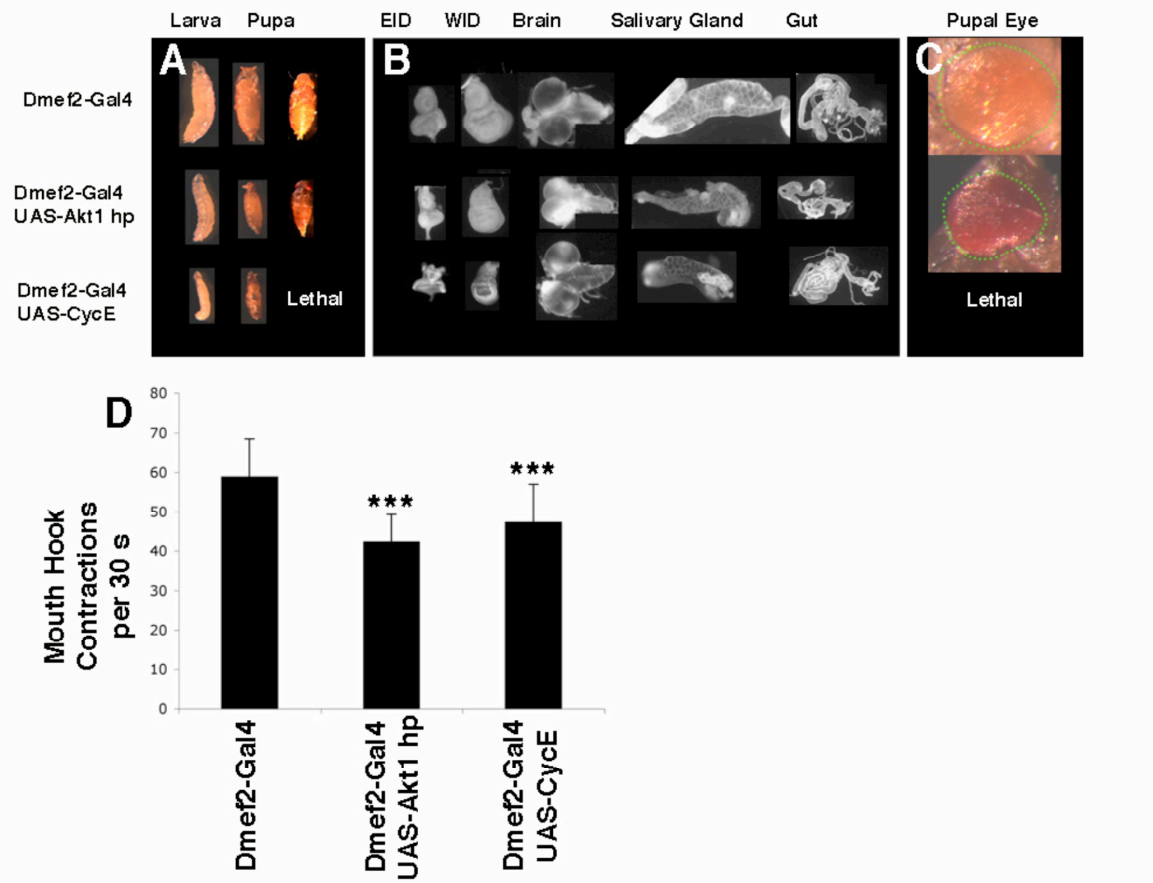

Fig. S8 Demontis and Perrimon

LEGEND OF SUPPLEMENTARY FIGURES

Figure S1. Insulin Receptor signaling and dMyc function are required for muscle growth. Body wall muscles from age-matched larvae carrying hypomorphic alleles of (A-B") *dmyc* (*diminutive*), and (C-D") the Insulin Receptor Substrate *chico*, stained with phalloidin (green) and DAPI (blue). Body wall muscles VL3 and VL4 are encircled in red. Both (B-B") dMyc and (D-D") Insulin receptor signaling are required for muscle growth and variation of nuclear size, in comparison with (A-A", C-C") matched heterozygous controls and wild type (not shown).

(F-F") Overexpression of *Cyclin E* (*CycE*) in muscles blocks muscle growth and variation of nuclear size, in comparison with control (E-E"). This indicates that the endoreplicative cell cycle is involved in ploidy variation, which is

necessary for muscle growth. Scale bars are 75 μm in (A-F), and 18.7 μm in (A'-F').

Figure S2. RNAi treatment against *dmyc*, *InR*, and *Akt1* impairs muscle growth and endoreplication. Phalloidin (green) and DAPI (blue) staining of body wall muscles from age-matched larvae, in which hairpins against (B-B") *dmyc*, (C-C") *InR*, or (D-D") *Akt1* are driven in muscles. *Dicer-2* (*Dcr2*) was co-expressed to increase potency of RNAi treatment (Dietzl et al., 2007). Knock-down of dMyc, InR, and Akt1 in all cases results in impaired muscle growth and endoreplication. Scale bars are 75 μm in (A-D), and 18.7 μm in (A'-D').

Figure S3. Tissue-specificity of transgene expression driven by Dmef2-Gal4 ad mhc-Gal4. Micrographs of overview of larvae in which *DsRed* expression has been driven with the (A) Dmef2-Gal4 and (B) mhc-Gal4 drivers. Auto-fluorescence (red channel) from wild-type larvae is analyzed as control (not shown). Immunofluorescence above background is detected in body wall muscles (somatic muscle lineage) but not in other endoreplicating tissues (including fat body (FB) and salivary glands (SG)), with both Dmef2-Gal4 and mhc-Gal4. Dmef2-Gal4 also drives transgene expression in cardiac and visceral muscle lineages, muscle (adepithelial) layer of wing imaginal discs (WID), and few cells in the brain (not shown). Fluorescence in the gut (G) arises from the underlying muscle layer. Note that mhc-Gal4 recapitulates the autonomous and non-autonomous phenotypes observed with Dmef2-Gal4, when sufficient trasgene expression is driven.

(C) *InR* overexpression in muscles (*Dmef2-Gal4 UAS-InR*) results in bigger larvae and pupae, and increases the size of most endoreplicating organs and, to a lesser extent, of imaginal discs. Compare with control (*UAS-PTEN*, Fig. 2), which does not significantly differ from *Dmef2-Gal4* control flies (not shown).

Figure S4. Overexpression of *foxo* and *dmnt* regulates myofiber growth and

nuclear ploidy by dMyc. (A-F") Micrographs of some of the genotypes quantified in Fig. 5. (A-A") Staining of body wall muscles of *Dmef2-Gal4* third instar larvae with phalloidin (green) and DAPI (blue). (B-B") The InR signaling repressor *foxo* was overexpressed in muscles using the Dmef2-Gal4 muscle driver. (B-B") Repression of InR signaling results in a significant decrease in the area of myofibers VL3 and VL4 (encircled in red) with concomitant reduction of nuclear area. (C-C") Repression of dMyc signaling, following overexpression of *dmnt*, impairs muscle growth and endoreplication. (D-E") Co-overexpression of *foxo* and *dmyc* (*dmyc tr2,* with tr2=transgene 2), impairs dMyc-dependent cell cycle progression, indicating that FOXO inhibits dMyc function (quantification in Fig. 5). (F-F") Co-expression of *dsRed* with *dmyc* does not impair dMyc function. Scale bars are 75 μm in (A-F), and 18.7 μm in (A'-F').

Figure S5. Expression pattern of the PG157-Gal4 driver. (A) View of dissected *PG157-Gal4 UAS-GFP* L3 wandering larvae, with internal organs exposed. (A') GFP fluorescence outlines the tissues where PG157-Gal4 drives transgene expression. GFP fluorescence is detected in salivary glands (sg, A,A'), cellular clusters in the brain (br, B,B'), trachea (tr, C,C'), and body wall muscles VI1 of the first abdominal segment and of thoracic segments (M, C,C'); (see Material and Methods for details).

Figure S6. Overview of genetic mosaics in muscles and quantification of the intensity of DAPI staining. Overexpression of (A) *GFP* alone, or *GFP* together with (B) *PTEN*, (C) *TSC1* and *TSC2*, (D) *dmyc*, (E) *dmyc* and *PTEN*, or (F) *dmyc* and *TSC1* and *TSC2* with PG157-Gal4. Transgene expression is driven in muscle VI1 (up, green due to co-expression of GFP), but not in neighboring muscles VL3 and VL4 (down, not green), where no transgene expression is driven. Muscle growth and endoreplication are modulated upon expression of *PTEN*, *TSC1* and *TSC2*, or *dmyc*. *PTEN*, and *TSC1* and *TSC2* antagonize dMyc activity. See Fig. 6 for a detailed analysis.

(G) Quantification of relative intensity of DAPI staining in genetic mosaics.

Transgene expression in muscle VI1 results in changes in the intensity of DAPI staining, that are normalized in each micrograph by comparison with muscles VL3 and VL4, where no transgene expression is driven. Values refer to the ratio of average intensity of DAPI staining per nucleus in muscle VI1 versus muscles VL3 and VL4, with n>9. Note that variation in the intensity of DAPI staining parallels with variation in nuclear size. dMyc promotes an increase in the intensity of DAPI staining, which is indicative of nuclear DNA content, that is antagonized by *PTEN*, and *TSC1* and *TSC2*.

Figure S7. Characterization of the FOXO/dMyc interaction.

(A) Densitometric analysis of dMyc protein levels from Western blot experiments upon modulation of InR signaling and *dmyc* overexpression in muscles with Dmef2-Gal4. dMyc protein levels are increased upon *dmyc* overexpression.

(B) FOXO and dMyc do not apparently co-immunoprecipitate. Immunoprecipitation of dMyc and FOXO proteins from S2R+ cells upon no transfection (mock), or transfected with pMT-foxo. Cell extracts were immunoprecipitated with mouse anti-dMyc antibodies; Input, mock, and immunoprecipitated materials were western blotted with rabbit anti-dMyc or anti-FOXO antibodies, as indicated. No significant co-immunoprecipitation of FOXO and dMyc is observed, suggesting that they might not physically interact.

(C, D) Analysis by qRT-PCR of *dmyc* and *foxo* mRNA levels, upon (C) overexpression and (D) RNAi treatment. *dmyc* and *foxo* overexpression were induced for 2 days, while RNAi treatment was performed for 3 days. Changes in *dmyc* and *foxo* mRNA levels are observed, consistent with the treatment that is performed. Levels of alpha-Tubulin84B are detected as normalization control.

Figure S8. Overexpression of *Cyclin E* in muscles non-autonomously regulates the growth of other internal organs. (A) Overview of L3 larvae and pupae. Overexpression of *Cyclin E* (*CycE*) in muscles (*Dmef2-Gal4 UAS-CycE*)

or RNAi-mediated knock-down of *Akt1* levels (*Dmef2-Gal4 UAS-Akt1 hp*) results in decreased body size, in comparison with matched controls (*Dmef2-Gal4*).

(B) Internal organs of L3 larvae were stained with the lipophilic dye FM4-64 to outline their size. Overexpression of *Cyclin E* or *Akt1* knockdown in muscles both result in decreased size of most internal organs, which are affected to different extents. Magnification is 3x (gut) and 10x. (C) The size of the eyes from pupae with decreased *Akt1* levels is also affected, in comparison with controls.

(D) Impairment of larval feeding behavior in flies overexpressing *Cyclin E* or with decreased levels of *Akt1* in muscles, in comparison with age-matched L3 control larvae ($n=25$, $p<0.001$). Transgene expression was driven at 25°C.