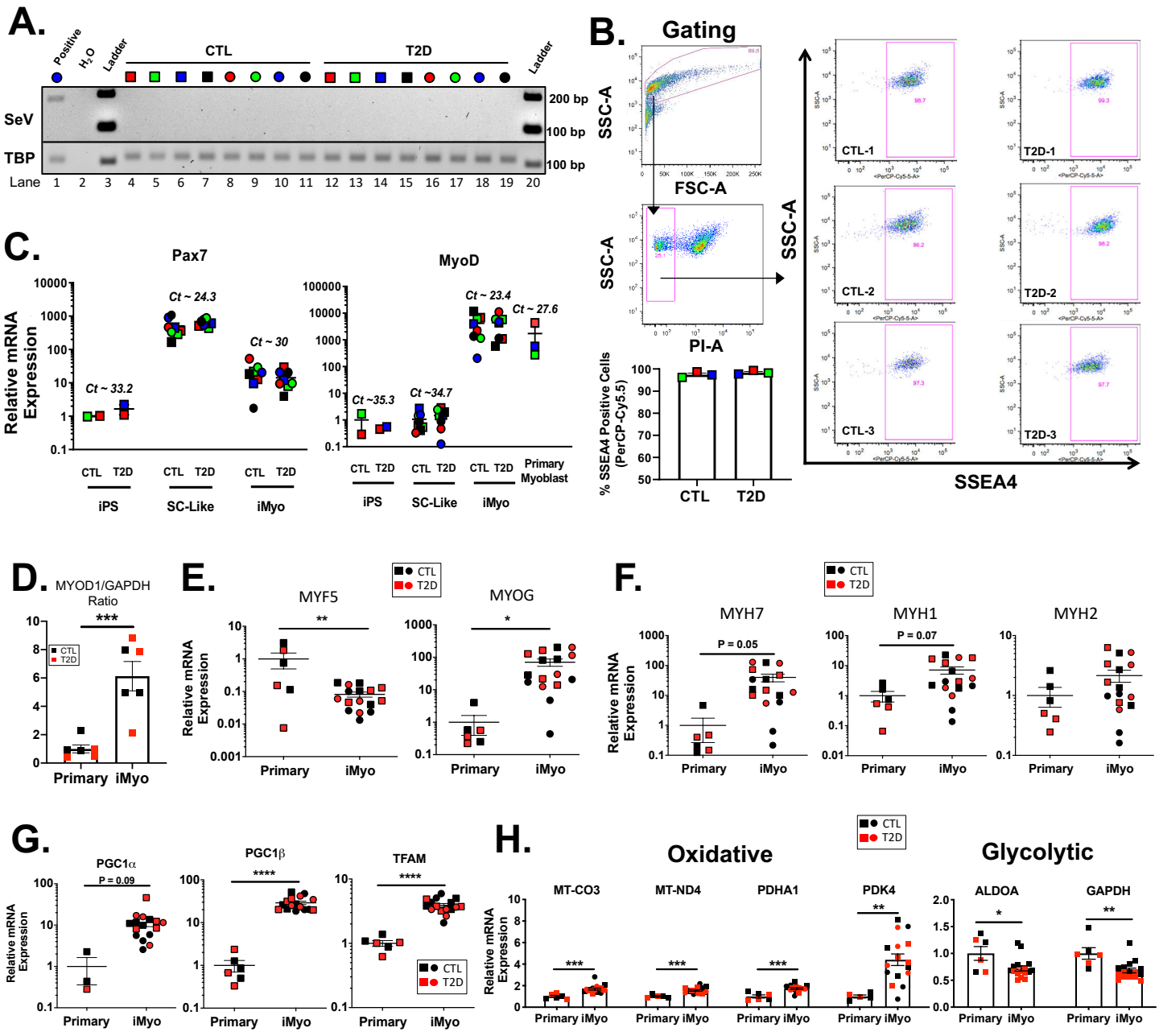
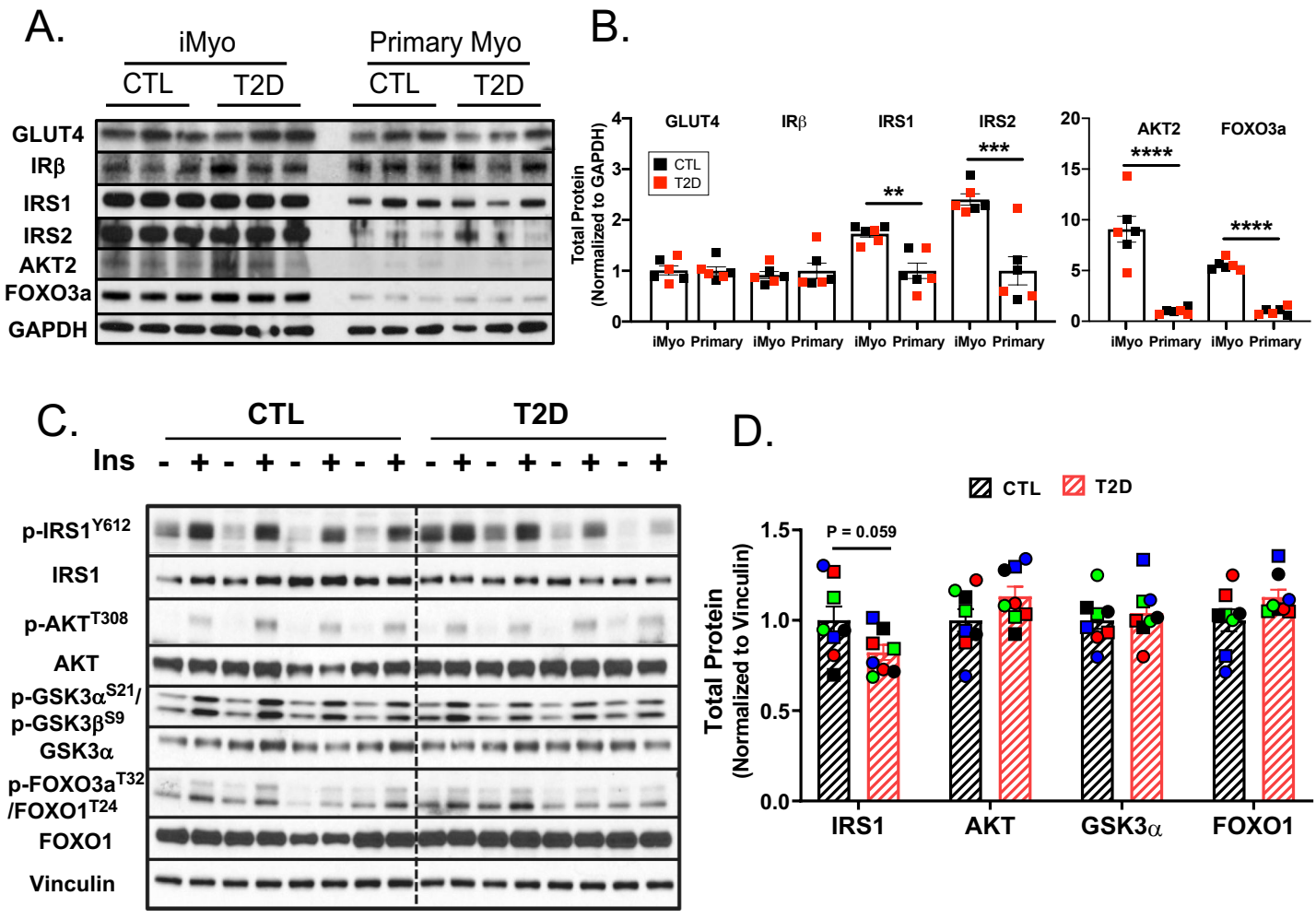
Figure S1. FACS and Gene Expression Analysis of iPSCs and Differentiated SC-like, iMyos and Primary Myoblasts, Related to Figure 1.



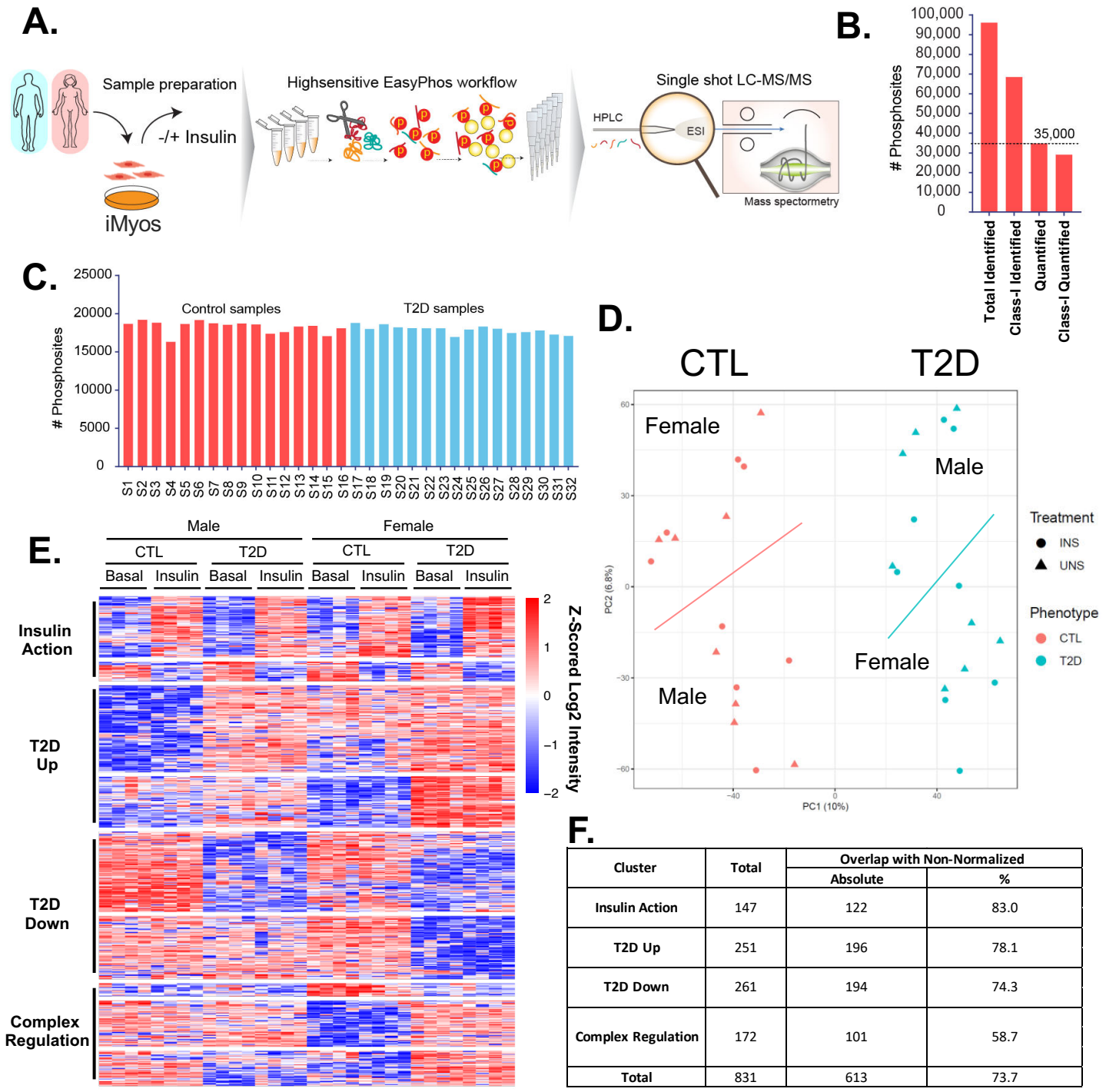
(A) RT-PCR analysis of iPSCs showing absence of Sendai virus genome after 16-23 passages. A low passage (p.1) iPSC cell line is used as positive control. (B) Gating strategy and FACS analysis of iPSCs from male subjects stained for SSEA4 (n = 3). (C) qPCR analysis of PAX7 and MYOD genes in iPSCs, SC-like, iMyos and primary myoblasts normalized to TBP and expressed as fold change over iPS (n = 2-8). (D) MYOD1 western blot quantification normalized to GAPDH (n = 6). (E-H) qPCR analysis of (E) myogenic factors MYF5 and MYOG, (F) myosin isoforms, (G) transcriptional regulators of mitochondrial biogenesis and (H) genes involved in oxidative and glycolytic metabolism. Male and female subjects are represented by squares and circles, respectively. Data are normalized to ribosomal subunit 18S and expressed as fold change over primary myoblasts (n = 3-16). All data are means ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, primary myoblast vs iMyos, Student's *t* test.

Figure S2. Comparative Protein Expression and Insulin Signaling Analysis in iMyos from Female Subjects, Related to Figure 2.



(A) Total expression and (B) quantification of key insulin signaling proteins in iMyos compared to primary myoblasts normalized to GAPDH. Data are means ± SEM, n = 6. ** P < 0.01, *** P < 0.001, **** P < 0.0001, Student's *t* test. (C) Insulin signaling in iMyos from female donors showing phosphorylation of IRS-1^{Y612}, AKT^{T308}, GSK3α^{S21}/GSK3β^{S9}, FOXO1^{T24}/FOXO3^{T32}. (D) Quantification of total IRS-1, AKT, GSK3α and FOXO1 normalized to Vinculin. Data are means ± SEM, n = 8.

Figure S3. Phosphopeptide Quantification and Principal Component Analysis (PCA) of the Phosphoproteomes from CTL and T2D iMyos, Related to Figure 3.

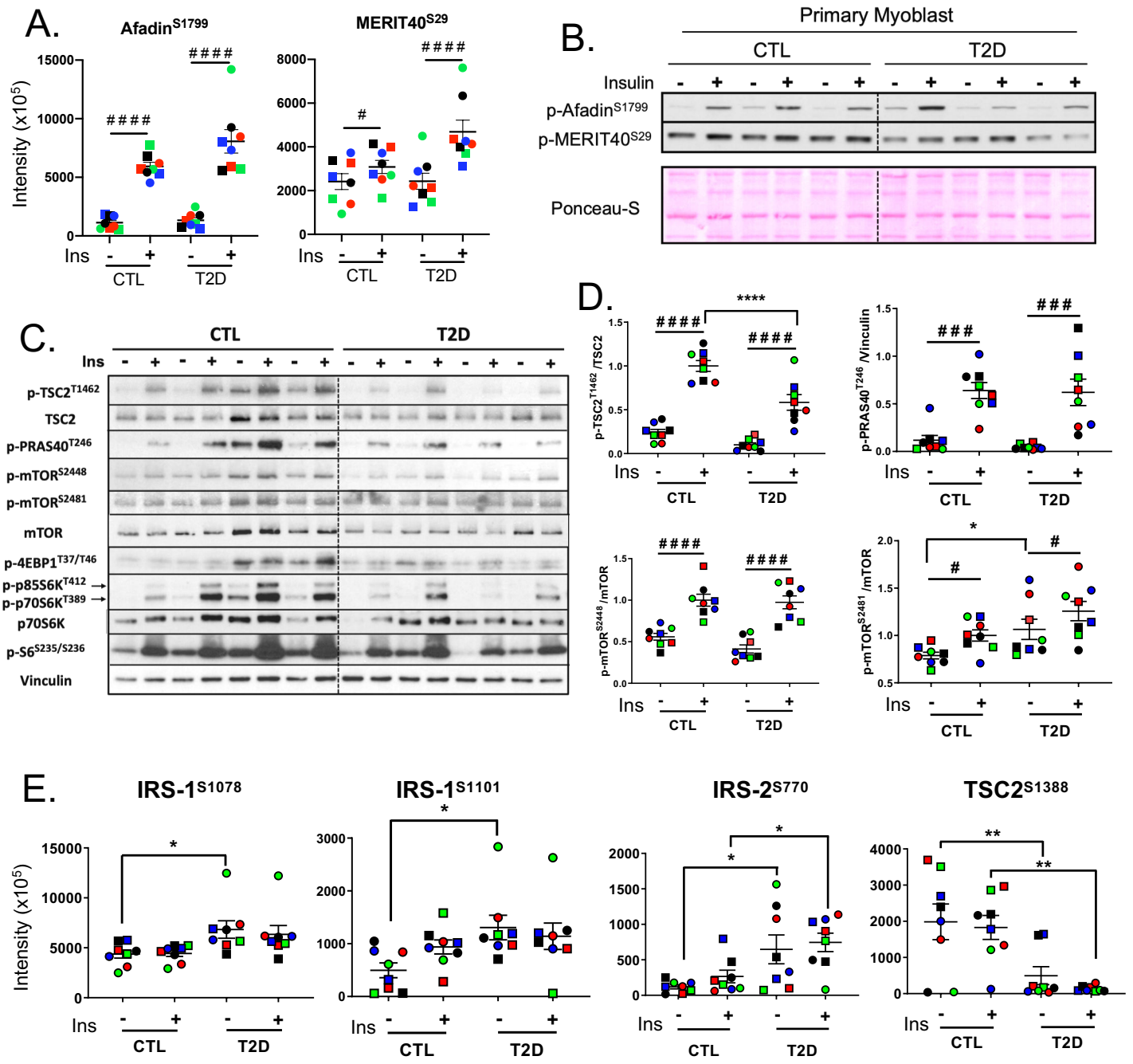


F.

Cluster	Total	Overlap with Non-Normalized	
		Absolute	%
Insulin Action	147	122	83.0
T2D Up	251	196	78.1
T2D Down	261	194	74.3
Complex Regulation	172	101	58.7
Total	831	613	73.7

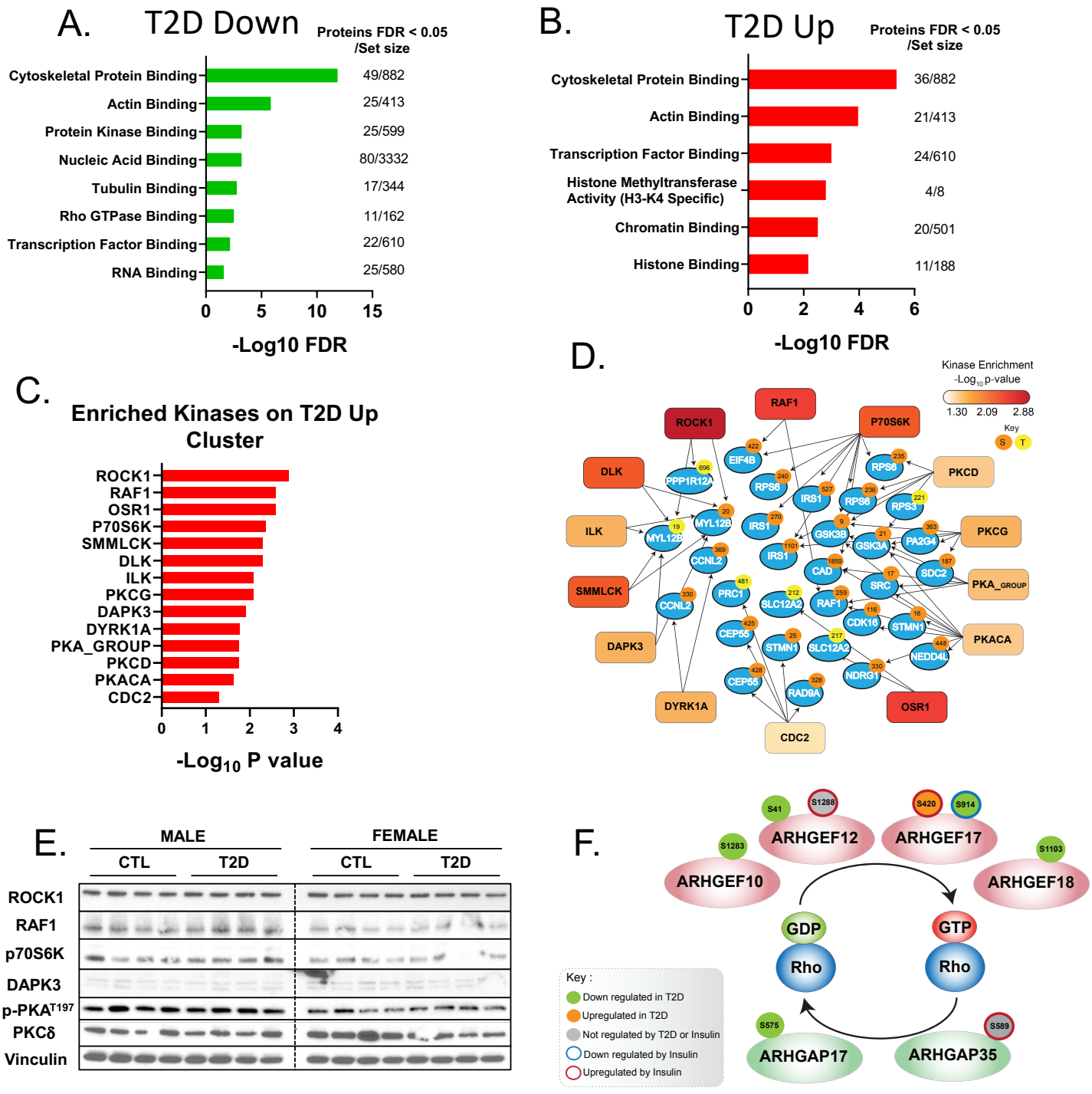
(A) Shematic of phosphoproteome workflow. (B) Average number of identified and quantified phosphopeptides by mass spectometry analysis. (C) Number of phosphosites quantified in all iMyo samples. (D) PCA plot showing separation of the phosphoproteomes by history of T2D, sex and insulin stimulation. (E) Hierarchical clustering analysis of phosphopeptides normalized to total protein levels by proteomics. (F) Table showing the number phosphosites on each cluster of the protein-normalized data. The number of sites that overlapped with non-normalized analysis are shown as absolute values and percentages. See also Table S2.

Figure S4. Insulin-Regulated Phosphosites in Primary Myoblasts and iMyos, Basal Changes on Phosphorylation of IRS1/2 and TSC2, Related to Figure 3.



(A-B) Site-specific phosphorylation of Afadin and MERIT40 in (A) iMyos by phosphoproteomics and in (B) primary myoblasts by western blot (n = 3). Data are means ± SEM of phosphosites intensity values (x10^5). # P <0.05, #### P <0.0001 basal vs insulin, Two-way ANOVA. (C) Validation of phosphoproteomics by immunoblot in iMyos from female subjects. (D) Western blot quantification of mTOR signaling showing TSC2^{T1462}, PRAS40^{T246}, mTOR^{S2448} and mTOR^{S2481}. Phosphorylated proteins are normalized by total protein expression. Data are means ± SEM, n = 8. # P < 0.05, ### P < 0.001, #### P < 0.0001 basal vs insulin, * P <0.05, **** P <0.0001 CTL vs T2D, Two-way ANOVA. (E) Analysis of phosphorylation of IRS-1^{S1078}, IRS-1^{S1101}, IRS-2^{S770} and TSC2^{S1388} by phosphoproteomics. Data are means ± SEM of phosphosites intensity values (x10^5). * P <0.05, ** P <0.01 CTL vs T2D, Two-way ANOVA.

Figure S5. Molecular Function (GO), Kinase Analysis and Expanded View of Rho GEFs and Rho GAPs, Related to Figure 4 and Figure 5.



(A and B). Enrichment analysis of overrepresented Molecular Function Gene Ontology terms of proteins with phosphosites (A) down-regulated or (B) up-regulated in T2D iMyos. (C) Putative regulatory upstream kinases enriched (P < 0.05) among proteins with up-regulated phosphosites in T2D iMyos. (D) Network indicating relationship between kinases and annotated substrates (Phosphosite Plus and RegPhos). Kinases are colored according to enrichment (-log10 P value). (E) Western blot of selected kinases in iMyos from CTL and T2D showing no major differences in expression. (F) Representation of Rho GEFs and GAPs whose phosphorylation was differentially regulated by T2D or insulin.

Table S3. Primer sequences used in the study, Related to STAR METHODS.

Gene	Primer Sequence	
TBP	F	TGATGCCTTATGGCACTGGACTGA
	R	CTGCTGCCTTTGTTGCTCTTCCAA
SOX2	F	GCCGAGTGGAAACTTTTGTCG
	R	GGCAGCGTGTACTTATCCTTCT
NANOG	F	TCCAACATCCTGAACCTCAG
	R	GACTGGATGTTCTGGGTCTG
OCT4	F	GTGGAGGAAGCTGACAACAA
	R	CAGGTTTTCTTTCCCTAGCT
PAX7	F	CGTGCTCAGAATCAAGTTCG
	R	GTCAGGTTCCGACTCCACAT
MYOD	F	CTCCAACTGCTCCGACGGCAT
	R	ACAGGCAGTCTAGGCTCGACAC
MYF5	F	GCCTGAAGAAGGTCAACCAG
	R	CCATCAGAGCAGTTGGAGGT
MYOG	F	AGATGTGTCTGTGGCCTTCC
	R	AGCTGGCTTCCTAGCATCAG
MYH7	F	CTTTGCTGTTATTGCAGCCATT
	R	AGATGCCAACTTTCCTGTTGC
MYH1	F	TTCATTGGGGTCTTGGACAT
	R	AACGTCCACTCAATGCCTTC
MYH2	F	AGAAACTTCGCATGGACCTAGA
	R	CCAAGTGCCTGTTCATCTTCA
PGC1a	F	GGACTCAAGTGGTGCAGTGA
	R	CTGCTAGCAAGTTTGCCTCA
PGC1b	F	CTGAAGAGGCGCTTTGAAGT
	R	GTGCTCAGAACACCGGTAGG
TFAM	F	GGGAAGGTCTGGAGCAGAG
	R	TGGACAACTTGCCAAGACAG
MT-CO3	F	TCCACTCCATAACGCTCCTC
	R	GTGGCCTTGGTATGTGCTTT
MT-ND4	F	TGAATCAACACAACCACCCACAGC
	R	AGTGGTTCACTGGATAAGTGGCGT
PDHA1	F	TGGTAGCATCCCGTAATTTTGC
	R	ATTCGGCGTACAGTCTGCATC
PDK4	F	GGAGCATTTCTCGCGCTACA
	R	ACAGGCAATTCTTGTCGCAAA
ALDOA	F	ATGCCCTACCAATATCCAGCA
	R	GCTCCCAGTGGACTCATCTG
GAPDH	F	TGGTATCGTGGAAGGACTCA
	R	TTCAGCTCAGGGATGACCTT
18S	F	GTTCCGACCATAAACGATGCC
	R	TGGTGGTGCCCTTCCGTCAAT
Sendai Virus	F	GGATCACTAGGTGATATCGAGC
	R	ACCAGACAAGAGTTTAAGAGATATGTATC