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Supplementary Materials for

A DGKζ-FoxO-ubiquitin proteolytic axis controls fiber size during skeletal muscle remodeling

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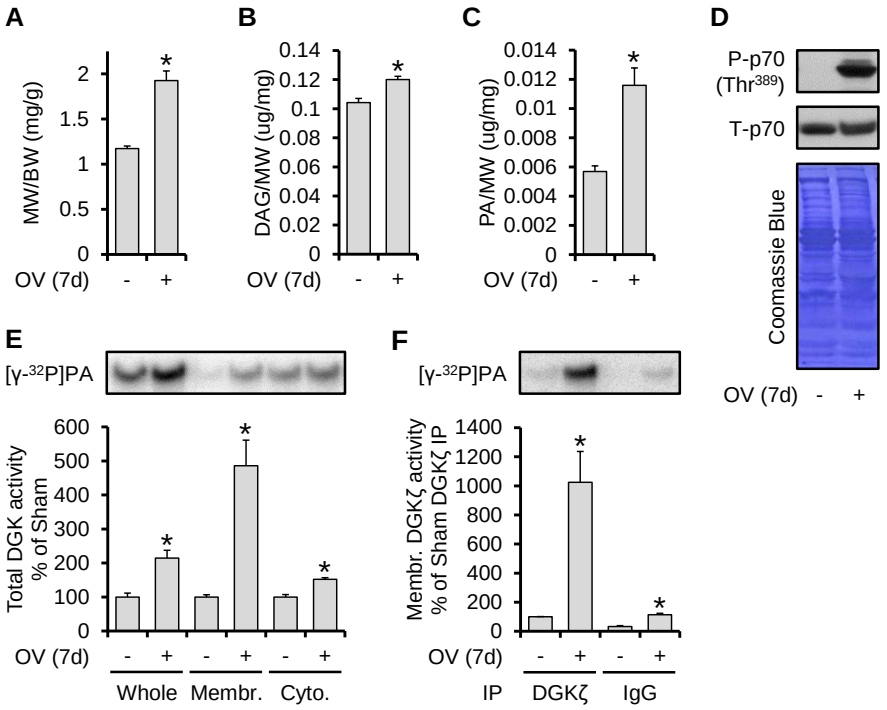

Figure S1. Mechanical overload activates DAG-PA-mTOR signaling and increases DGKζ activity. Rats were subjected to mechanical overload (OV+) or sham (OV-) surgery and the plantaris muscles (*n*) were collected at 7 days post-surgery. (**A**) Muscle weight (MW) to body weight (BW) ratio. *n* = 6-8 from 3-4 rats per group. (**B-C)** TLC-based measurements of DAG (*n* = 3 from 3 rats per group) (**B**) and PA (*n* = 4 from 3-4 rats per group) (**C**). (**D**) Western blotting to detect phosphorylated (P) and total (T) p70. *n* = 4 from 3-4 rats per gorup. (**E-F**) DGK activity assay in whole homogenate (Whole), membrane fraction (Membr.), or cytosolic fraction (Cyto.) (*n* = 3-4 from 2-3 rats per group) (**E**) or in immunoprecipitates (IP) from the membrane fraction (*n* = 3 from 2-3 rats per group) (**F**). Values were expressed as mean + s.e.m. **P* < 0.05 compared to sham (within the same fraction in E or IP in F), Student's *t*-test.

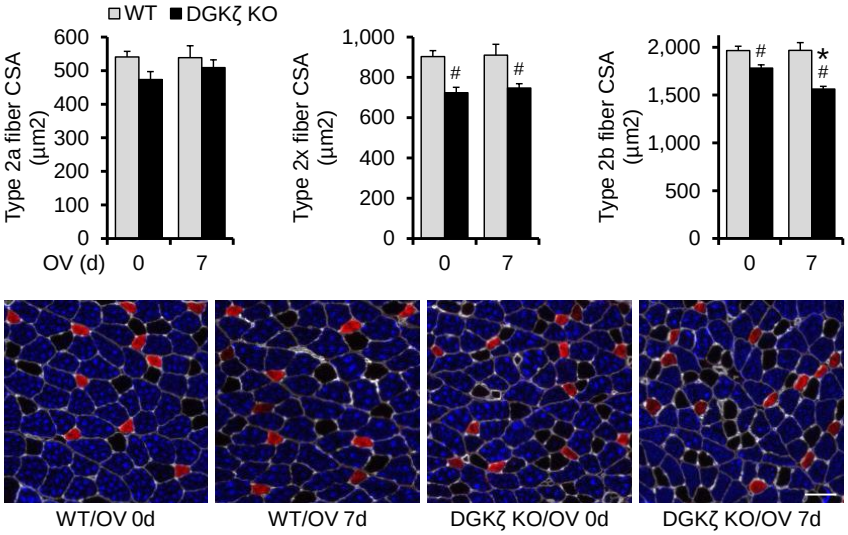

Figure S2. DGKζ preserves type 2b fiber size during mechanical overload. WT and DGKζ KO mice were subjected to mechanical overload (of the extensor digitorum longus muscles; OV 7d) or sham (OV 0d) surgery. The muscles (*n*) were collected at 7 days post-surgery and subjected to immunohistochemistry on cross-sections with antibodies against laminin and type 2a myosin heavy chain (MHC) or type 2b MHC to measure CSA in type 2a fibers, type 2x fibers, and type 2b fibers. *n* = 6-8 from 3-4 mice per group. The lower panel shows representative images (white: laminin; red: type 2a MHC; black: type 2x MHC; blue: type 2b MHC; Scale bar: 50μm). Values were expressed as mean + s.e.m. **P* < 0.05 compared to sham within the same genotype, #*P* < 0.05 compared to WT within the same surgery, 2-way ANOVA.

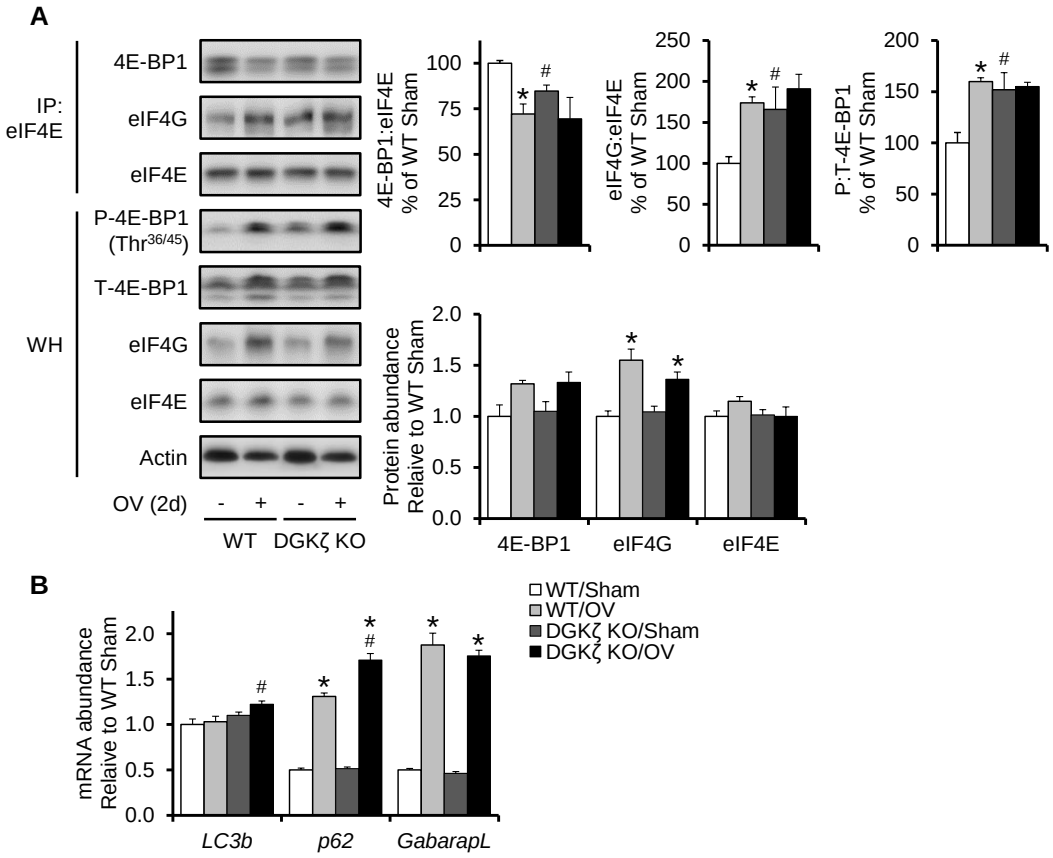

Figure S3. The effects of DGKζ KO on mTOR signaling events and FoxO target gene expression after the onset of mechanical overload. WT and DGKζ KO mice were subjected to mechanical overload (OV+) or sham (OV-) surgery and the plantaris muscles (*n*) were collected at 2 days (A) or 1 day (B) post-surgery. (**A**) Whole homogenates (WH) and immunoprecipitates (IP) of eIF4E were subjected to Western blotting to detect the indicated proteins. *n* = 5-6 from 3 mice per group. (**B**) qRT-PCR to measure mRNA expression of the indicated genes. *n* = 6-8 from 3-4 mice per group. Values were expressed as mean + s.e.m. **P* < 0.05 compared to sham within the same genotype, #*P* < 0.05 compared to WT within the same surgery, 2-way ANOVA.

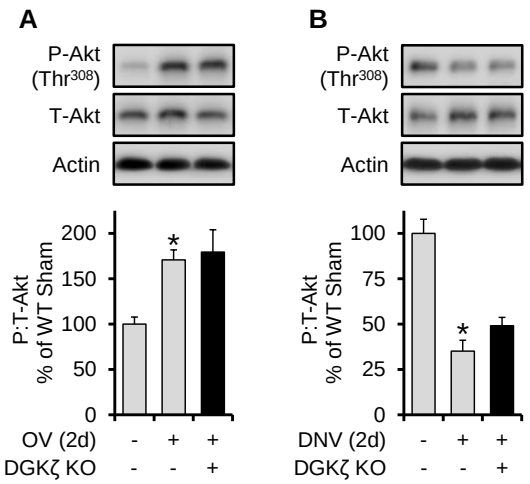

Figure S4. The effects of DGKζ KO on the phosphorylation of Akt. WT (DGKζ KO-) and DGKζ KO mice were subjected to mechanical overload (OV+), denervation (DNV+), or sham (OV- or DNV-) surgery. The plantaris (**A**) or tibialis anterior (**B**) muscles (*n*) were collected at 2 days post-surgery and subjected to Western blotting to detect phosphorylated (P) and total (T) Akt. n = 3-6 from 3-4 mice per group. Values were expressed as mean + s.e.m. **P* < 0.05 compared to sham within the same genotype, 1-way ANOVA.

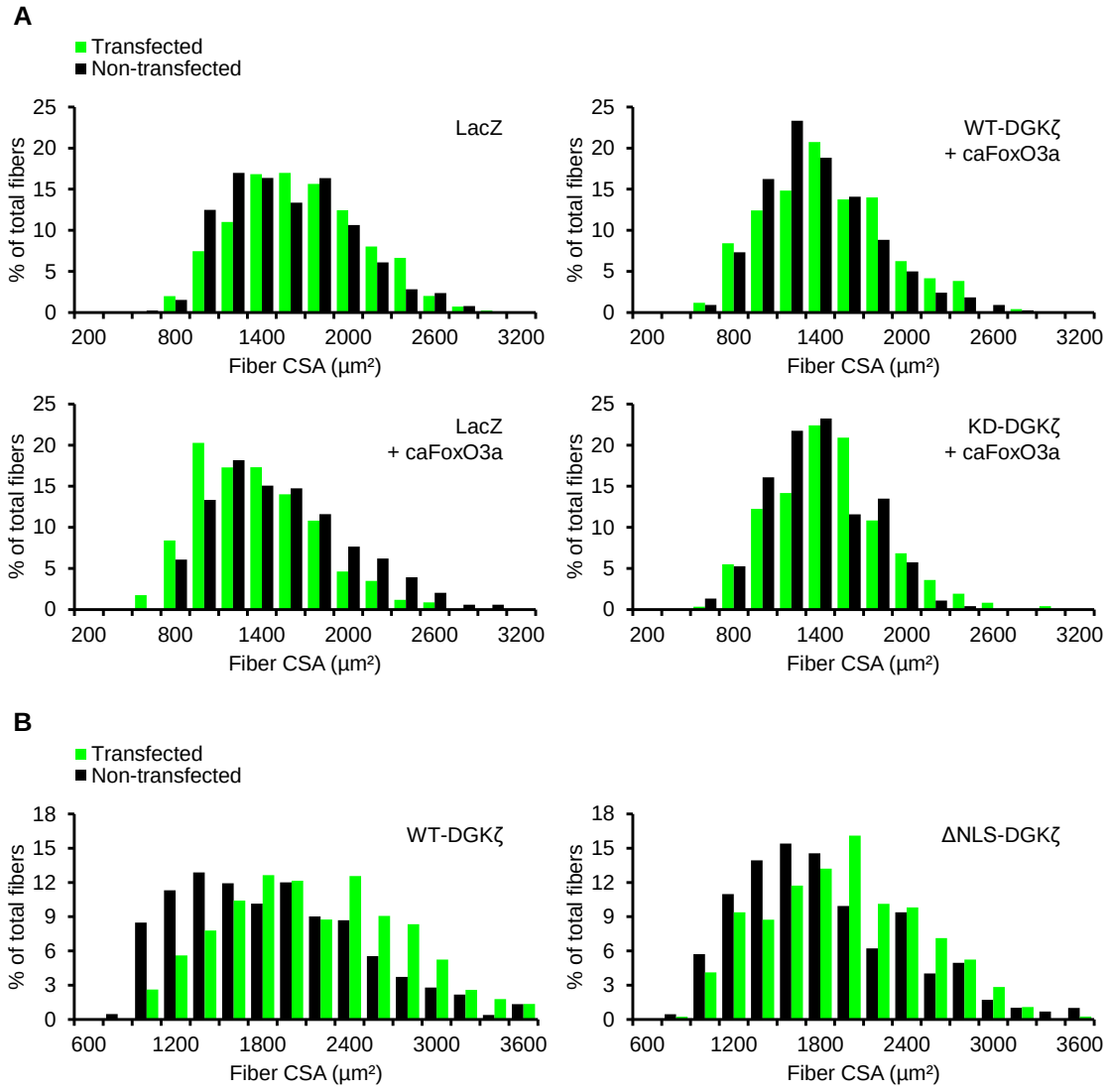

Figure S5. The effects of DGKζ overexpression on the distribution of fiber size under various conditions. Tibialis anterior muscles (*n*) were transfected with LacZ, HA-tagged WT-DGKζ, or HA-tagged kinase dead (KD)-DGKζ ± caFoxO3a (**A**), or with FLAG-tagged WT-DGKζ or FLAG-tagged nuclear localization signal mutated (ΔNLS)-DGKζ (**B**), and collected at 7 days post-transfection. Cross-sections of the muscles were subjected to immunohistochemistry to measure CSA of the transfected and non-transfected fibers, and the distribution of the CSA was displayed on a histogram. A, $n = 4$ from 2-4 mice per group; B, $n = 6$ from 6 mice per group. Mean values are shown.

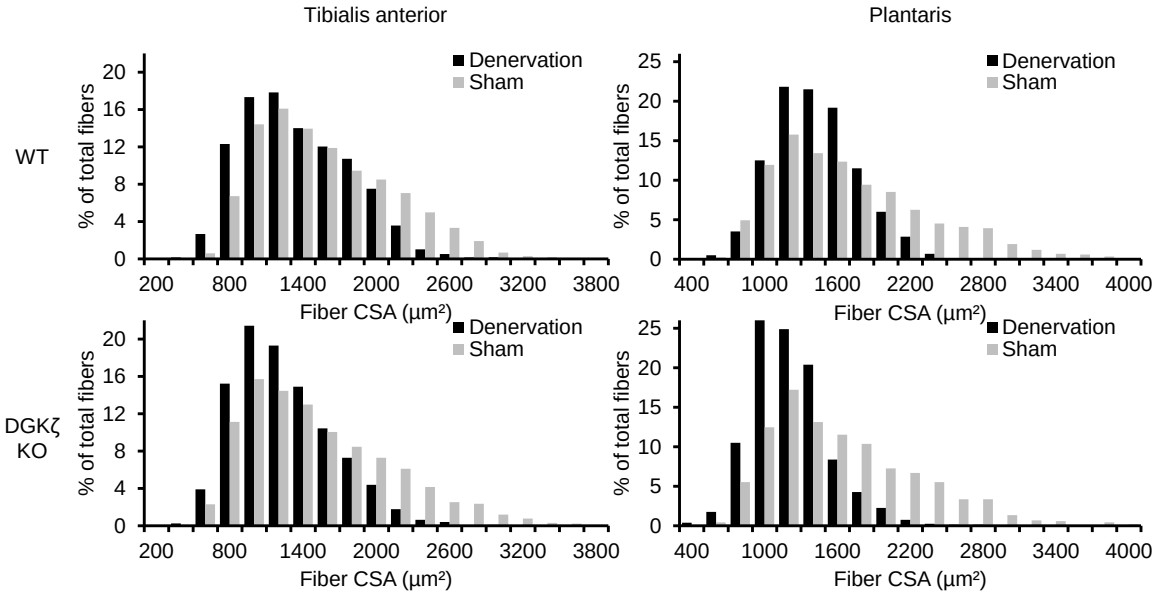

Figure S6. The effects of denervation on the distribution of fiber size in WT and DGKζ KO muscles. WT and DGKζ KO mice were subjected to denervation or sham surgery, and the tibialis anterior and plantaris muscles (*n*) were collected at 7 days post-surgery. Cross-sections of the muscles were subjected to immunohistochemistry to measure CSA, and the distribution of the CSA was displayed on a histogram. *n* = 3-6 from 3-6 mice per group. Mean values are shown.

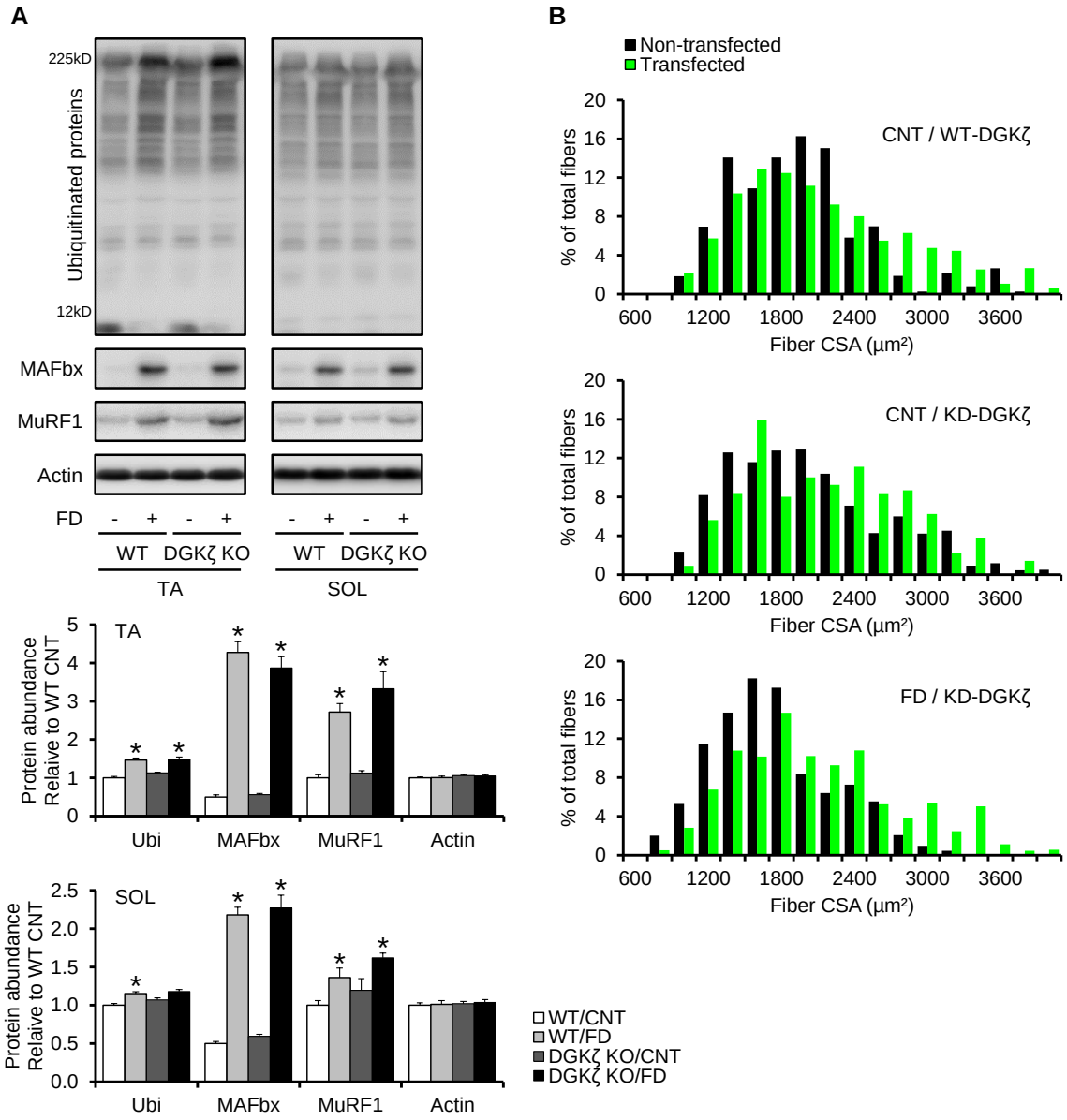

Figure S7. The effects of DGKζ KO on the activation of the UPS during food deprivation. (**A**) WT and DGKζ KO mice were subjected to food deprivation (FD+) or control (FD-) condition. The tibialis anterior (TA) and soleus (SOL) muscles (*n*) were collected at 2 days post-FD and subjected to Western blotting to detect the indicated proteins. *n* = 4-6 from 3 mice per group. (**B**) Tibialis anterior muscles (*n*) from WT mice were transfected with HA-tagged WT-DGKζ or HA-tagged kinase dead (KD)-DGKζ immediately before being subjected to 2 days of food derivation (FD) or the control (CNT) condition. Cross-sections of the muscles were subjected to immunohistochemistry to measure CSA of the transfected and non-transfected fibers, and the distribution of the CSA was displayed on a histogram. *n* = 4 from 2-4 mice per group. Values were expressed as mean (+ s.e.m.). *P < 0.05 compared to control within the same genotype, 2-way ANOVA.

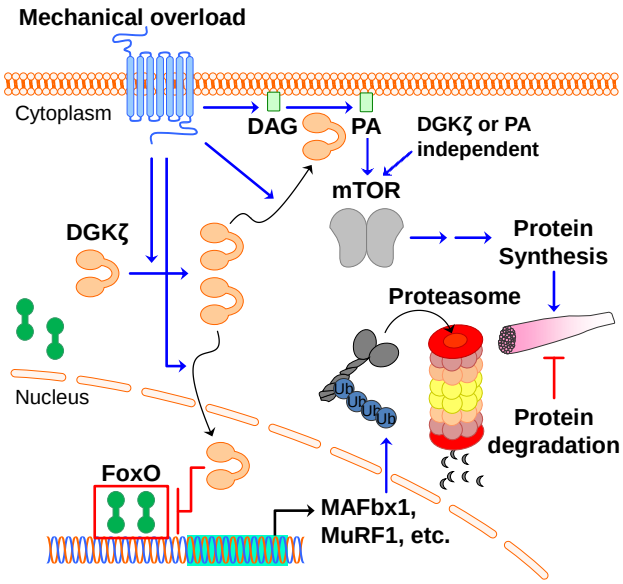

Figure S8. A proposed mechanism through which DGKζ promotes skeletal muscle hypertrophy in response to mechanical overload. Mechanical overload increases the abundance of DGKζ while inducing its membrane and nuclear translocation. The increase in the amount of membrane-associated DGKζ promotes the synthesis of PA from DAG which is also increased by mechanical overload, and thus contributes to the activation of mTOR signaling and protein synthesis. In the nucleus, DGKζ inhibits the activity of FoxO and the expression of FoxO target genes, such as MAFbx1 and MuRF1, to prevent excessive ubiquitin-proteasome-system-dependent proteolysis during mechanical overload. The resulting net positive balance between protein synthesis and protein degradation ultimately leads to muscle hypertrophy.

Table S1. Muscle weight, body weight, and tibia length.

Figure	Muscle		WT/Sham	WT/OV 3d	WT/OV 7d	KO/Sham	KO/OV 3d	KO/OV 7d
2A		MW (mg)	16.98 ±0.16	22.75 ±0.38	28.89 ±0.92	14.61 ±0.51	18.73 ±0.75	22.78 ±1.25
		BW (g)	24.2 ±0.36	24 ±0.31	23.9 ±0.66	22.85 ±0.36	21.22 ±0.45	22.7 ±0.41
			WT/Sham	WT/DNV	KO/Sham	KO/DNV		
6C	TA	MW (mg)	32.24 ±0.59	27.72 ±0.48	31.07 ±0.54	22.72 ±0.38		
	EDL	MW (mg)	7.18 ±0.09	7.43 ±0.12	6.27 ±0.11	5.65 ±0.14		
	GAST	MW (mg)	84.31 ±1.73	68.39 ±1.57	80.07 ±1.42	58.07 ±1.42		
	PLT	MW (mg)	11.03 ±0.23	10.37 ±0.32	9.93 ±0.13	7.79 ±0.19		
	SOL	MW (mg)	5.54 ±0.13	5.14 ±0.16	5.85 ±0.13	4.82 ±0.11		
		BW (g)	17.39 ±0.22	18.13 ±0.22	17.36 ±0.21	17.04 ±0.31		
			WT/CNT	WT/FD	KO/CNT	KO/FD		
7B	TA	MW (mg)	45.08 ±0.63	37.73 ±0.84	39.28 ±2.13	32.87 ±1.9		
	EDL	MW (mg)	10.12 ±0.33	8.66 ±0.12	8.42 ±0.32	7.1 ±0.23		
	GAST	MW (mg)	120.1 ±4.19	103.12 ±2.69	102.6 ±5.2	81.88 ±4.49		
	PLT	MW (mg)	16 ±0.48	13.7 ±0.21	13.55 ±0.58	11.25 ±0.69		
	SOL	MW (mg)	7.77 ±0.22	6.8 ±0.22	6.78 ±0.47	5.4 ±0.18		
		TL (mm)	17.1 ±0.08	17.1 ±0.11	16.7 ±0.22	16.43 ±0.26		

CNT, control; DNV, denervation; EDL, extensor digitorum longus; FD, food deprivation; GAST, gastrocnemius; KO, knock out; OV, overload; PLT, plantaris; SOL, soleus; TA, tibialis anterior; WT, wild type; All values were expressed as mean + s.e.m.