Online Supplemental Data

Cyclophilin A is required for Angiotensin II-induced p47phox Translocation to Caveolae in Vascular Smooth Muscle Cells

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Running title: CyPA regulates p47phox translocation to caveolae

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Supplemental Data

Figure I. Cytosolic p47phox protein expression in WT and KO-VSMC. Quantitative analysis of cytosolic p47phox expression in WT and KO-VSMC using Image J (NIH). Data are mean±SEM. *P>0.05 vs WT-0 minute (n=5).

Figure II. Immunofluorescence analysis of p47phox in WT and KO-VSMC. A-L, Representative images of WT and KO-VSMC immunostained with rabbit anti-p47phox (red color) and FITC-phalloidin to visualize cell cytoskeleton structure. **M,** AngII-induced p47phox plasma membrane translocation was quantified using Adobe Photoshop CS3. **N,** CyPA expression in WT and KO-VSMC was confirmed by Western blot. Data is presented as average±SEM. *P<0.05 vs WT-0 minute (n=3).

Figure III. AngII induced p47phox translocation to the caveolae. A, Caveolae (35% sucrose) and non-caveolae (45%) fractions from WT and KO-VSMC were separated using sucrose gradient fractionation. p47phox and CyPA expression in corresponding fractions were measured by Western blot. Caveolin-1 was used as caveolae marker. **C** and **D,** Caveolae (35% sucrose) and non-caveolae (45%) were prepared from KO-VSMC and Flag-CyPA overexpressed KO-VSMC. p47phox and Flag expression in corresponding fractions were measured by Western blot.

Figure IV. p47phox and CyPA expression in the nuclear fractions and at the plasma membrane. A, Quantitative analysis of p47phox and CyPA colocalization (merged image) in the nucleus was quantified by Adobe Photoshop CS3. At least 25 cells from 5 different fields per experiment for 3 independent experiments are used for statistics (n=3, *p<0.05 vs control). Data are mean±SEM. (*p<0.05 versus control). **B,** Nuclear p47phox and CyPA expression was measured by Western blot following subcellular fractionation (n=3). **C,** Quantitative analysis of p47phox and CyPA colocalization (merged image) at the plasma membrane was quantified by Adobe Photoshop CS3. At least 25 cells from 5 different fields per experiment for 3 independent experiments are used for statistics (n=3, *p<0.05 vs control). Data are mean±SEM. (*p<0.05 versus control).

Figure V. AngII induced p47phox and CyPA association. **A,** p47phox and CyPA expression in AngII-treated RASMC were measured by Western blot. **B** and **C,** Total cell lysates of Flag-CyPA constitutively over-expressed VSMC (VSMC-Tg) were immunoprecipitated (IP) with anti-Flag antibody and immune complexes were blotted for p47phox and Flag reactivity. p47phox and Flag-CyPA expression were measured in total cell lysates (TCL, lower panel). (* p<0.05 versus vehicle, n=3). **D,** p47phox and CyPA expression in methyl-β-cyclodextrin (MβCD) and/or cholesterol (Chol) treated RASMC were measured by Western blot.

Figure VI. p47phox and CyPA expression. Protein expression were measured in cytochalasin B-treated RASMC total cell lysate (TCL).

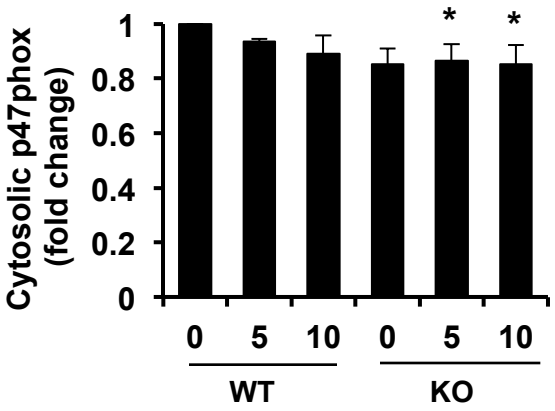
Figure VII. p47phox and CyPA expression. A, Protein expressions were determined in Rottlerin-treated RASMC total cell lysate (TCL). **B,** The domain structures of p47phox used for lentiviral transduction experiments; PX-phox homology domain, SH3-Src homology 3 domain, AIR-Auto inhibitory region, PRR-proline rich region.

Figure VIII. PPIase activity regulates AngII-induced p47phox and CyPA association. A, Protein expression in CsA-treated total cell lysates (TCL) was measured by Western blot. **B,** WT-CyPA or R55A-CyPA expression in transduced RASMC were measured by Western blot. **C** and **D,** AT1R stable HeLa cells were transfected with WT-Flag-CyPA & R55A-Flag-CyPA using Fugene 6 for 48 hour, then treated with AngII (10^{-7} mol/L) for 10 minutes. TCL were immunoprecipitated (IP) with anti-Flag antibody and immune complexes were blotted for p47phox and Flag antibody (the upper panel). Protein expressions in TCL were measured by Western blot (the lower panel).

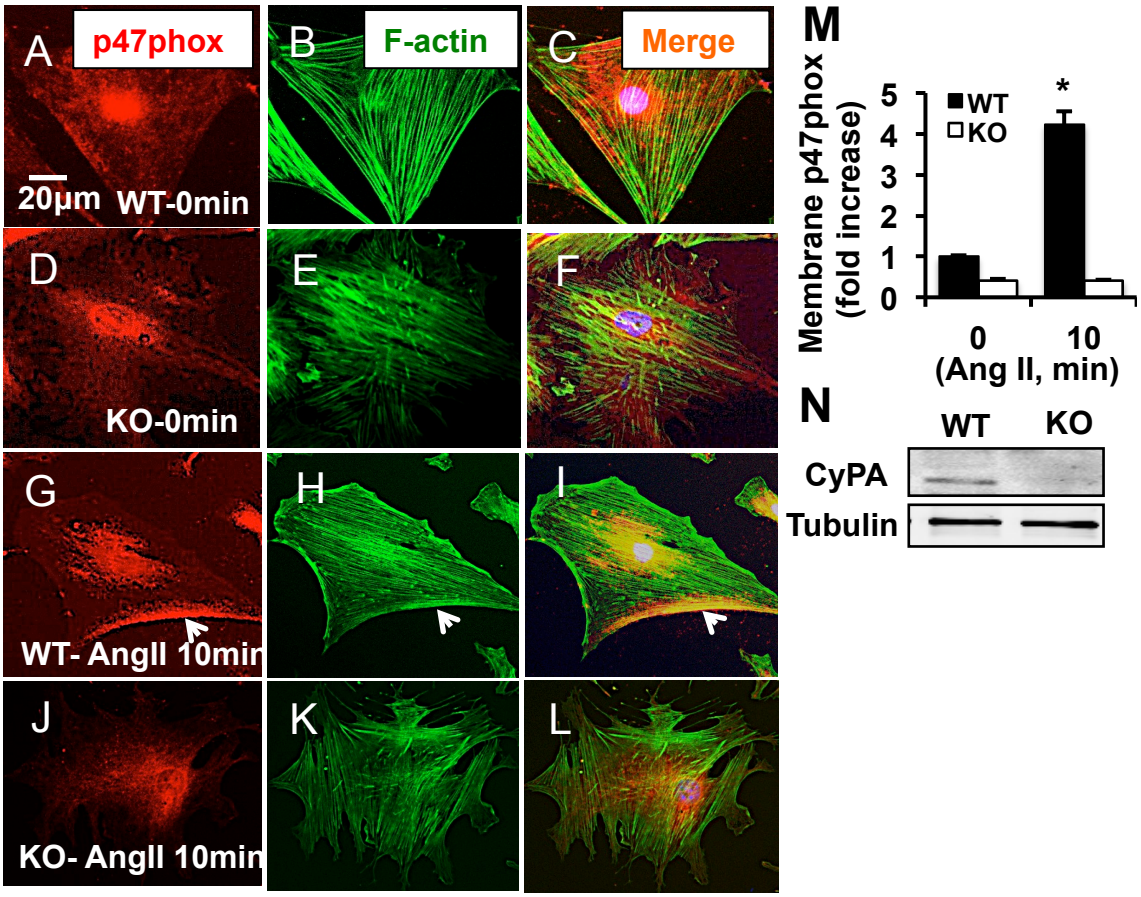
Figure IX. CyPA-PPIase activity regulates AngII induced p47phox translocation to the caveolae. A-D, Caveolae and non-caveolae fractions of RASMC pretreated with CsA (1μmol/L) for 1 hour were separated by sucrose gradient centrifugation. P47phox and CyPA expression were measured by Western blot. Caveolin-1 was used as caveolae marker. Percent (%) distribution of p47phox and CyPA in the caveolae fractions were analyzed using Image J (NIH). Data is presented as average±SEM. (*$p<0.05$ versus control).

Figure X. Schematic model of CyPA regulated ROS production in VSMC. CyPA regulates ROS production in VSMC; **(1)** Actin cell cytoskeleton polymerization, **(2)** it interacts with p47phox in basal as well as AngII stimulated conditions and **(3)** it supports p47phox translocation to the caveolae.

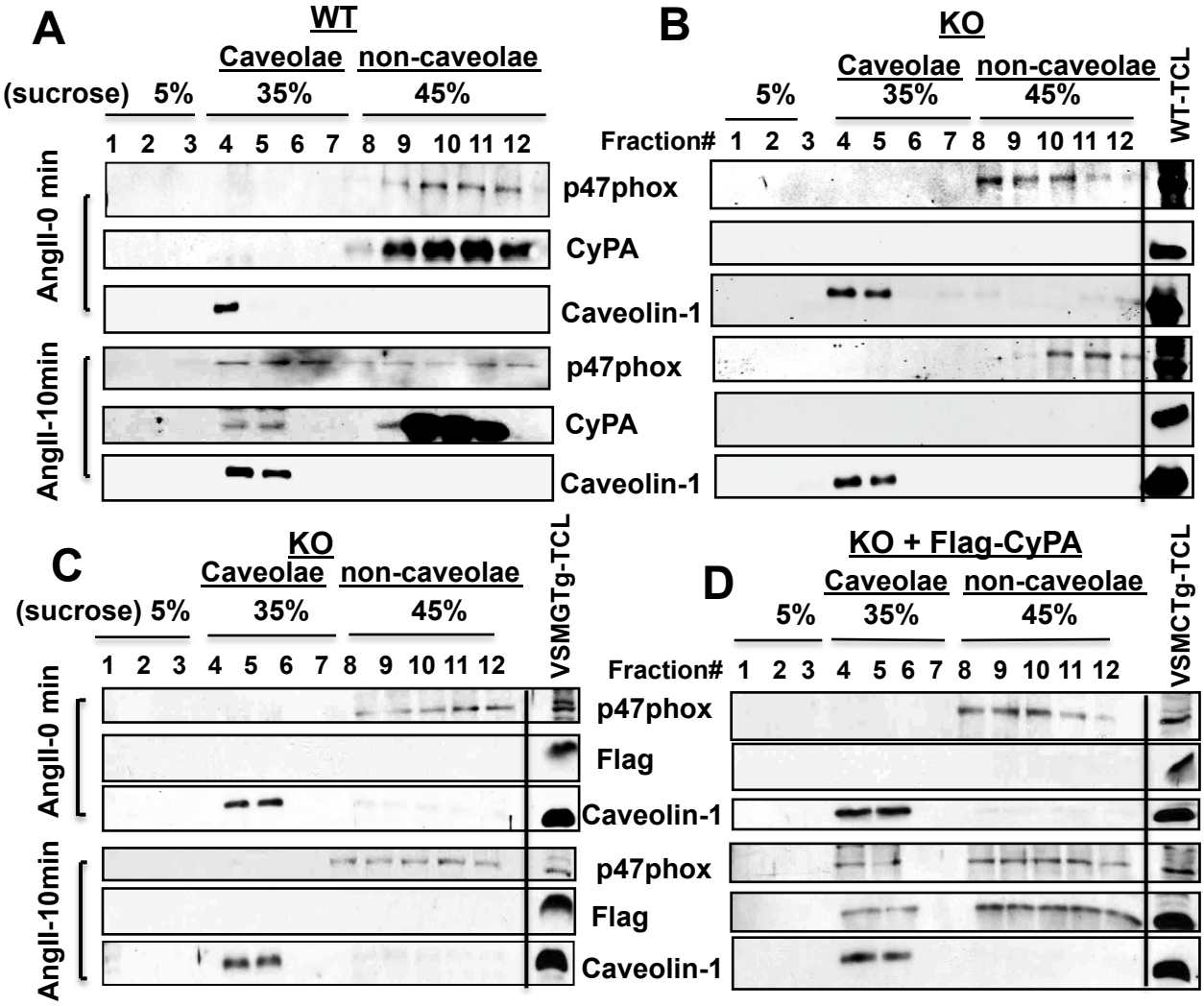
Supplementary Figure I



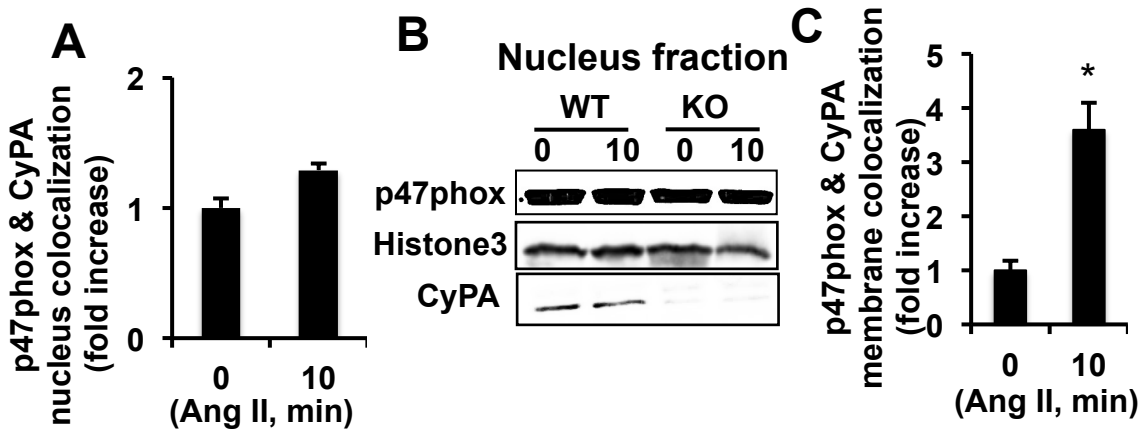
Supplementary Figure II



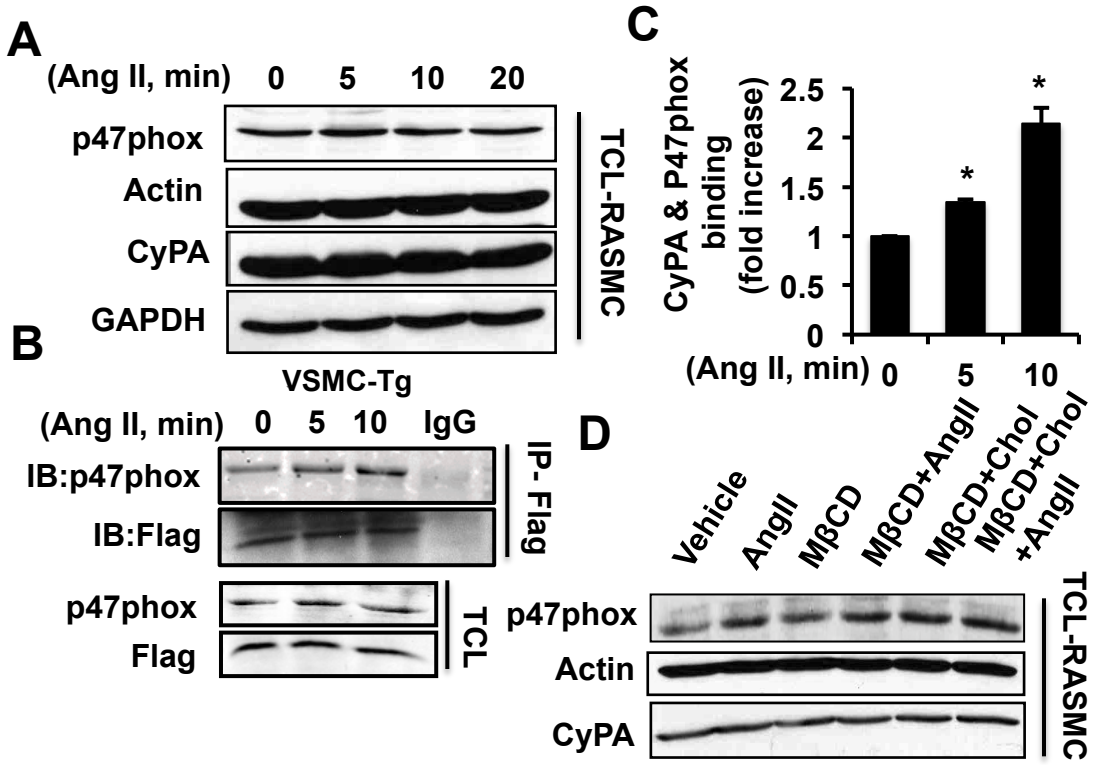
Supplementary Figure III



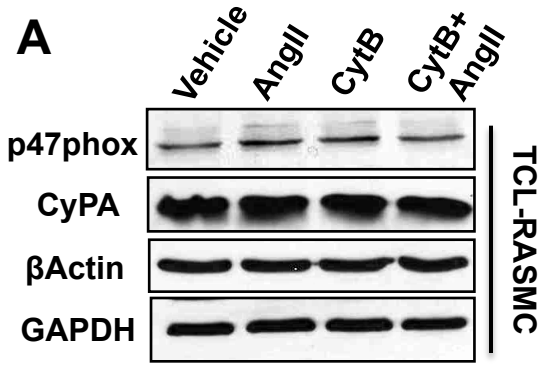
Supplementary Figure IV



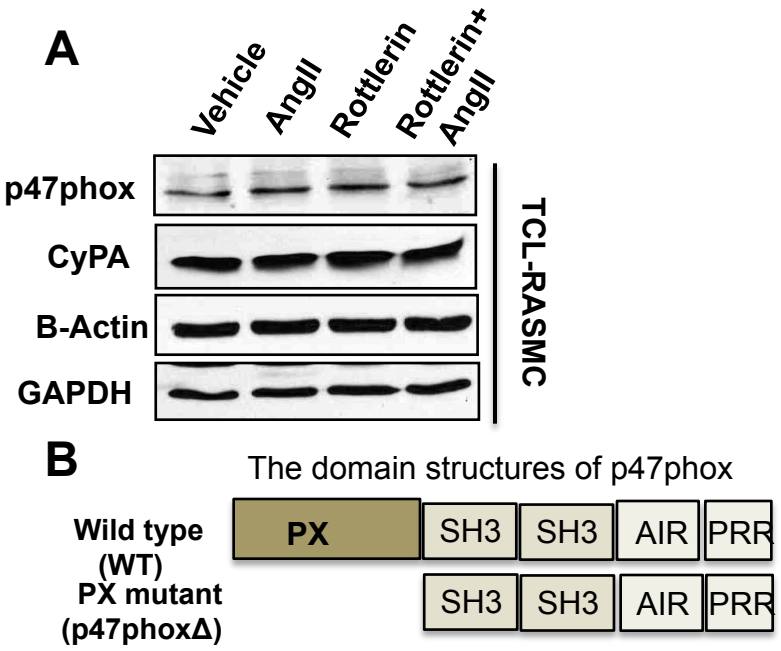
Supplementary Figure V



Supplementary Figure VI



Supplementary Figure VII



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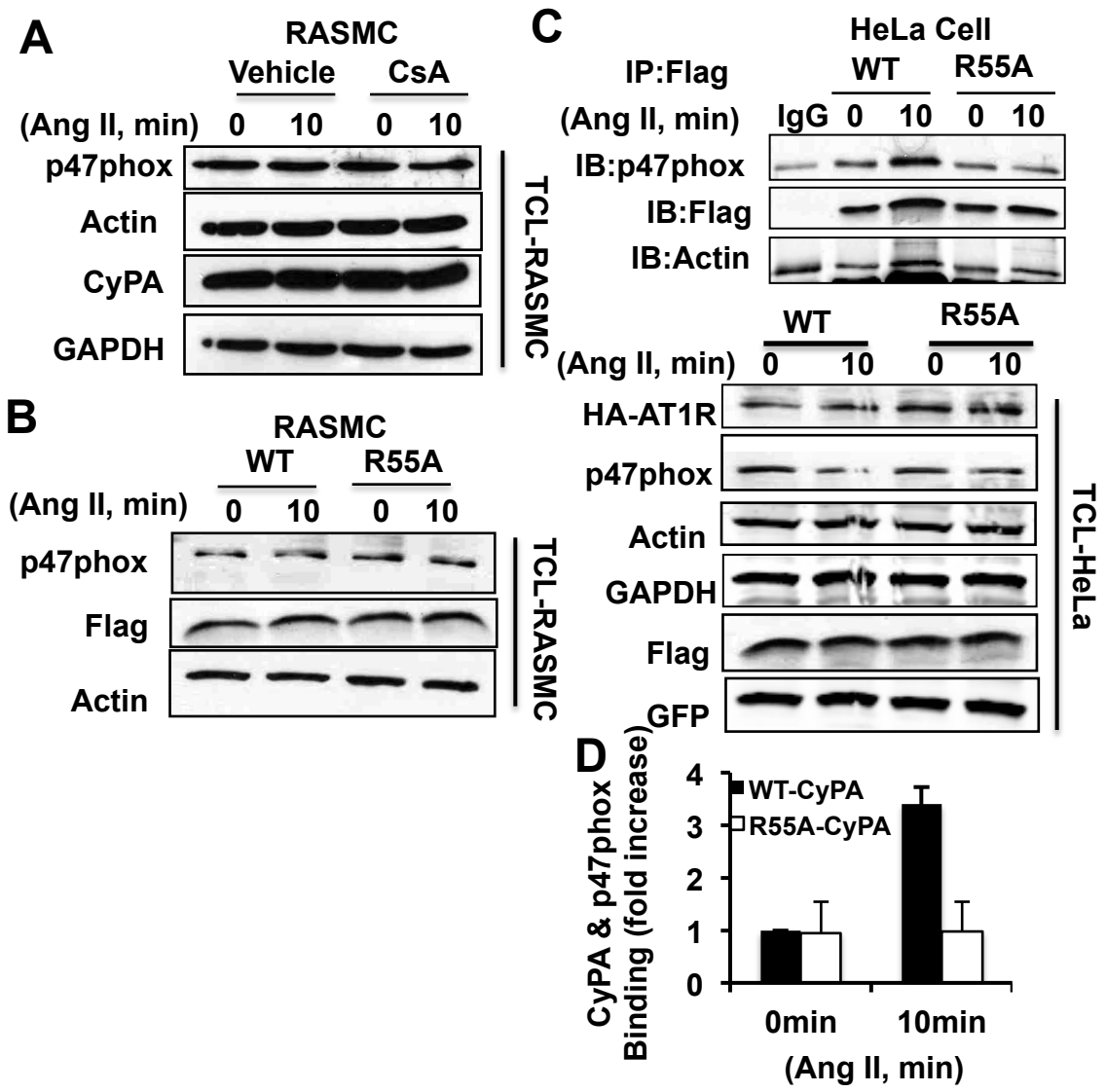
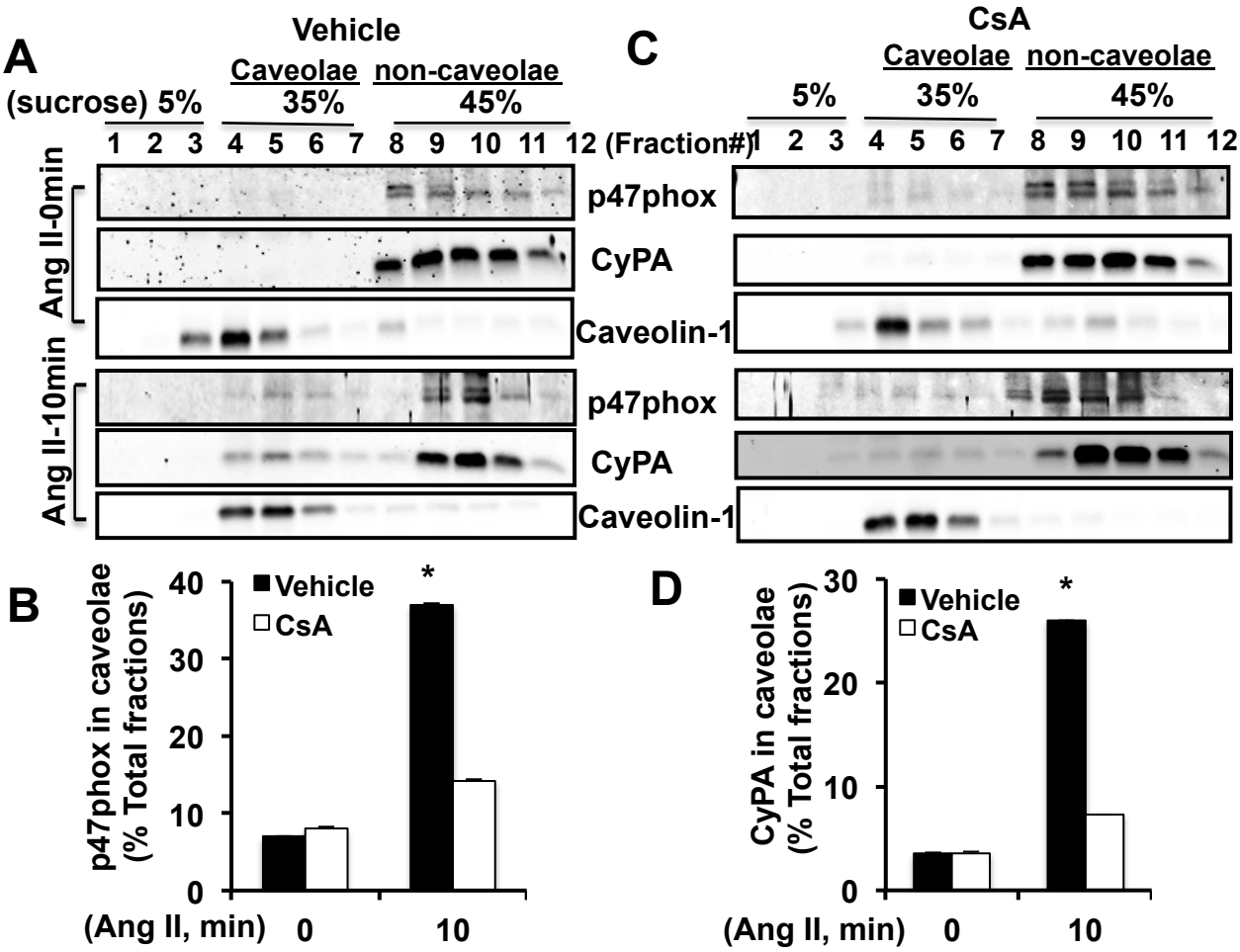


Supplementary Figure IX



Supplementary Figure X