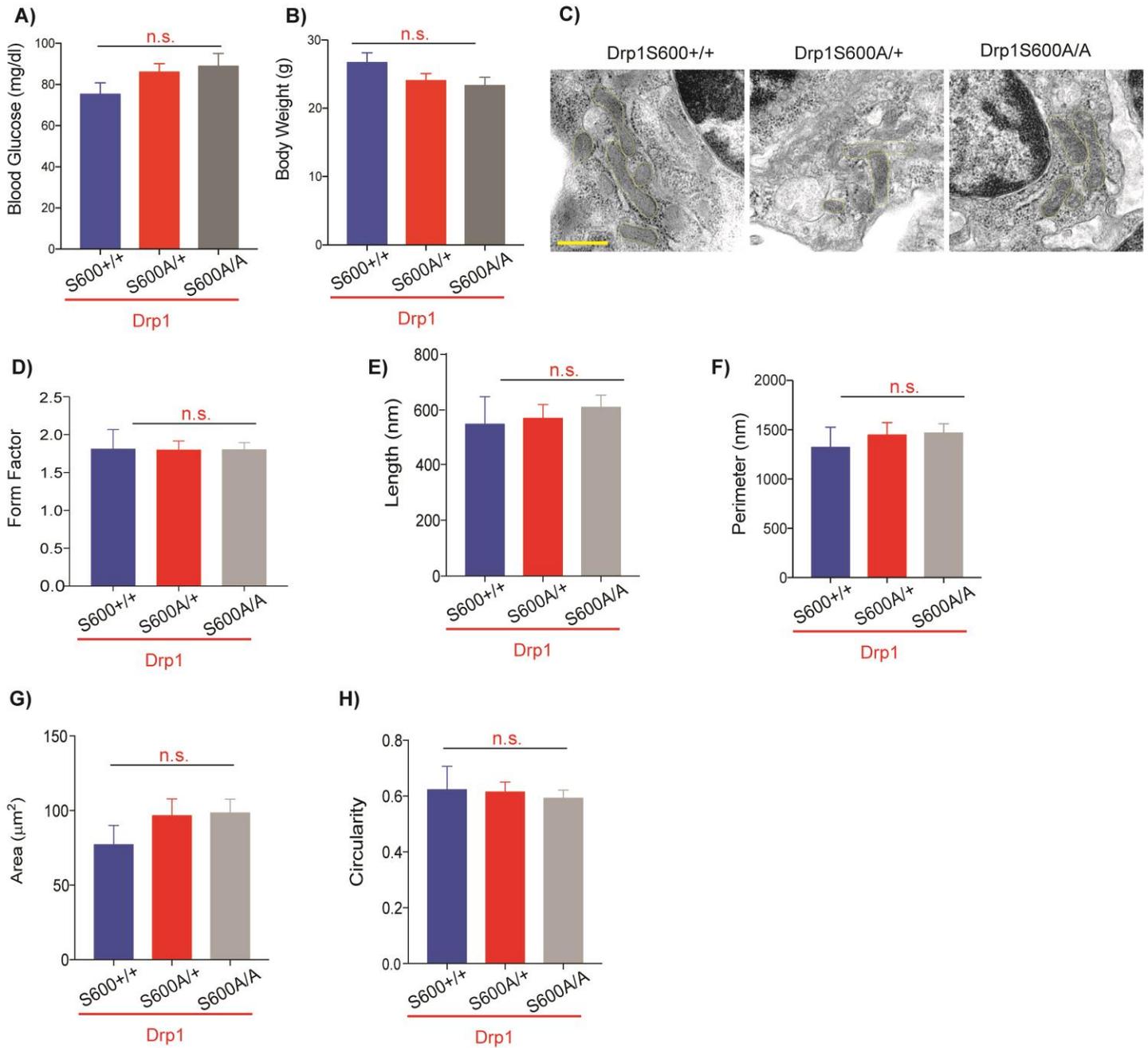


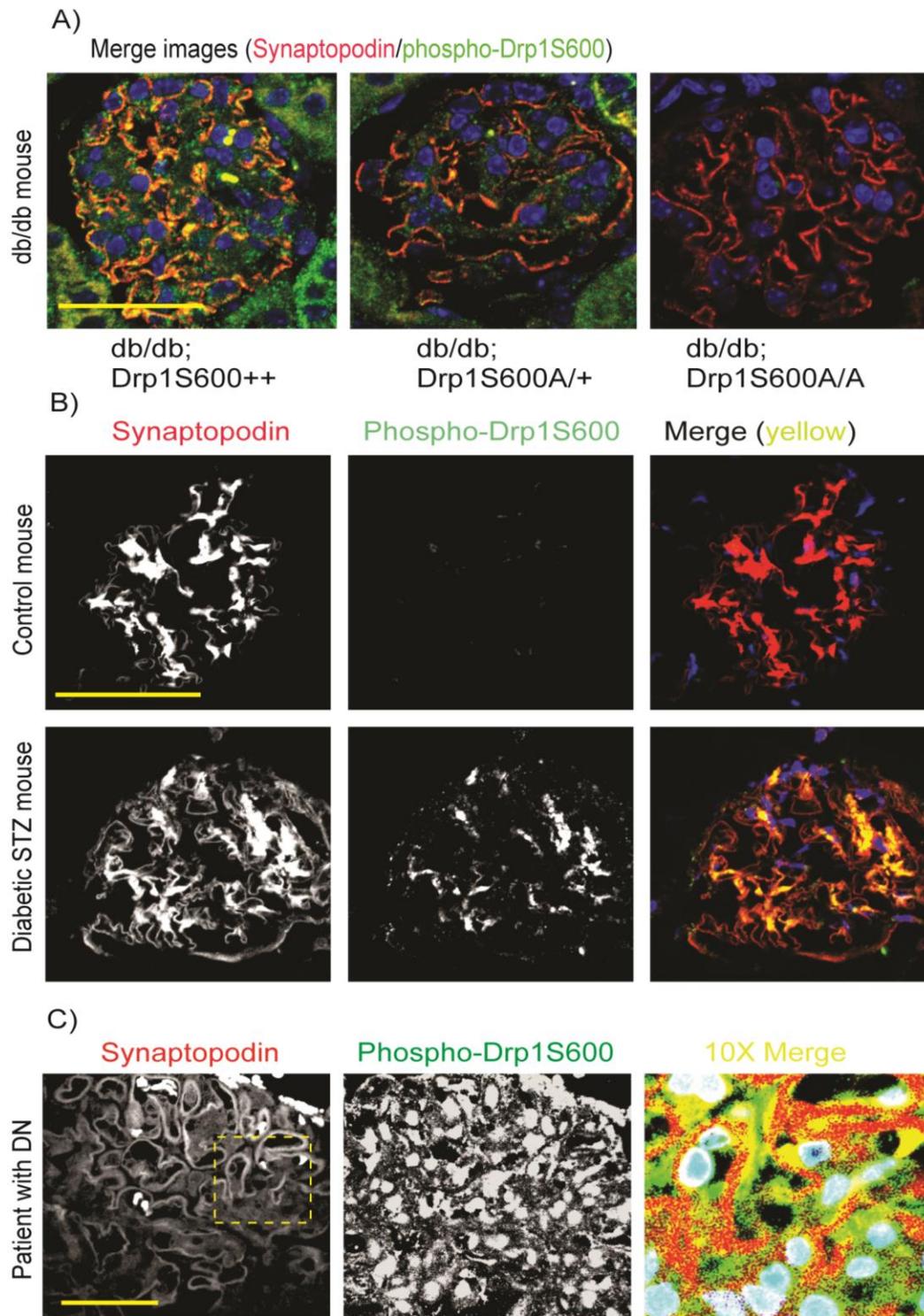
Drp1S600 phosphorylation regulates mitochondrial fission and progression of nephropathy in diabetic mice

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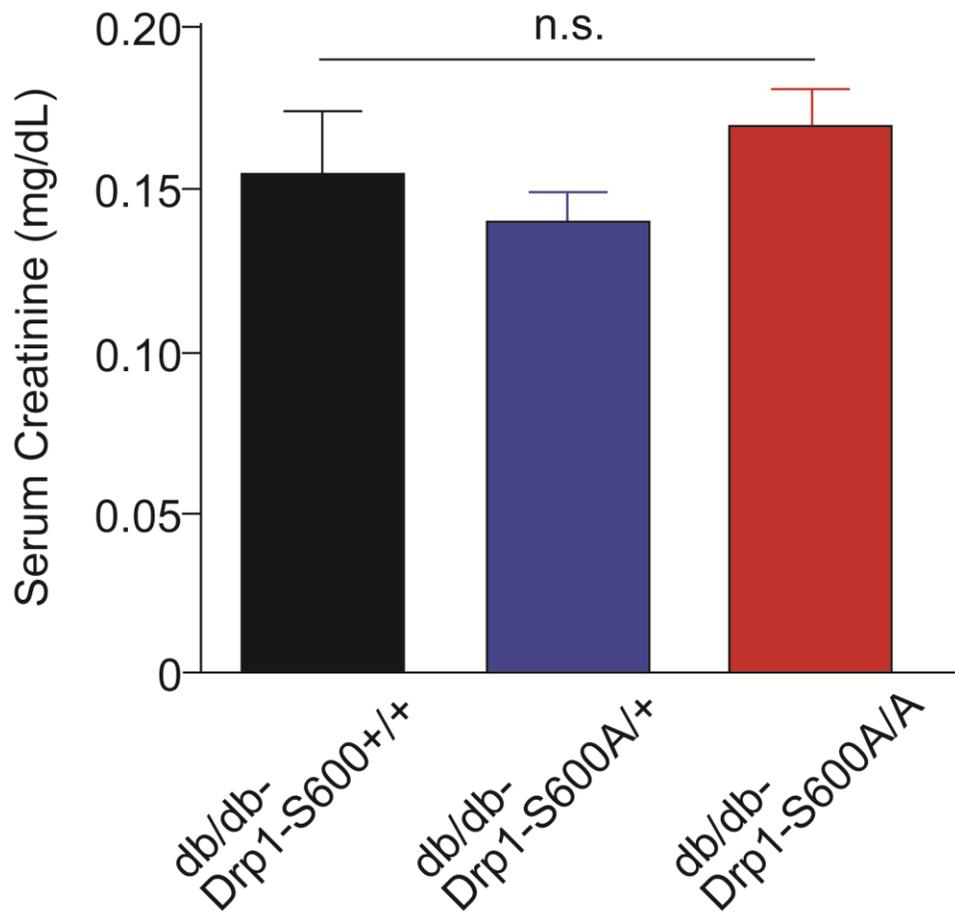
Supplemental Figures Addendum



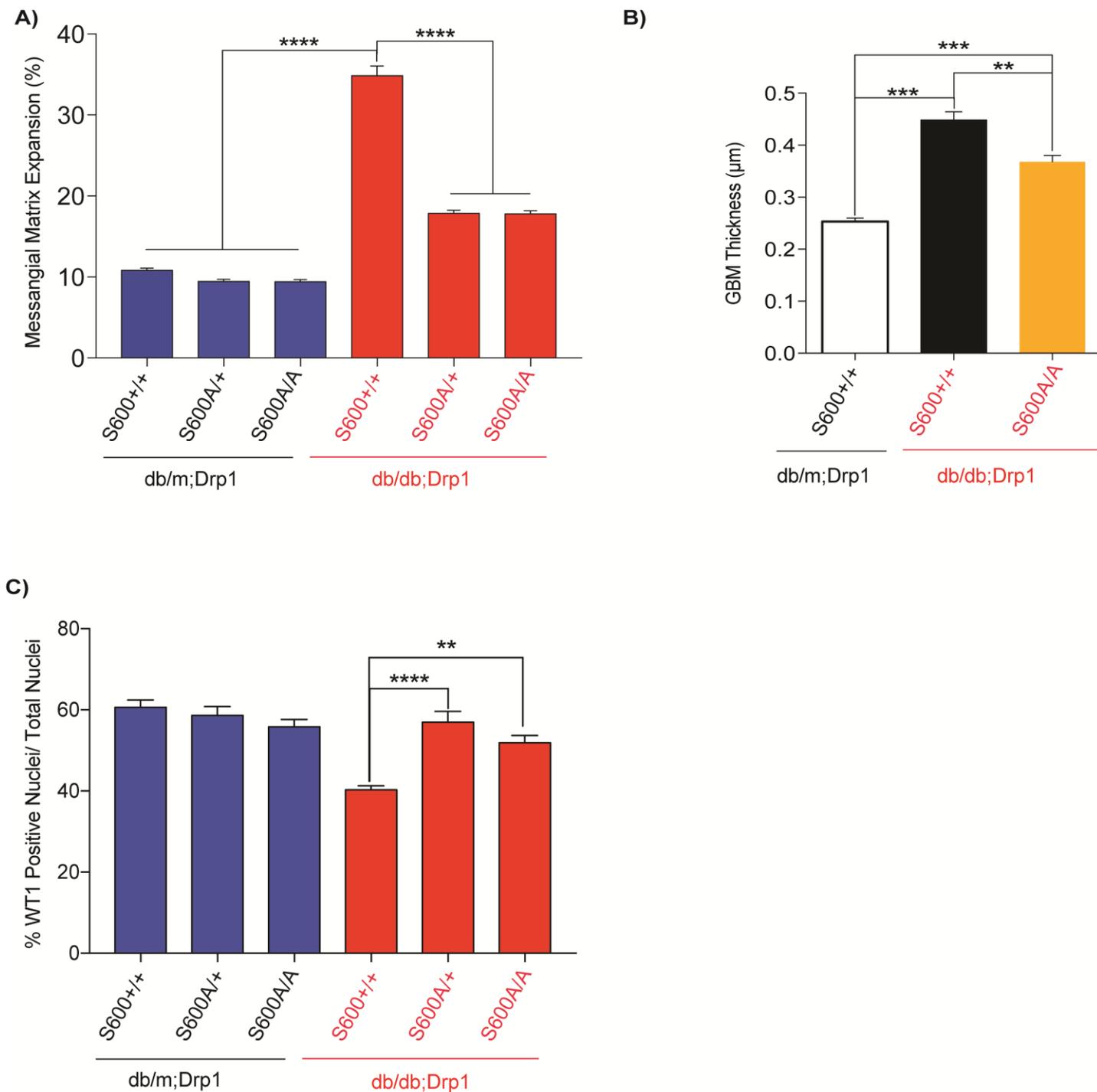
Supplemental Figure 1. *Drp1S600A* knockin does not have a distinctive phenotype. The *Drp1S600A* mutation was characterized in the homozygous and heterozygous states within the C57Bl/6J background. **(A)** Blood glucose and **(B)** body weight at 20 weeks of age. **(C)** TEM micrographs illustrating mitochondrial morphology of glomerular podocytes at 20 weeks of age. Analyses of mitochondria from TEM micrographs: **(D)** Form factor **(E)** length **(F)** perimeter **(G)** area and **(H)** circularity. Representative images are from a sampling of 3 animals. Statistical analyses were performed using one way-analysis of variance (one-way ANOVA) followed by Tukey's multiple comparisons test. A $p < 0.05$ considered to be statistically significant. Results are presented as $\text{mean} \pm \text{SEM}$ ($n=3/\text{group}$). ns= $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



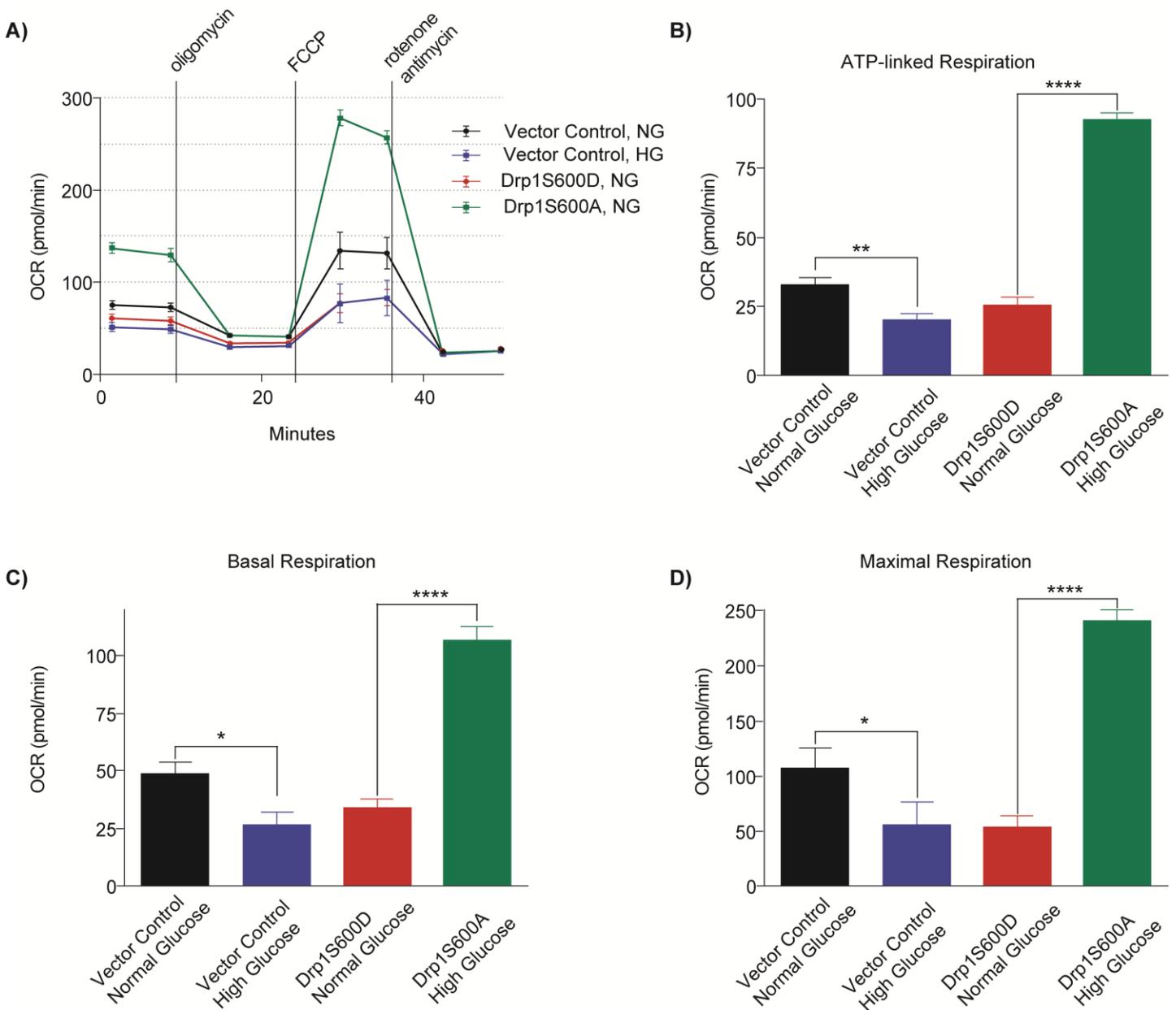
Supplemental Figure 2. Increased Drp1S600 phosphorylation in DN. Immunostaining for phospho-Drp1S600 and synaptopodin. **(A)** All 3 Drp1S600 mutant genotypes in our db/db model of Type 2 diabetes. **(B)** Streptozotocin treated mice. **(C)** Biopsy from a patient with DN. Scale bar denotes 50µm. Representative images are from a sampling of 3-5 animals or patients.



Supplemental Figure 3. Serum creatinine was not significantly changed in diabetic mutant mice. Serum from diabetic mutant mice was collected and analyzed for creatinine. Statistical analyses were performed using one way-analysis of variance (one-way ANOVA) followed by Tukey's multiple comparisons test. A $p < 0.05$ considered to be statistically significant. Results are presented as mean \pm SEM (n=3/group).



Supplemental Figure 4. Morphometric data from diabetic *Drp1S600A* knockin mice. (A) Mesangial matrix expansion at 20 weeks of age. Kidney histology sections were PAS stained. **(B)** TEM micrographs were used to determine the glomerular basement membrane (GBM) thickness. **(C)** Paraffin embedded sections of kidney were stained for podocyte nuclei (WT1) and all nuclei (DAPI). Percent of WT1 positive nuclei per total number of glomerular nuclei are shown. Statistical analyses were performed using one way-analysis of variance (one-way ANOVA) followed by Tukey's multiple comparisons test. A $p < 0.05$ considered to be statistically significant. Results are presented as mean \pm SEM ($n = 5-8$ /group). ns= $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

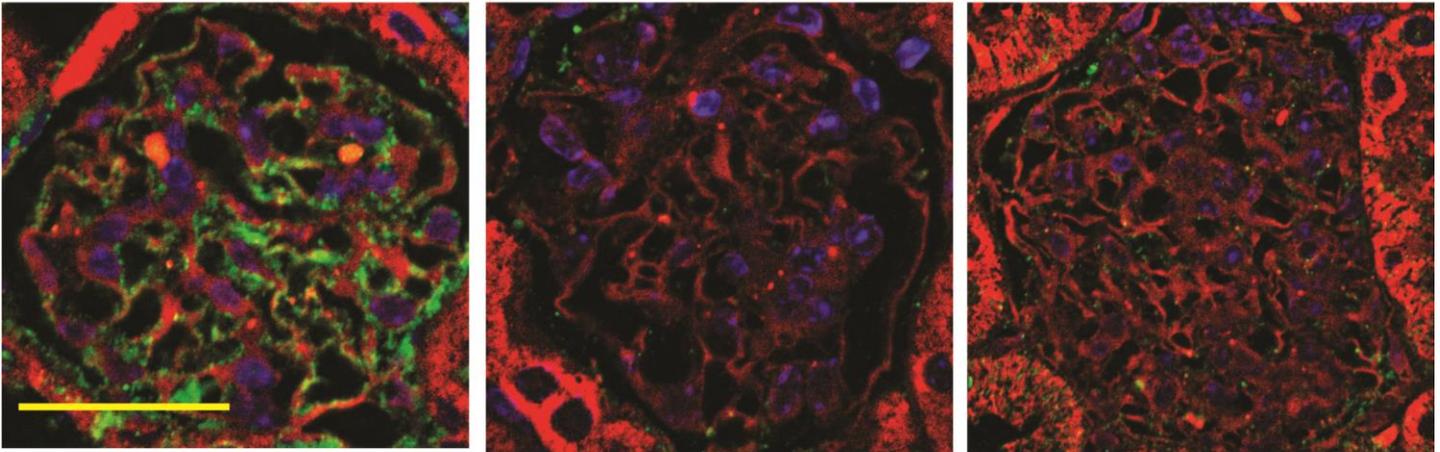


Supplemental Figure 5. Seahorse oxygen consumption rate (OCR) analysis of podocytes. Cultured podocytes containing empty vector, Drp1S600D, or Drp1S600A were treated with normal glucose (5mM, NG) or high glucose (25mM, HG) for 48 hours. **(A)** Cells were subjected to OCR measurements. **(B)** ATP-linked respiration. **(C)** Basal respiration. **(D)** Maximal respiration. Statistical analyses were performed using one way-analysis of variance (one-way ANOVA) followed by Tukey's multiple comparisons test. A $p < 0.05$ considered to be statistically significant. Results are presented as mean \pm SEM (n=5-8/group). ns= $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Merged Images

Red: Synaptopodin

Green: Nitrotyrosine

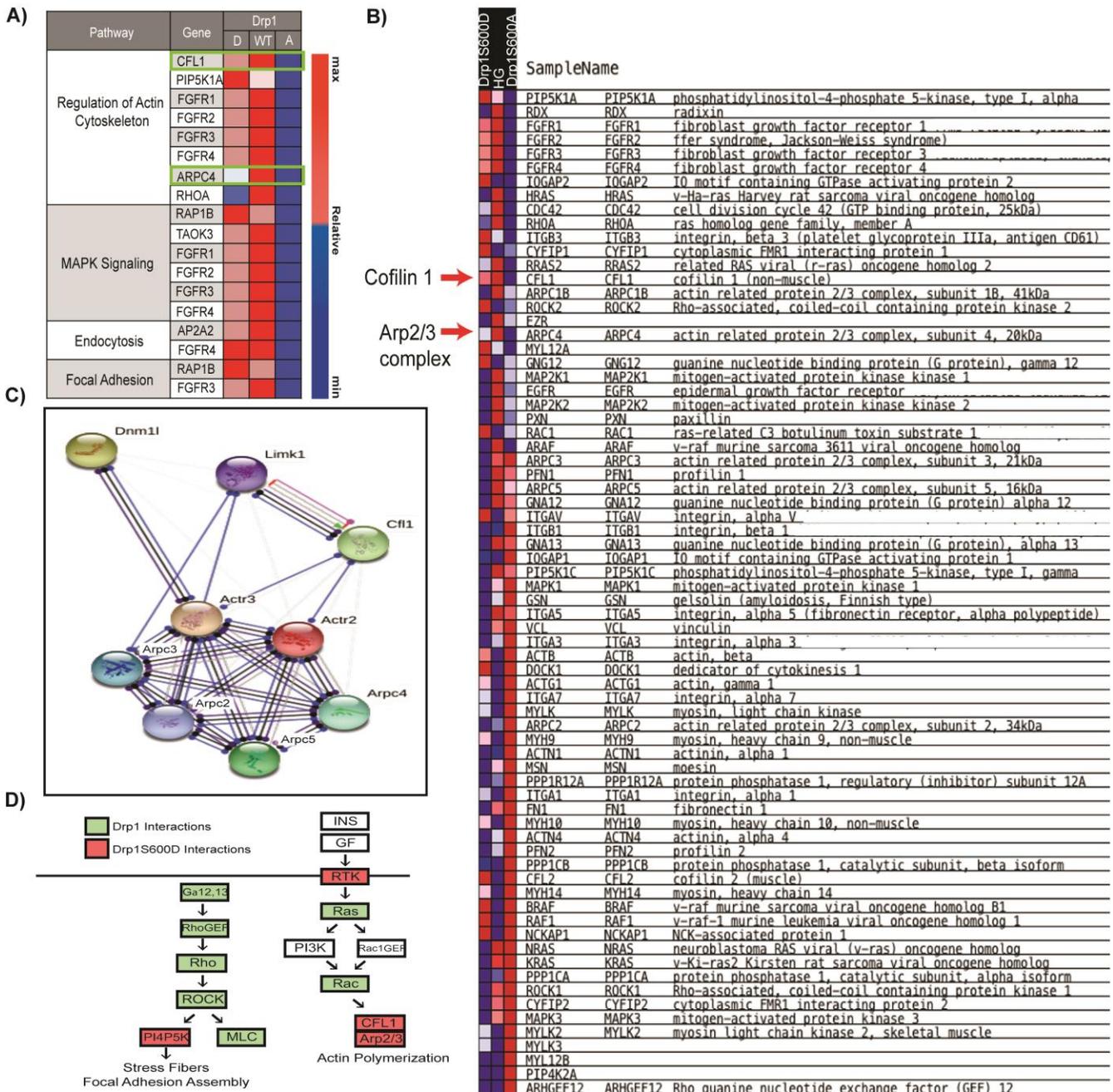


Drp1S600+/+

Drp1S600A/+

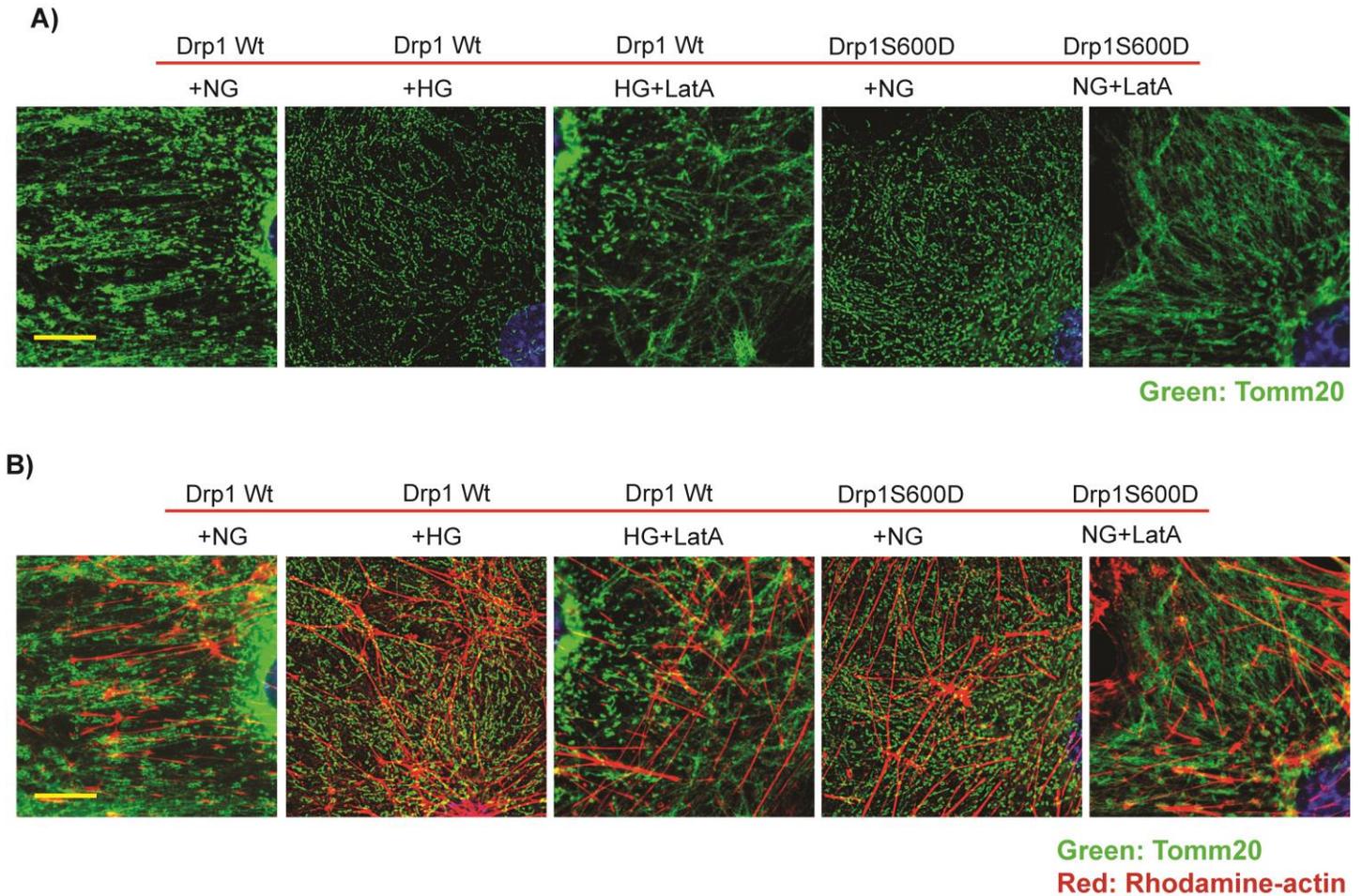
Drp1S600A/A

Supplemental Figure 6. Increased nitrotyrosine staining in diabetic glomeruli is significantly attenuated in heterozygous and homozygous *Drp1S600A* mice. Immunofluorescent staining with synaptopodin and the oxidative stress marker, nitrotyrosine. Scale bar denotes 50 μ m. Representative of 3 mice.

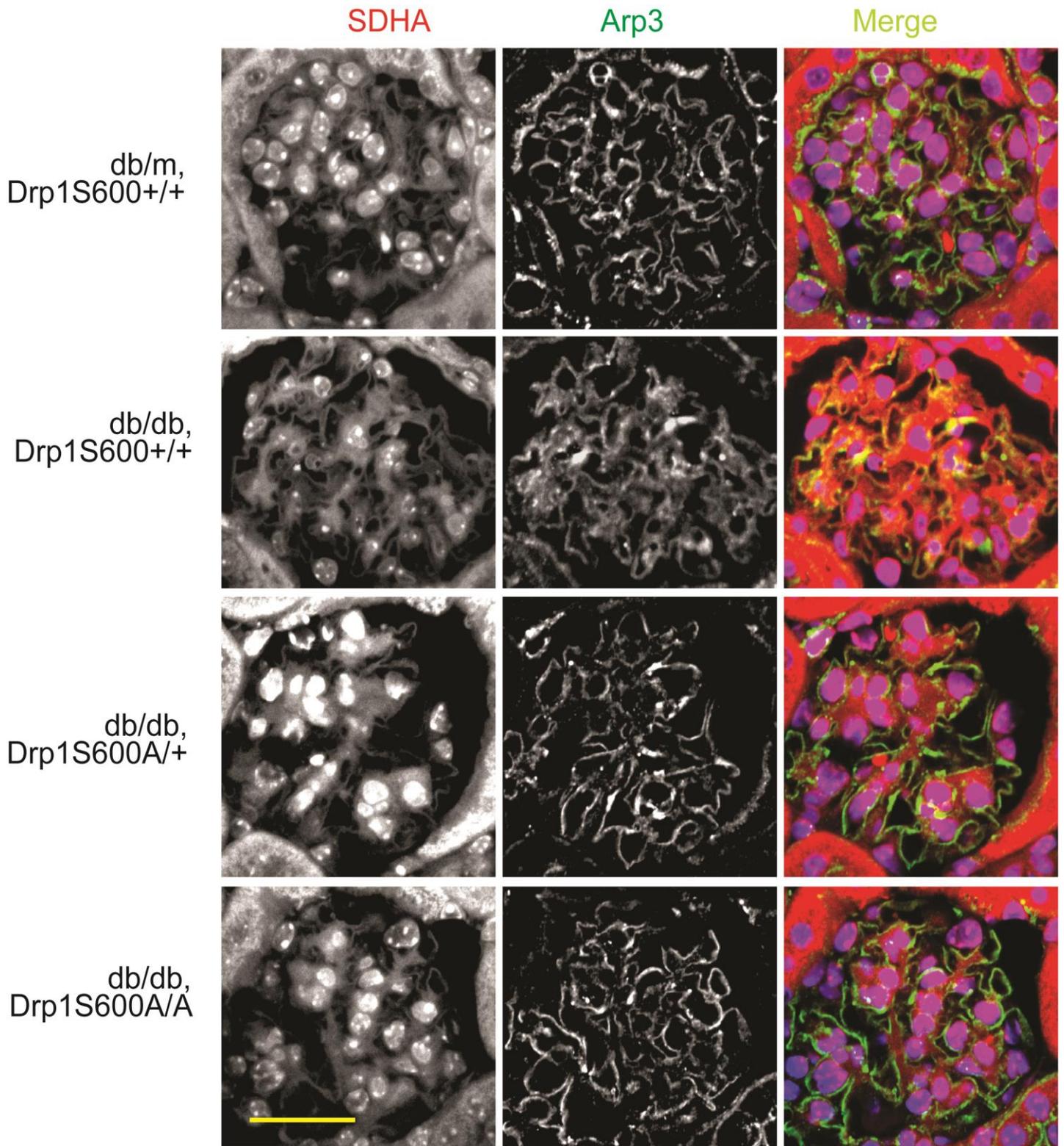


Supplemental Figure 7. Actin cytoskeleton pathway is a top hit for phospho-Drp1S600. In vitro differentiated podocytes stably expressing FLAG-tagged versions of Drp1 (*wild-type*) and Drp1-S600A were treated with HG (25 mM) for 48 hours. FLAG-tagged Drp1-S600D were cultured under normal glucose. Cells were cross-linked, immunoprecipitated, and subjected to mass spectrophotometry. KEGG Pathway analysis of protein interaction partners was performed. **(A)** Selected genes in pathways shown and illustrated by color (red, high; blue, low) for the degree of change in each of the 3 constructs tested. **(B)** Expanded list of selected genes in pathways shown and illustrated by color (red, high; blue, low) the degree of change in each of the 3 constructs tested. **(C)** String protein analysis of protein molecular action was performed, blue=binding, black=reaction, purple=catalysis, green=activation, red=inhibition, light blue=phenotype, pink=post-translational modification, gold=transcriptional regulation. An arrowed line is positive interaction, blocked line is a negative interaction, and rounded line is unknown interaction. **(D)** Focus on “Regulation of actin cytoskeleton” genes illustrating strong interaction of the phospho-S600 at CFL1 (cofilin1), ACTR3, ARP2/3 (actin related proteins 2/3), RTK (receptor tyrosine kinase), and PI4P5K (Phosphatidylinositol-4-Phosphate 5-Kinase).

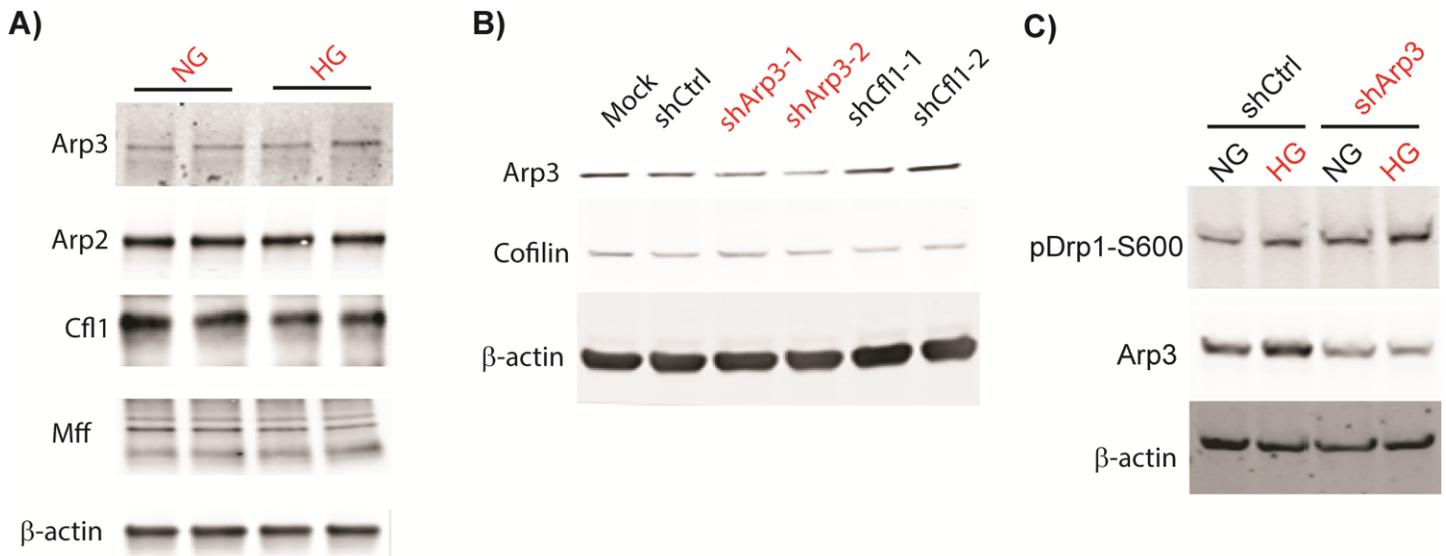
Cultured podocytes



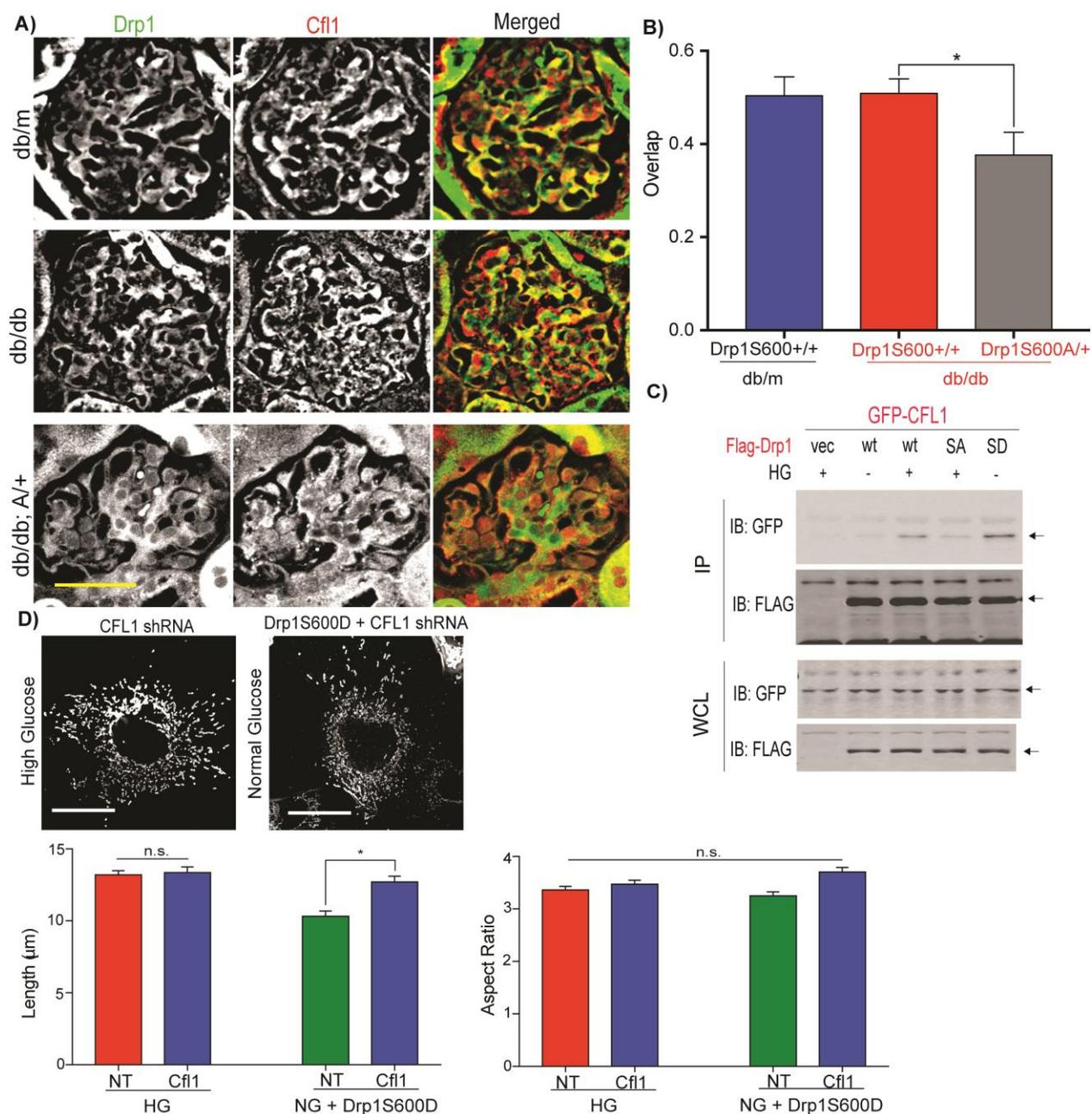
Supplemental Figure 8. Actin cytoskeleton interacts with phospho-Drp1S600 and mitochondria. *In vitro* differentiated podocytes with the empty vector control or stably expressing Drp1-S600D were employed for biochemical and morphological analysis. Podocytes were immunostained with TOMM20 (green) for mitochondria and rhodamine-phalloidin (red) for actin. From left to right cells were cultured under normal glucose (NG), high glucose (HG), HG with latrunculin A (HG + LatA), NG, and NG + LatA. First 3 panels are empty vector podocytes and final 2 panels are podocytes stably expressing Drp1S600D. HG and 50nM latrunculin A were used for 48 hrs. **(A)** Mitochondrial staining without the actin to emphasize mitochondrial network. **(B)** Mitochondria and actin shown together to visualize network. Images are representative of 3-4 replicates of cell cultures.



Supplemental Figure 9. *Arp3* localizes with mitochondria in diabetic *db/db* mice, but not when mutant *Drp1S600A* is present. Immunohistochemistry against the mitochondrial marker, SDHA (red), and Arp3 (green). Scale bar denotes 50µm. Representative of 3 mice.



Supplemental Figure 10. Cytoskeletal proteins and Mff are not increased in expression by high glucose culture. Cultured podocytes were treated with normal glucose (5mM, NG) or high glucose (25mM, HG) for 48 hours and cellular lysates probed by Western blot for indicated proteins. (A) Western blot analysis of cytoskeletal proteins and Mff. (B) Western blot of podocytes showing effective knockdown of Arp3 and cofilin 1 by shRNA. (C) Western blot for Drp1S600 phosphorylation in Arp3 knockdown cells. Representative of 3 independent trials.



Supplemental Figure 11. Phospho-Drp1S600 Drp1 and cofilin 1. **(A)** Immunofluorescent staining of kidney paraffin sections. Representative images of stained kidney derived of four mouse strains are shown. Sections were stained against total Drp1 (grey-scale, green in merge) and cfl1 (grey-scale, red in merge) Scale= 50 μ m. **(B)** Colocalization analysis using Pearson's Correlation analysis of total Drp1 and mitochondria determined from images represented in **A**. **(C)** Podocytes with empty vector (vec), FLAG-tagged *wild-type* Drp1 (wt) cultured under normal and HG, FLAG-tagged-Drp1-S600A (SA) under HG, and FLAG-tagged Drp1-S600D (SD) under normal glucose were employed. Cells were also transiently transfected with GFP-CFL1. Top panels are immunoprecipitation (IP) material against the FLAG-tag with subsequent immunoblotting against GFP and FLAG-tag. Bottom panels are the starting whole cell lysates (WCL). **(D)** ShRNA knockdown of cfl1 in podocytes (native and S600D) followed by mitochondrial staining with TOMM20. Under each respective image are shown quantitation of the observed mitochondrial network: mitochondrial length and then aspect ratio. Scale bar=25 μ m. Representative images are from a sampling of 3 animals or cell cultures. Statistical analyses were performed using one way-analysis of variance (one-way ANOVA) followed by Tukey's multiple comparisons test. A $p < 0.05$ considered to be statistically significant. Results are presented as mean \pm SEM (n=3/group). ns= $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.