Supplementary Materials

The calcium-dependent protease calpain-1 links TRPC6 activity to podocyte injury

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Running title: **Podocyte TRPC6 activates calpain**

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Supplementary Figure legends

Supplementary Figure 1: Cortical calpain mRNA expression in control and adriamycin-treated rats

Wistar rats were injected with vehicle (Control) or adriamycin (ADRIA) to induce adriamycin nephropathy, a model for human FSGS. Cortical mRNA expression of various calpains was determined. * $p < 0.05$ compared to vehicle treated control rats, n=2 rats/group.

Supplementary Figure 2: Talin and calpain co-localise in podocytes

Glomerular Talin was co-stained with synaptopodin, representative images of healthy controls **(A)** and FSGS patients **(B)**. Synaptopodin was stained in combination with an *in situ* zymography for calpain activity in sections from healthy controls **(C)** and FSGS patients **(D)**.

Supplementary Figure 3: TRPC6 is overexpressed in podocytes in FSGS and adriamycin nephropathy

TRPC6 was co-stained with synaptopodin, representative images of control human kidney **(A)**, FSGS patient **(B)**, control rat **(C)** and ADRIA treated rat **(D)**.

Supplementary Figure 4: Calpain inhibition in an animal model for human FSGS improves renal outcome

Wistar rats were injected with vehicle (Control) or adriamycin (ADRIA) to induce adriamycin nephropathy, a model for human FSGS. Animals were treated with vehicle or calpeptin for 6 weeks after which they were sacrificed. Glomerular injury was approximated using the albumin/creatinin ratio (**A**) and urinary IgG excretion (**B**). Serum calpain activity was assessed (**C**). A calpain activity assay was performed on the supernatant of the sections, normalized to surface area of the section (**D**). The podocyte injury marker desmin was co-stained with synaptopodin and its expression was quantified by immunofluorescence (**E**). * $p < 0.05$ compared to vehicle treated control rats. $^{\$}$ $p < 0.05$ compared to vehicle treated ADRIA rats. † $p < 0.05$ compared to calpeptin treated vehicle rats, n=8 rats per treatment group.

Supplementary Figure 5: TRPC6-dependent Ca^{2+} influx and calpain-1 in cultured podocytes.

The effect of adriamycin (ADRIA) on calpain-1 expression compared to vehicle treated cells (Vehicle) was detemined in mouse podocytes using real-time quantitative RT-PCR analyses (**A**). Intracellular Ca^{2+} concentration was determined by Fura-2 ratiometry. Stably transfected TRPC6 knockdown ($TRPC6^{KD}$) and scrambled (Scram) cultured podocytes were pre-treated for 24h with vehicle or ADRIA. After removal of the specific media, cells were exposed to 100 µM OAG to activate TRPC6, and Ca^{2+} influx was measured (**B**). Differentiated immortalized cultured podocytes were transfected with non-targeting scrambled (Scram) or calpain-1 ($CPN\text{-}1^{KD}$) siRNA. Subsequently, they were treated with vehicle or adriamycin (ADRIA) for 24 hours. TRPC6 mRNA expression was determined using real-time quantitative RT-PCR analyses (**C**). * $p < 0.05$ compared to healthy controls. Results are representative of two separate experiments, with n=4 per treatment group.

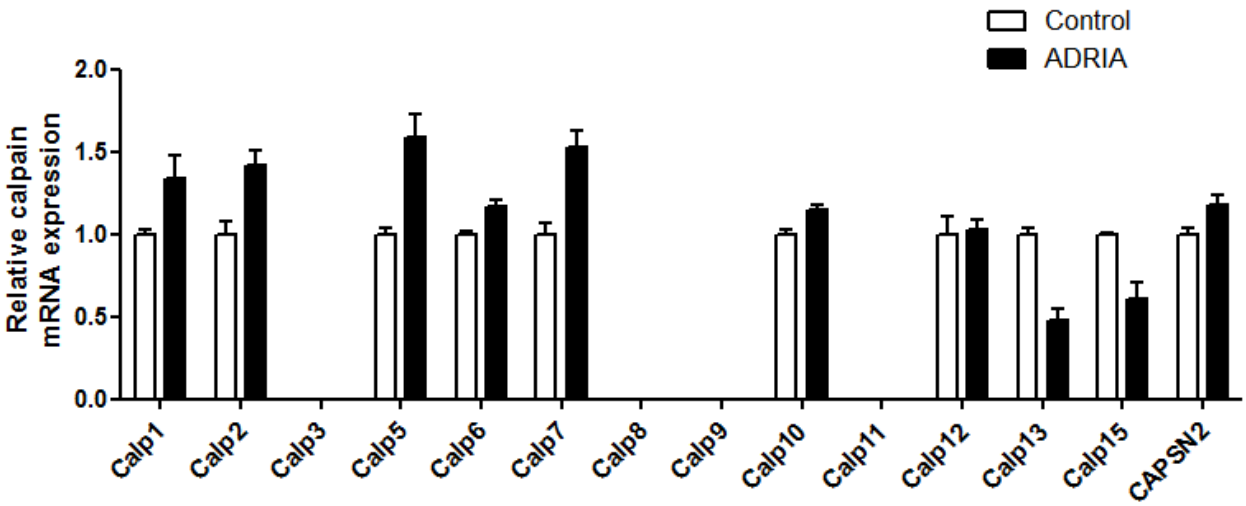
Supplementary Figure 6: Calpain activity in injured cultured podocytes and experimental as well as human FSGS is not of mitochondrial origin.

Calpains are also expressed in mitochondria. Therefore, we the mitochondrial calpain activity was also determined. No increase in calpain activity was found in mitochondria isolated from kidney cortex of FSGS patients (n=3) versus controls (n=5) (**A**). Similar results were obtained for kidneys from adriamycin-treated rats versus vehicle-treated controls, n=8 rats/group (**B**) and vehicle, adriamycine (ADRIA)- or 1-oleoyl-2-acetyl-sn-glycerol (OAG)-treated cultured mouse podocytes, representative of two experiments, with n=4/group (**C**).

Supplementary Figure 7: Schematic overview of the link between calpain-1 and TRPC6 in podocyte injury

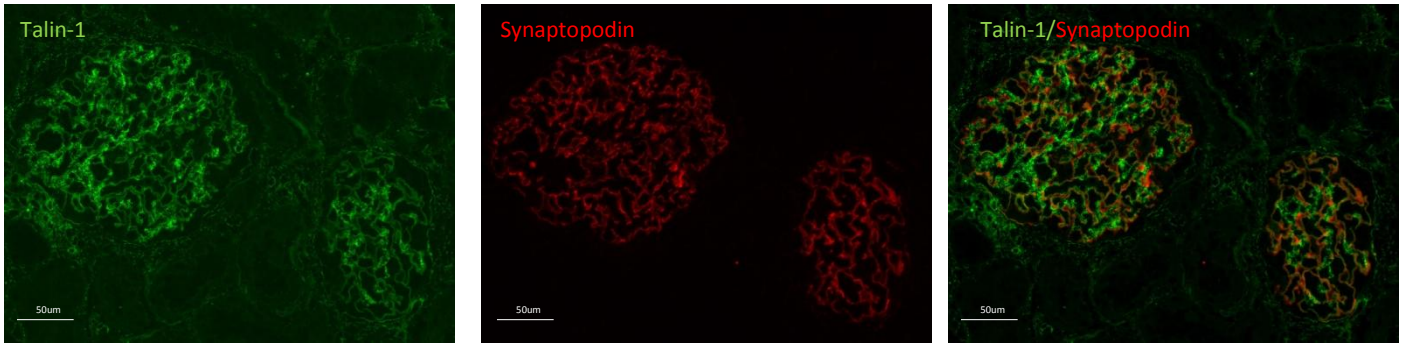
Ca^{2+} influx through TRPC6 activates the calcineurin-NFAT pathway which leads to further TRPC6 transcription and expression. Based on our findings, TRPC6-dependent Ca^{2+} influx also activates calpain-1, which in turn can activate calcineurin. Activation of calpain-1 leads to breakdown of structural proteins like Talin-1 and calpastatin which causes enhanced cell motility, and eventually podocyte effacement and cell death. Whether calpain-1 has an effect on TRPC6 itself, as a possible negative feed-back mechanism, needs to be further elucidated.

Suppl Figure 1



Suppl Figure 2

A



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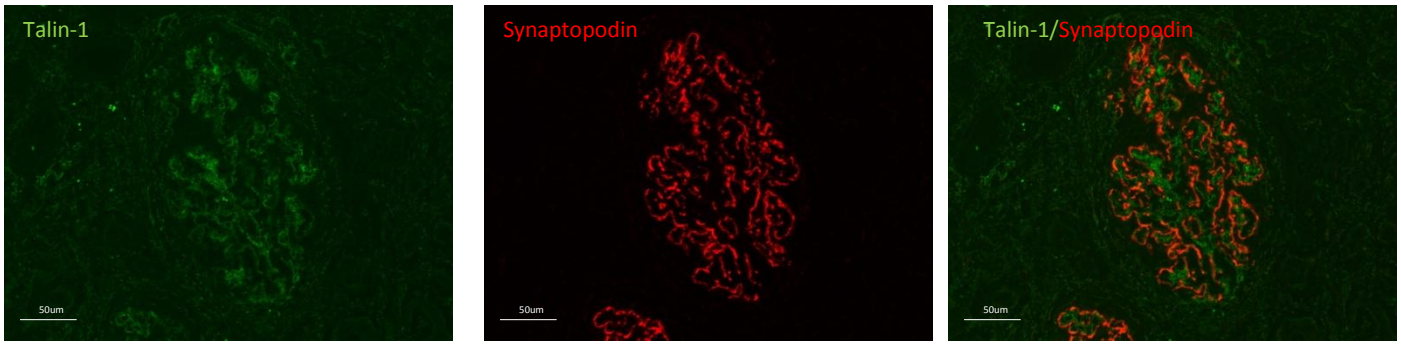

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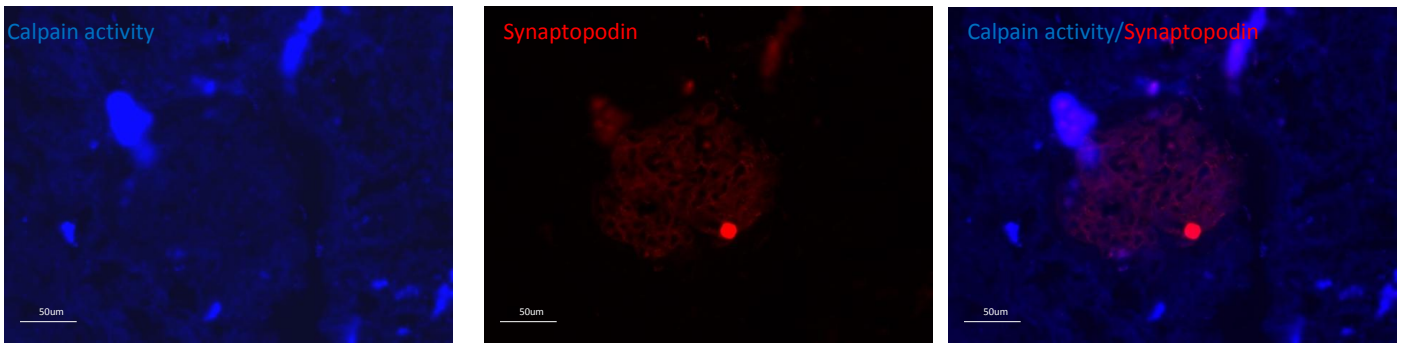

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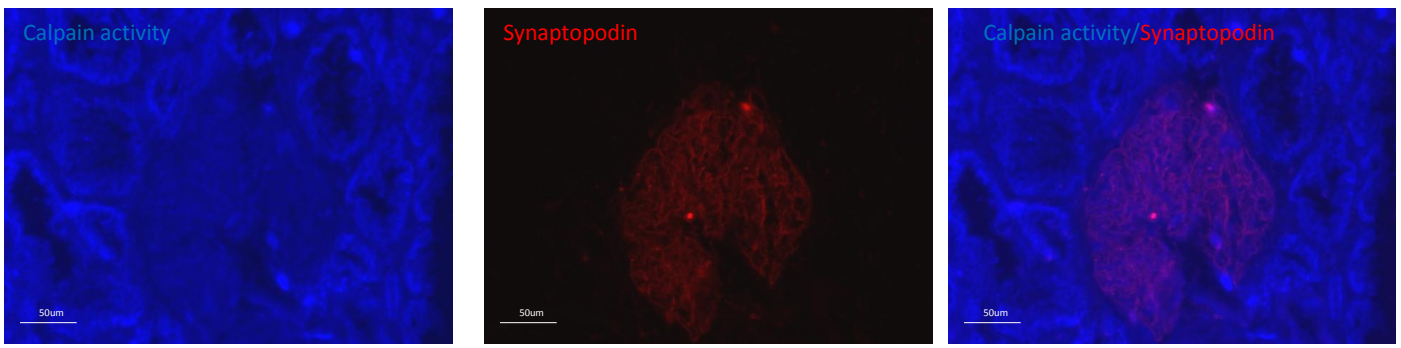
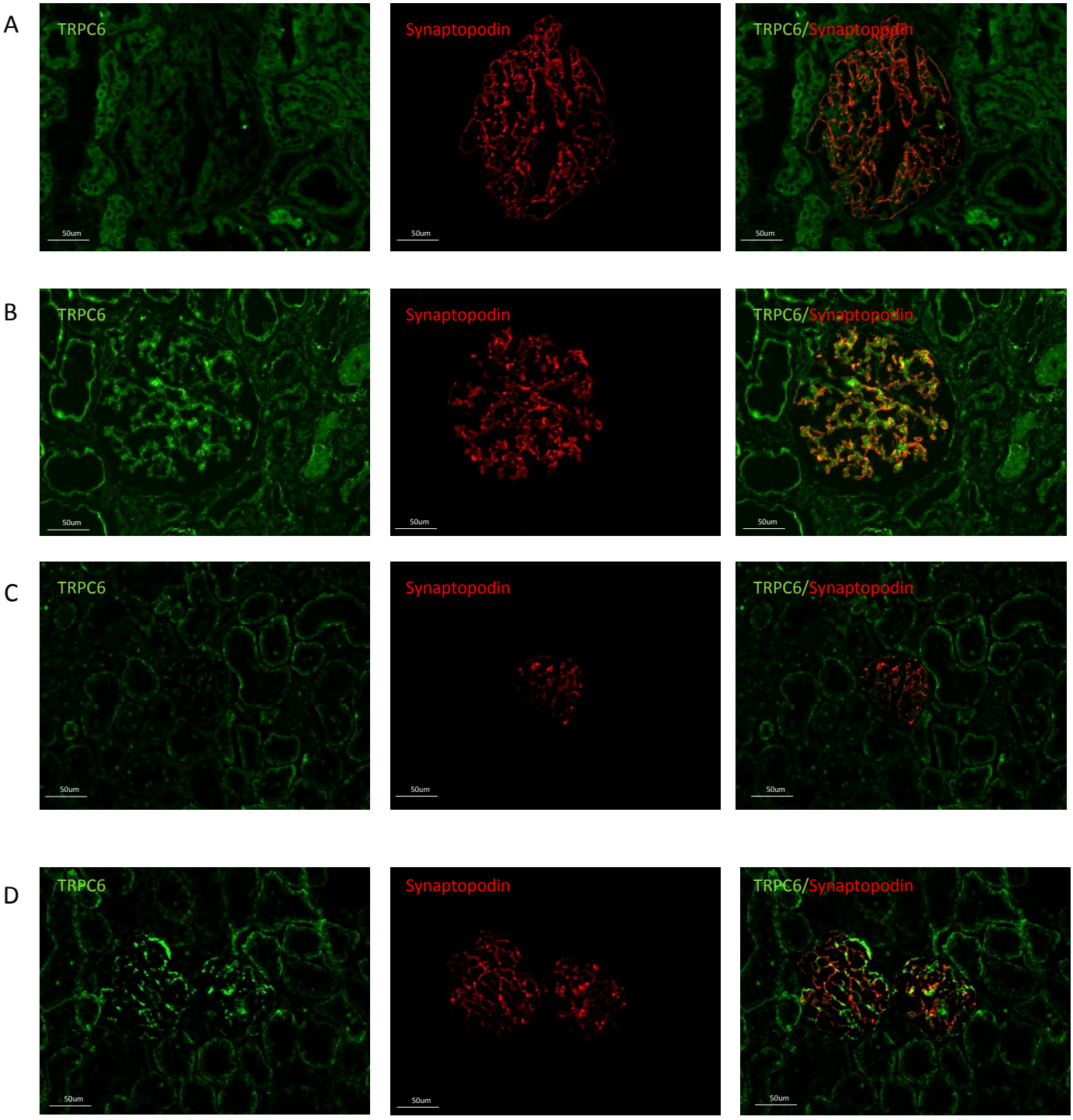
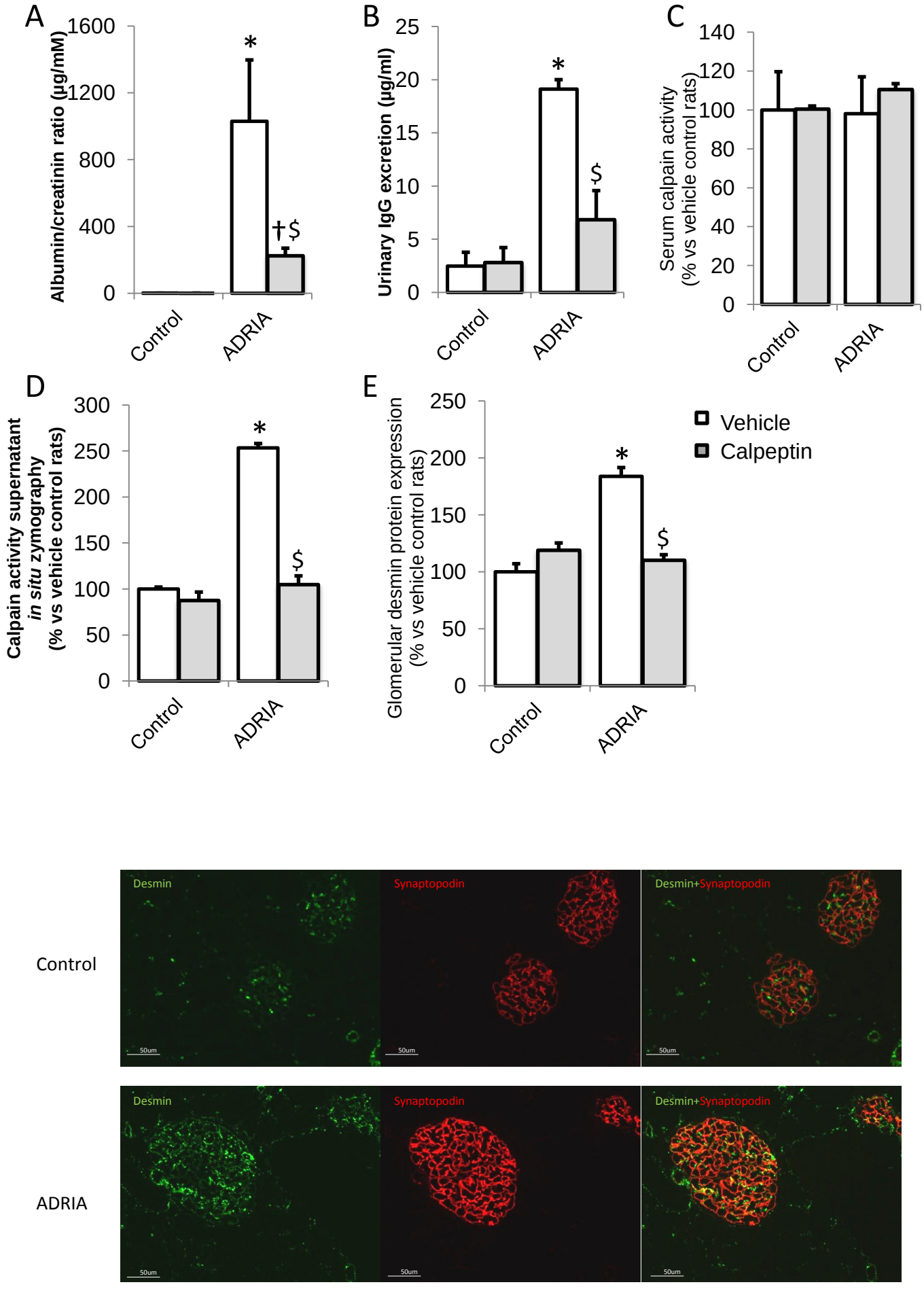


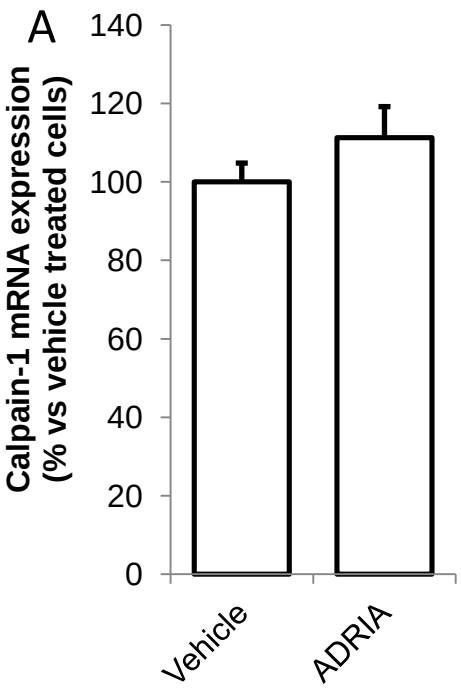
Suppl Figure 3

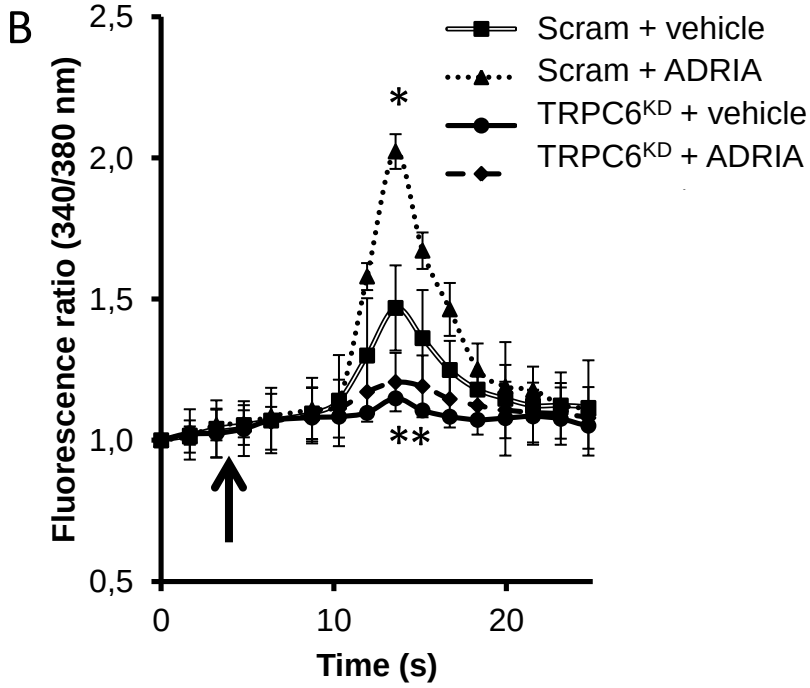


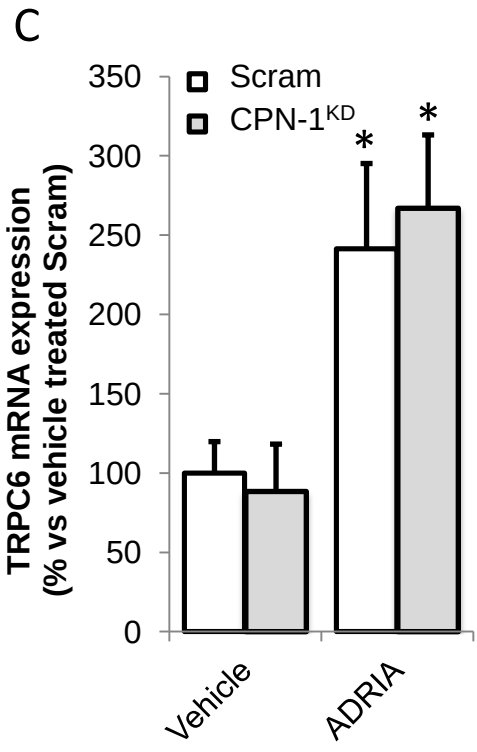
Suppl Figure 4



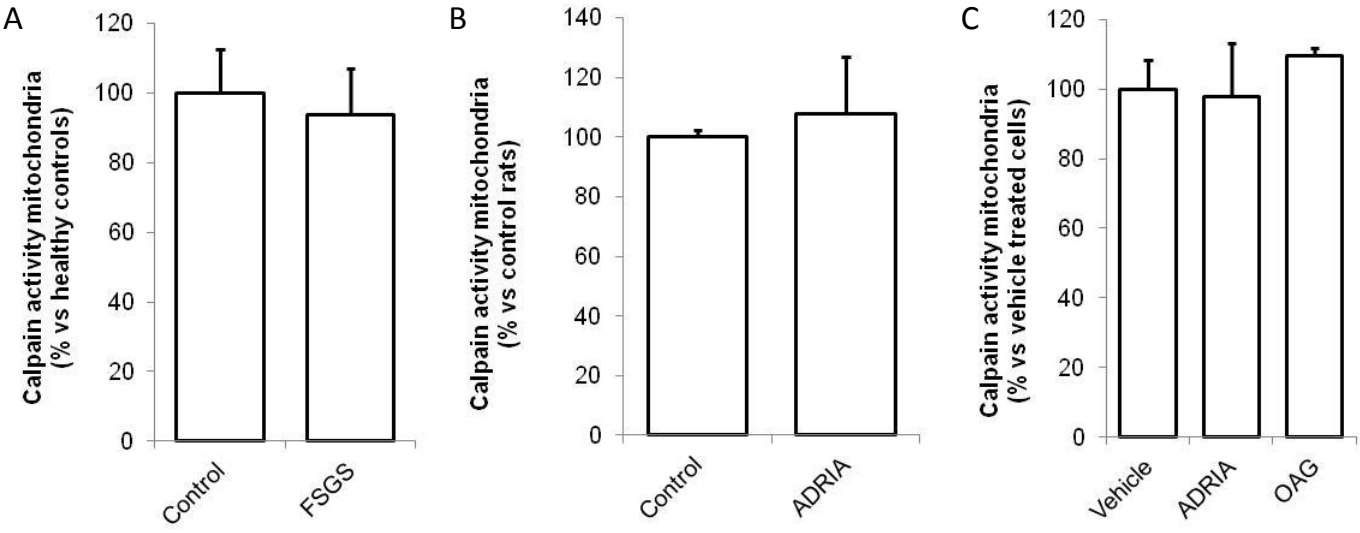
Suppl Figure 5







Suppl Figure 6



Suppl Figure 7