Online Supplementary Information

TABLE S-I: PRIMERS FOR CLONING, GENOMIC PCR & QUANTITATIVE RT-PCR

Name	Sequence	Comments
EGFP-forward	ACACCCTGGTGAACCGCAT	50 nM; Ta=60ºC
EGFP-reverse	CCATGATATAGACGTTGTGGCTGTT	50 nM; Ta=60ºC
Microglobulin-forward	GCTATCCAGAAAACCCCTCAA	50 nM; Ta=60ºC
Microglobulin-reverse	CATGTCTCGATCCCAGTAGACGGT	50 nM; Ta=60ºC
PMCA4a forward	TCAACACCTTGATGTAAAACTGGTTCCTAGC	300 nM; Ta=69ºC
PMCA4a reverse	GACGACTCTGATTGCCCACAGGAG	300 nM; Ta=69ºC
PMCA4b reverse	TATGGAACAGTTTGACGACTCTGATCTGAG	300 nM; Ta=69ºC
PMCA4b forward	AGTGACAAAGAGGACATCAGCAGGGATACTGAG	300 nM; Ta=69ºC
PMCA4e forward	ACACCTTGATGTAAAACTGGTTCCTAGCTC	300 nM; Ta=69ºC
PMCA4e reverse	AACAGTTTGACGACTCTGATTCTCACTCAC	300 nM; Ta=69ºC
5’UTR forward	GCTACTCTGAAACTTATTGCACGAG	Ta=60ºC
Mid-cDNA reverse	CTTCACTGGCCATGGGCTCAATG	Ta=60ºC
Mid-cDNA forward	TCATTGAGCCCATGGCCAGTGAAG	Ta=60ºC
3’UTR reverse	CCACCGTCACATACAGATGG	Ta=60 ºC
PMCA4-exon-3-forward (Genomic PCR)	AACGTGATACCTCCAAAAAGGCCCAAG	Ta=65ºC Amplicon = 816 bp
PMCA4-exon-4-reverse (Genomic PCR)	AATTTTTGCTCCAGTTCGATGCGACTC	Ta=65ºC Amplicon = 816 bp

Table-S-I Legend: Note: f, forward; r, reverse; Ta, annealing temperature; EGFP, enhanced green fluorescent protein; all qRT-PCR primer pairs generated single amplicon peaks.

TABLE S-II: Genes Modulated in P4KO vs. P4WT cells (Early G_1 Stage)

Fold Change	p-value	Symbol	Definition
47.5	2.39E-09	Casp1	Caspase 1 (ICE); down regulates c-myc; inflammatory.
39.4	**6.38E-09**	**Rgs16**	**Regulator of G-protein signaling 16 (Rgs16); inhibits p38MAPK; NFAT suppresses Rgs16**
21.2	6.38E-09	Gpnmb	Glycoprotein (transmembrane) nmb (Gpnmb)
69.1	**8.95E-09**	**Dcn**	**Decorin; tumor suppressor; reduces tumor metastases**
14.2	1.24E-08	Adm	Adrenomedullin (Adm)
30.6	1.24E-08	Pdpn	Podoplanin; transmembrane glycoprotein
20.9	1.49E-08	Lce1f	Late cornified envelope 1F
40.2	1.54E-08	LOC100048554	PREDICTED: similar to monocyte chemoattractant protein-2 (MCP-2).
23.5	1.54E-08	Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2 (Serpinb2)
-6.6	3.81E-08	Rcan2	Regulator of Calcineurin 2 mRNA
-3.6	4.02E-05	E2f2	E2F transcription factor 2 mRNA

TABLE S-III: Genes Known to be Implicated in G_1 Phase Arrest

(P4KO vs. P4WT cells in early G_1 stage)

Fold Change	Symbol	Definition
69.1	**Dcn**	**decorin (Dcn); tumor suppressor; reduces tumor metastases**
5.2	Igf1	insulin-like growth factor 1; regulates MAPK/Akt pathway
5	Itga5	integrin alpha 5 (fibronectin receptor alpha)
4.2	Egfr	epidermal growth factor receptor; tumor metastases
3.9	Fgf2	fibroblast growth factor 2
3.4	Ngf	nerve growth factor
3.3	Dusp1	dual specificity phosphatase 1; ER stress induced apoptosis
2.8	Csf1	colony stimulating factor 1; induces NFATc1 degradation
2.1	Akap12	A kinase (PRKA) anchor protein 12; cell growth-related protein
2.1	Cdkn2A	cyclin-dependent kinase inhibitor 2A; p16(INK4a); tumor suppressor
-3.6	E2f2	E2F transcription factor 2; tumor suppressor
-3.5	Mcm7	minichromosome maintenance deficient 7; chromosomal DNA replication factor
-3.2	Mybl2	Myeloblastosis oncogene-like 2 (Mybl2); B-Myb; S and G2/M phase regulation
-3	Notch1	Notch gene homolog 1; tumor suppressor effect in myeloid leukemia
-2.8	Rbl1	retinoblastoma-like 1 (p107); cell cycle regulation
-2.5	Pim1	proviral integration site 1; regulates S phase entry
-2.2	Mtbp	Mdm2, transformed 3T3 cell double minute p53 binding protein; Myc regulator
-2.2	Cdkn1B	cyclin-dependent kinase inhibitor 1B; p27 or Kip1; regulates cell cycle exit
-2.1	Cdkn1C	cyclin-dependent kinase inhibitor 1C (p57 or Kip2); regulator of cell cycle exit

TABLE S-IV: NFAT Facilitator Proteins, NFAT pathway modulators and NFAT Targets

(P4KO vs. P4WT in early G_1 stage)

Fold Change	p-value	Gene Name	Definition
69.1	**8.95E-09**	**Dcn**	**Decorin (Dcn), mRNA.**
39.4	**6.38E-09**	**Rgs16**	**Regulator of G-protein signaling 16 (Rgs16), mRNA.**
-6.6	3.81E-08	Rcan2	Regulator of calcineurin 2 (Rcan2), transcript variant 1, mRNA.
-1.8	0.002574978	Rcan1	Regulator of calcineurin 1 (Rcan1), transcript variant 2, mRNA.
-3.6	4.02E-05	E2f2	E2F transcription factor 2 (E2f2), mRNA.

TABLE S-V: Genes Modulated in PMCA4a-Rescued P4KO at Late G_1/S Stage

(PMCA4a-rescued P4KO vs. P4KO)

FoldChange	p-value	Symbol	Definition
-44.6	**2.5E-10**	**Tfap2b**	**AP-2β,** Activating enhancer-binding protein 2 beta, transcript variant 2 (inhibits Myc transactivation, inhibits cell cycle)
-15.5	4.7E-09	Angpt2	Angiopoietin 2 (disrupts vascular remodeling)
-24.7	8.02E-09	Pcdh17	Protocadherin (cell-cell connections)
-8.2	4.09E-08	Tfap2a	AP-2α, Activating enhancer-binding protein 2 alpha (transcription activator/repressor; mutations cause branchio-oculo facial syndrome)
-1.27	**NS**	**Dcn**	**Decorin**
+1.1	**NS**	**Rgs16**	**Rgs16**
+4.9	1.68E-07	Tnxb	Tenascin XB (matrix maturation during wound healing)
+7.8	2.15E-07	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (vascular calcification)
+4.8	2.74E-07	Slitrk5	SLIT and NTRK-like family, member 5 (axonogenesis)
+9.1	5.56E-07	Igf2bp3	Insulin-like growth factor 2 mRNA binding protein 3 (represses translation of insulin-like growth factor-2)
+4.9	7.26E-07	Rragb	Ras-related GTP binding B (Ras-homologous GTPases; signal transducers)

TABLE S-VI: Genes Modulated in PMCA4b-Rescued P4KO at Late G_1/S Stage

(P4b-rescued P4KO vs. P4KO)

Fold Change	p-value	GeneName	Definition
24.4	1.85E-25	Ptprd	Protein tyrosine phosphatase, receptor type, D (Ptprd), transcript variant a, mRNA.
14.9	1.97E-21	Gjb3	Gap Junction protein beta 3 mRNA
32.0	2.03E-21	Anxa8	Annexin A8 mRNA
-136.8	6.80E-24	Dkk3	Dickkopf homolog 3 (Xenopus laevis) (Dkk3), mRNA.
-50.4	**8.51E-24**	**Cdkn2b**	**Cyclin-dependent kinase inhibitor 2B** (**p15**; **inhibits CDK4**) (**Cdkn2b**), mRNA.
-13.4	8.72E-24	Loxl1	Lysyl oxidase-like 1 (Loxl1), mRNA.
-23.9	8.72E-24	Fgf10	Fibroblast growth factor 10 (Fgf10), mRNA.
-22.3	1.37E-22	Pdgfra	Platelet derived growth factor receptor, alpha polypeptide (Pdgfra), transcript variant 1, mRNA.
-9.2	4.87E-22	Sema3f	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3 F (Sema3f), mRNA.
-2.4	**1.59E-13**	**Dcn**	**Decorin (Dcn), mRNA.**
-18.5	**3.07E-19**	**Rgs16**	**Regulator of G-protein signaling 16 (Rgs16), mRNA.**

TABLE S-VII: Genes Modulated in P4KO at Late G_1/S Stage

(P4KO vs. P4WT)

Fold Change	p-value	GeneName	Definition
39.4	1.20E-24	Thy1	Thymus cell antigen 1, theta (Thy1), mRNA.
33.0	7.76E-22	Ly6a	Lymphocyte antigen 6 complex, locus A (Ly6a), mRNA.
64.7	1.12E-20	Serpinf1	Serine (or cysteine) peptidase inhibitor, clade F, member 1 (Serpinf1), mRNA.
5.2	2.00E-20	Igfbp4	Insulin-like growth factor binding protein 4
7.4	2.82E-20	Pcdh21	Protocadherin 21 (Pcdh21), mRNA.
9.3	**3.24E-20**	**Dcn**	**Decorin (Dcn), mRNA.**
4.6	5.75E-20	Nnmt	Nicotinamide N-methyltransferase (Nnmt), mRNA.
7.6	6.68E-20	Spon2	Spondin 2, extracellular matrix protein (Spon2), mRNA.
15.1	**2.00E-18**	**Rgs16**	**Mus musculus regulator of G-protein signaling 16 (Rgs16), mRNA.**
-7.2	6.68E-20	Dlx1	Distal-less homeobox 1 mRNA
-5.8	3.12E-17	Tuft1	Tuftelin 1 mRNA

TABLE S-VIII: Genes Modulated in PMCA4b-Rescued P4KO at Late G_1/S Stage

(PMCA4b-rescued P4KO vs. Vector-control P4KO)

FoldChange	p-value	Symbol	Definition
22.5	1.48E-24	Pacrg	Park2 co-regulated mRNA
9.0	5.59E-21	Gpc1	Glypican 1 mRNA
-28.0	1.29E-24	Ogn	Osteoglycin (Ogn), mRNA.
-15.3	1.29E-24	Ppap2b	Phosphatidic acid phosphatase type 2B (Ppap2b), mRNA.
-17.4	3.56E-24	Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide (Atp1b1), mRNA.
-7.3	4.31E-22	Dusp1	Dual specificity phosphatase 1 (Dusp1), mRNA.
-15.3	**5.55E-22**	**Dcn**	**Decorin (Dcn), mRNA.**
-22.5	5.69E-22	Cdkn2b	Cyclin-dependent kinase inhibitor 2B (**p15, inhibits CDK4**) (Cdkn2b), mRNA.
-7.7	8.59E-22	Loxl1	Lysyl oxidase-like 1 (Loxl1), mRNA.
-7.9	9.58E-22	Ier3	Immediate early response 3 (Ier3), mRNA.
-3.9	**9.25E-14**	**Rgs16**	**Regulator of G-protein signaling 16 (Rgs16), mRNA.**

A

PMCA4-b (alternatively spliced variant) = **VASHSDSPLPSLETPV**
PMCA4-a (classical exon-20 of 181 nt) = AVTSPPVG**NQSRQTVP**
PMCA4-e (novel exon-20 of 191 nt) = AVTSPPVG**SE**

B

Peptide used for PMCA4b Antibody = **SH**SD**SPL**P**S**L**ETPV**
Peptide used for PMCA4a/e Antibody = **QT**E**A**PL**K**R**V**RENMTQH**LD**

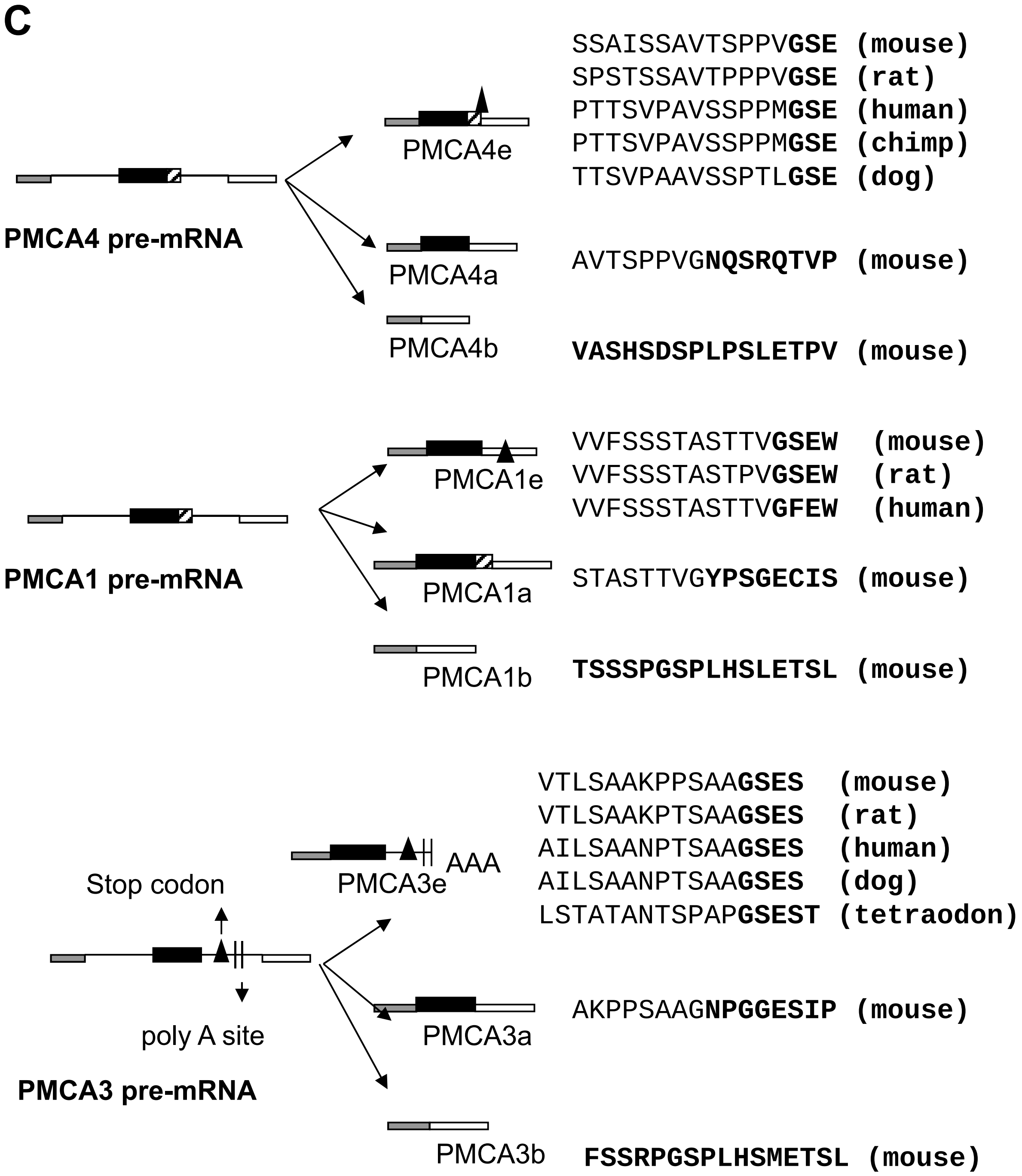


Figure S-I. **(A)** Predicted amino acid sequences at the C-terminus of the cloned splice variants. Bold letters denote exon-20 or exon-21 encoded residues. **(B)** Sequences of peptides used to generate chicken polyclonal Abs against PMCA4b and PMCA4a/e splice variants. Bold letters denote residues conserved between mouse and rat PMCA4. **(C)** Schematic representation and relevant sequences of alternatively spliced PMCA genes. Lines show introns, rectangles are exons, and filled triangles are in-frame stop codons. Sequences at the carboxy tails of resulting splice variants are displayed. Bold letters show residues expressed as a result of exon inclusion or exclusion. NB: alternative splicing at site C of PMCA1 and PMCA3 is more complex than shown. The schematic emphasizes instead the similarities between ‘e’ variants of PMCA1, PMCA3 and PMCA4.

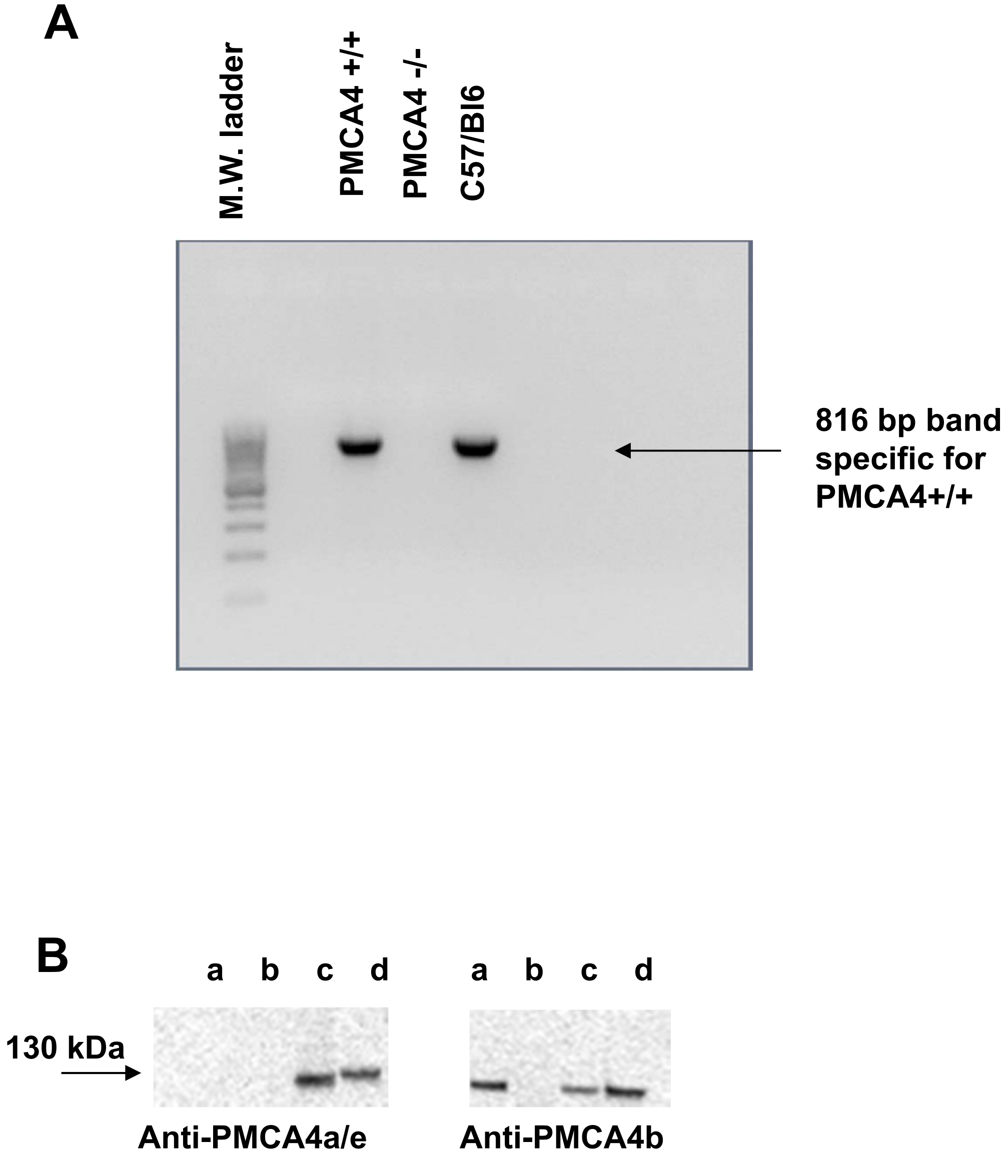


Figure S-II. Validation of PMCA4a/e and PMCA4b specific antibodies (A) Genomic PCR using exon-3 and exon-4 specific primers confirmed that the Neyses lab-generated PMCA4-/- mouse hearts (in which exons-2 and -3 have been deleted) lack these PMCA4 exons from their genome. **(B)** Immunoblots of microsomal protein extracts made from (a) C57bl6 heart, (b) PMCA4-/- mouse heart, (c) C57bl6 stomach and (d) C57bl6 brain were probed with affinity purified chicken polyclonal antibodies raised against peptides specific for PMCA4b and PMCA4a/e. Note that the PMCA4a/e antibody did not give a band for normal mouse heart (lane a) presumably because of negligible PMCA4a expression in the heart.

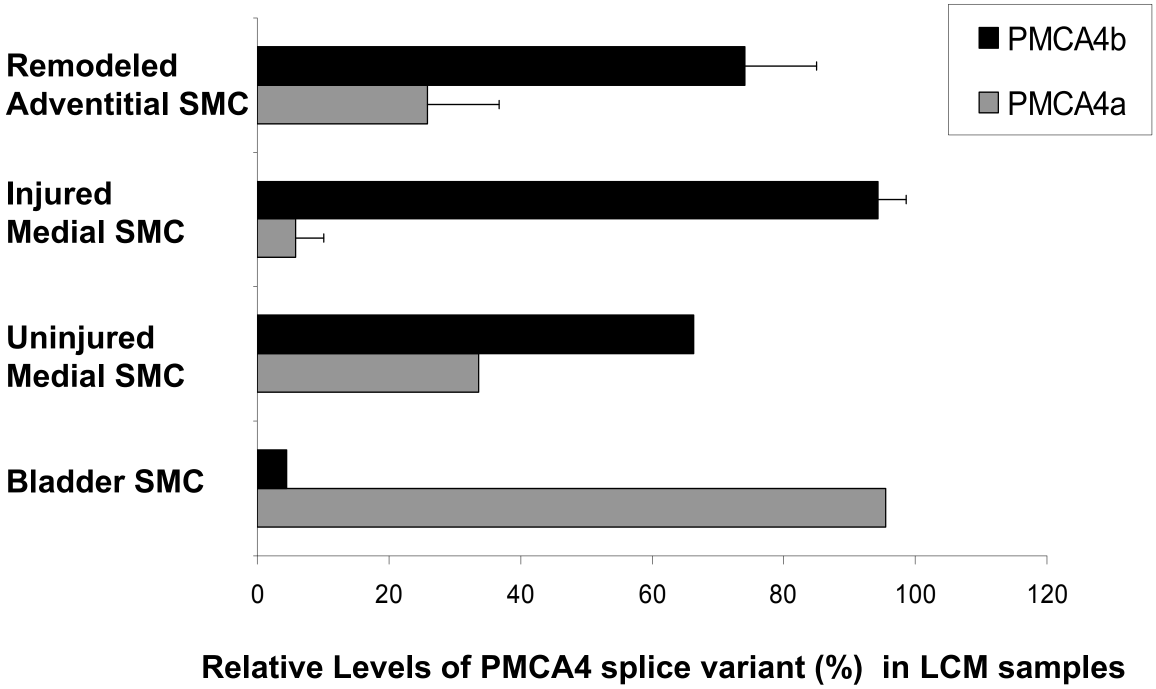
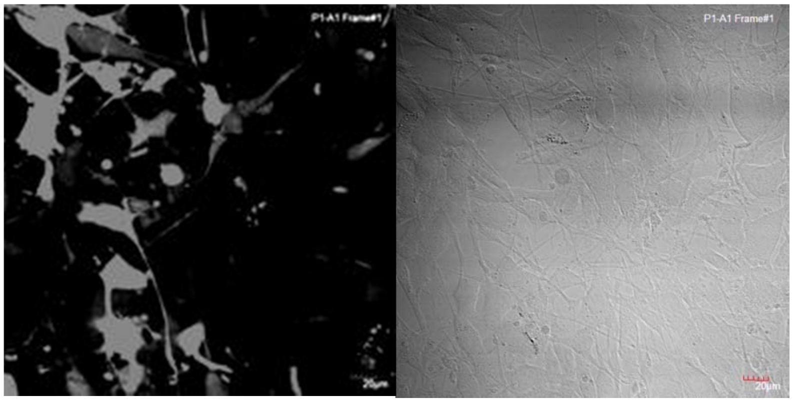
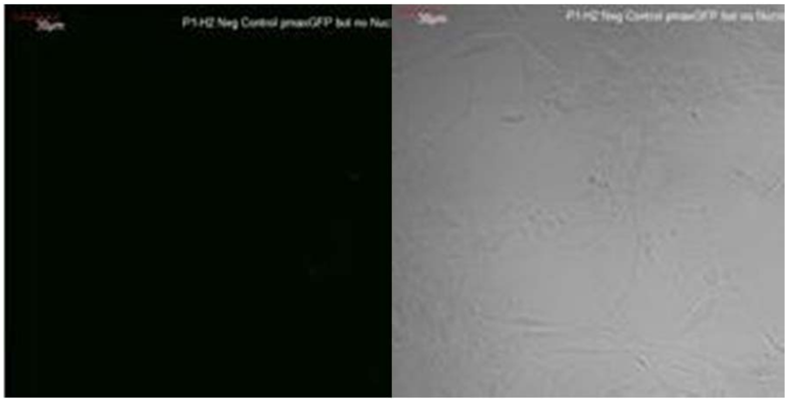


Figure S-III. **Laser capture microdissection** (LCM) was used to obtain uninjured media (n=1), injured media (n=3) and remodeled adventitia regions (n=2) from mouse carotid artery cross-sections (8-10 wk old male; day-9 post injury). Bladder smooth muscle region (n=1) was used to show that the LCM protocol could demonstrate high relative PMCA4a abundance observed in Fig1A. These microdissected regions were used to isolate RNA, make cDNA and carry out qRT-PCR for microglobulin (housekeeping gene) and PMCA4a and PMCA4b to calculate microglobulin-normalized relative proportions of PMCA4a:4b splice variants in the various arterial regions.



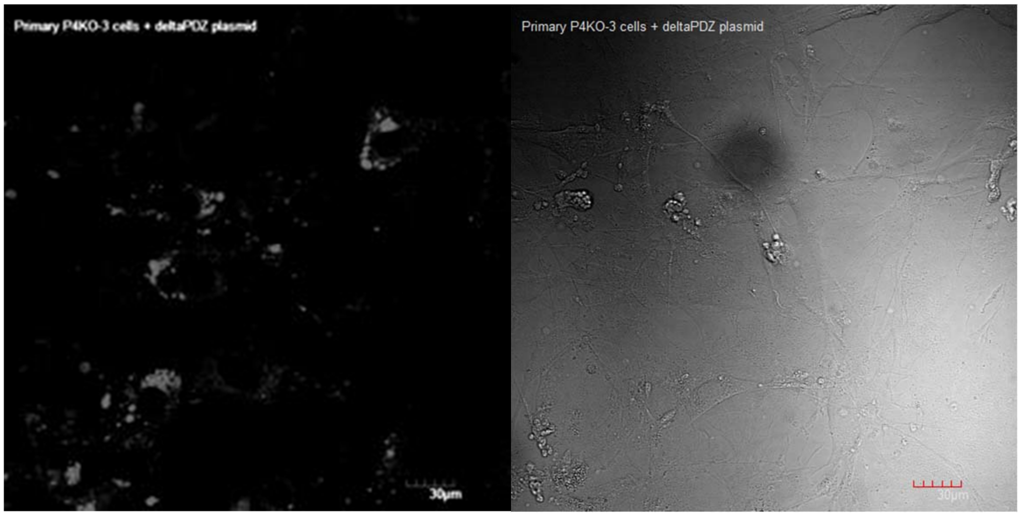
CA-137 electroporation parameters: 67% efficiency
Mean of 4 fields of view; > 100 cells counted; not much cell death

CM-137 electroporation parameters: 100% efficiency
Mean of 3 fields of view ~50 cells counted; significant cell death

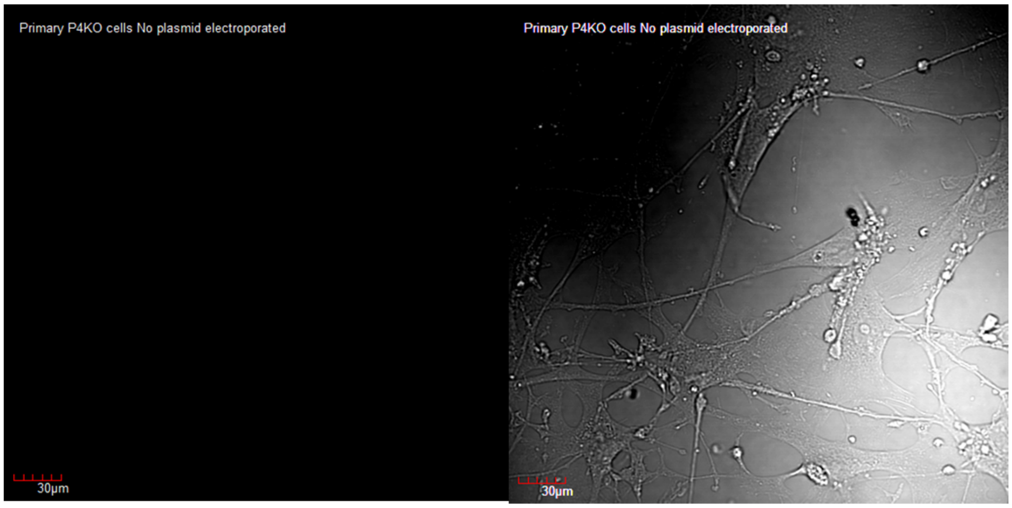


Negative Control: No electroporation parameters used; cells incubated with maxGFP plasmid in cuvette

Figure S-IV. **Optimization of Electroporation Parameters**. maxGFP plasmid, mouse primary carotid artery smooth muscle cells, Lonza P1 Nucleofection Mix and Lonza 4D Nucleofector were used.

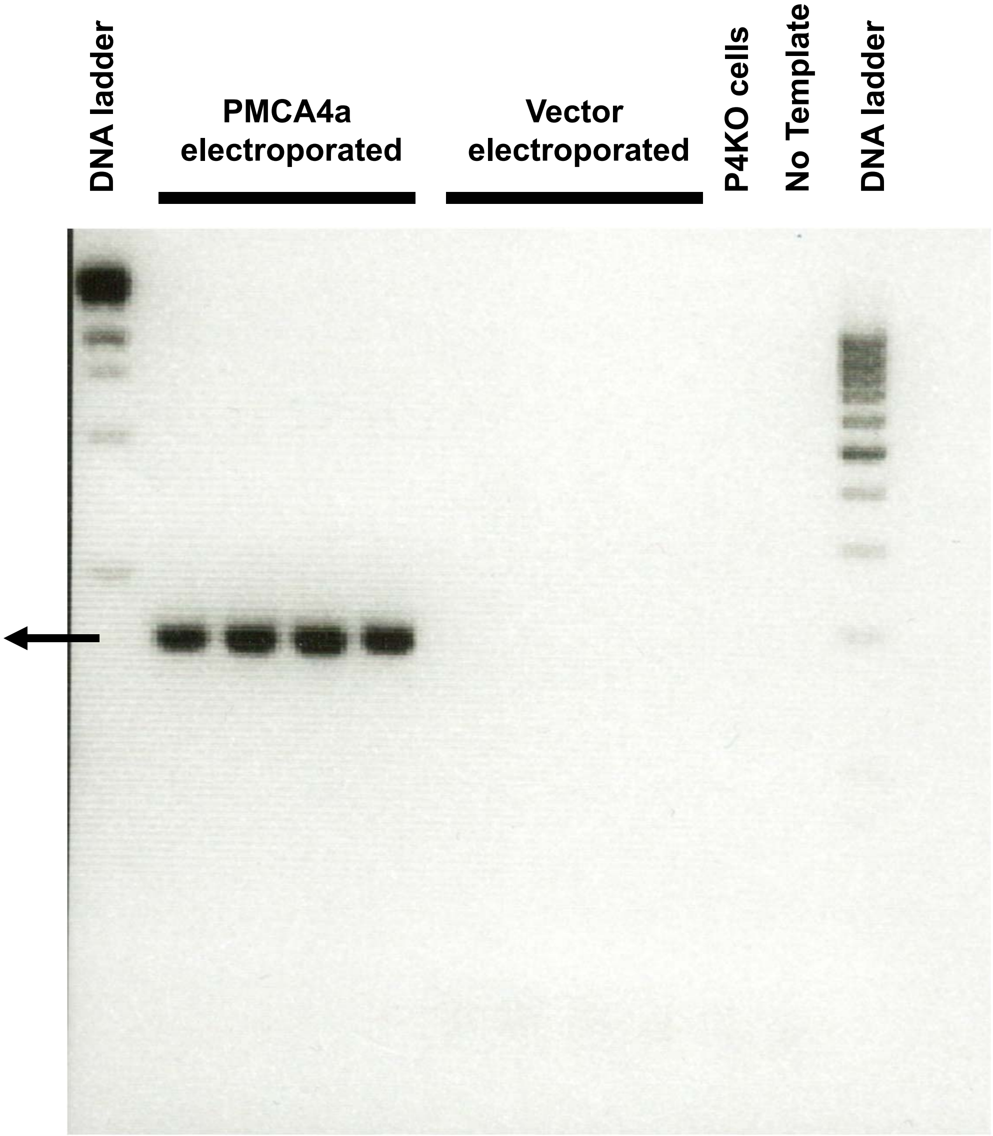


EGFP-huPMCA4b-Δ-PDZ construct electroporated into P4KO cells



Unelectroporated P4KO cells

Figure S-V. **Validation of electroporation of PMCA4 cDNA into VSMCs**. EGFP-tagged human PMCA4b-Δ-PDZ cDNA construct was electroporated into P4KO primary carotid artery smooth muscle cells using the Lonza 4D Nucleofector. Electroporated cells were seeded on cover slips, allowed to recover for 24 h, fixed with paraformaldehyde, washed with PBS, mounted on slides and used for confocal imaging.



PMCA4a specific amplicon band: RT-PCR primers designed to detect PMCA4 exon9-exon10 junction which is absent from P4KO cells

Figure S-VI. **RT-PCR validation of electroporation of PMCA4 cDNA into VSMCs**. P4KO cells carry a genetic deletion whereby exon-9 has been removed and exon-8 splices to exon-10 in mature PMCA4 mRNA. Mouse PMCA4a cDNA construct encodes exon-9 joined to exon-10. RT-PCR primers designed to detect this exon-9-exon-10 junction were used to screen cDNA made from P4KO cells, P4KO cells rescued with vector and P4KO cells rescued with mouse PMCA4a cDNA (cells were subjected to puromycin selection for 14 days after electroporation). Agarose gel electrophoresis showed exon-9-exon-10 junction only in those P4KO cells which received PMCA4a cDNA.

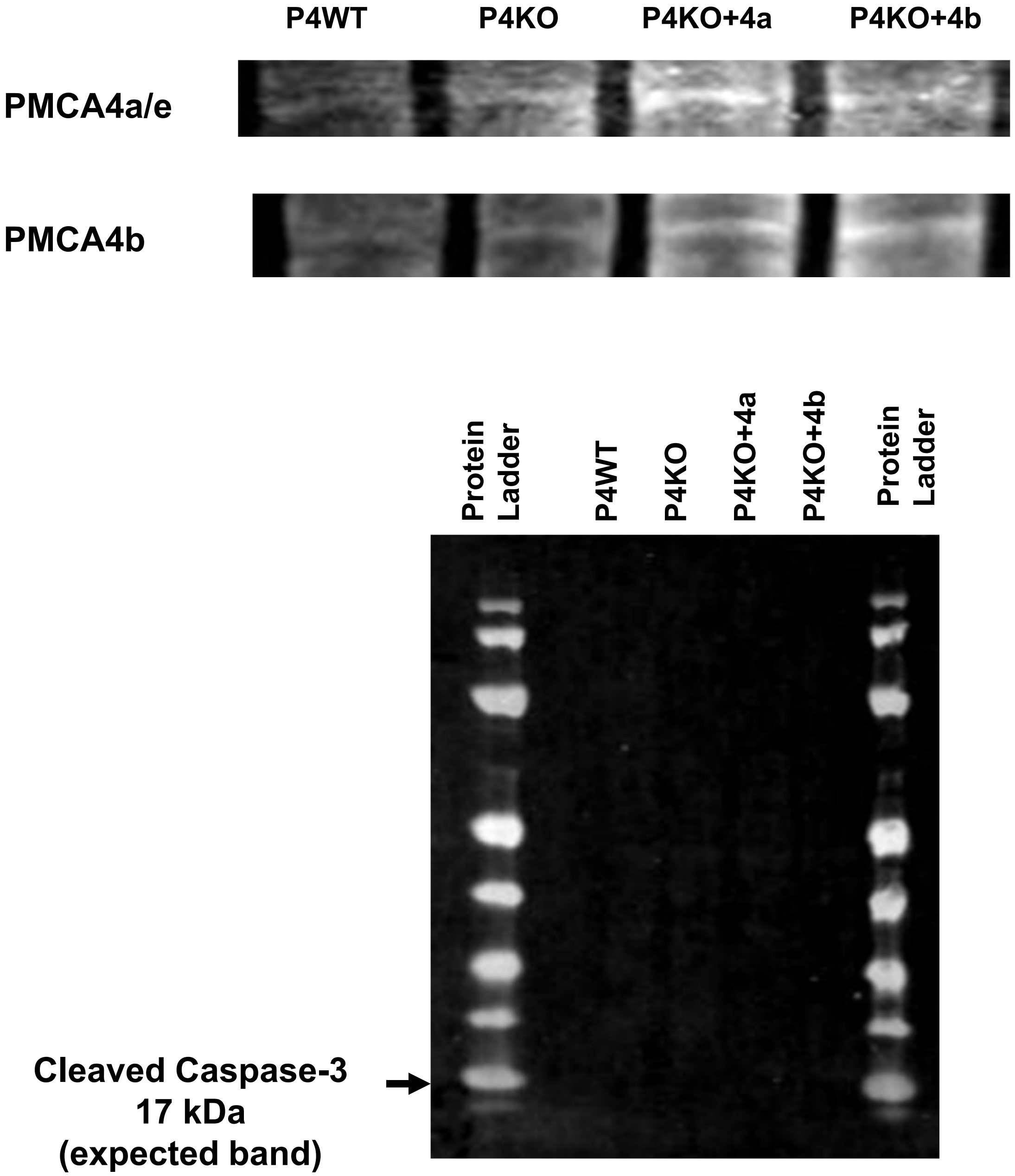


Figure S-VII. **PMCA4a/e, PMCA4b and cleaved Caspase-3 Western blots.** Whole cell protein extracts made from P4WT, P4KO, P4KO+4a and P4KO+4b rescued primary carotid VSMCs were used for immunoblots probed with anti-PMCA4a/e, anti-PMCA4b or anti-cleaved Caspase-3 (apoptosis marker) antibodies. LiCor's IR680 fluorescent dye conjugated secondary antibodies (anti-chicken for PMCA4a/e and PMCA4b and anti-rabbit for cleaved Caspase-3) were used and blots were imaged with the Odyssey fluorescent scanner (LiCor, NE). PMCA4a was overexpressed 1.5-fold in P4KO+4a cells while PMCA4b was overexpressed 1.8-fold in P4KO+4b cells as compared to P4KO cells. There was no cleaved Caspase-3 band visible which signifies absence of any apoptosis in these cells.

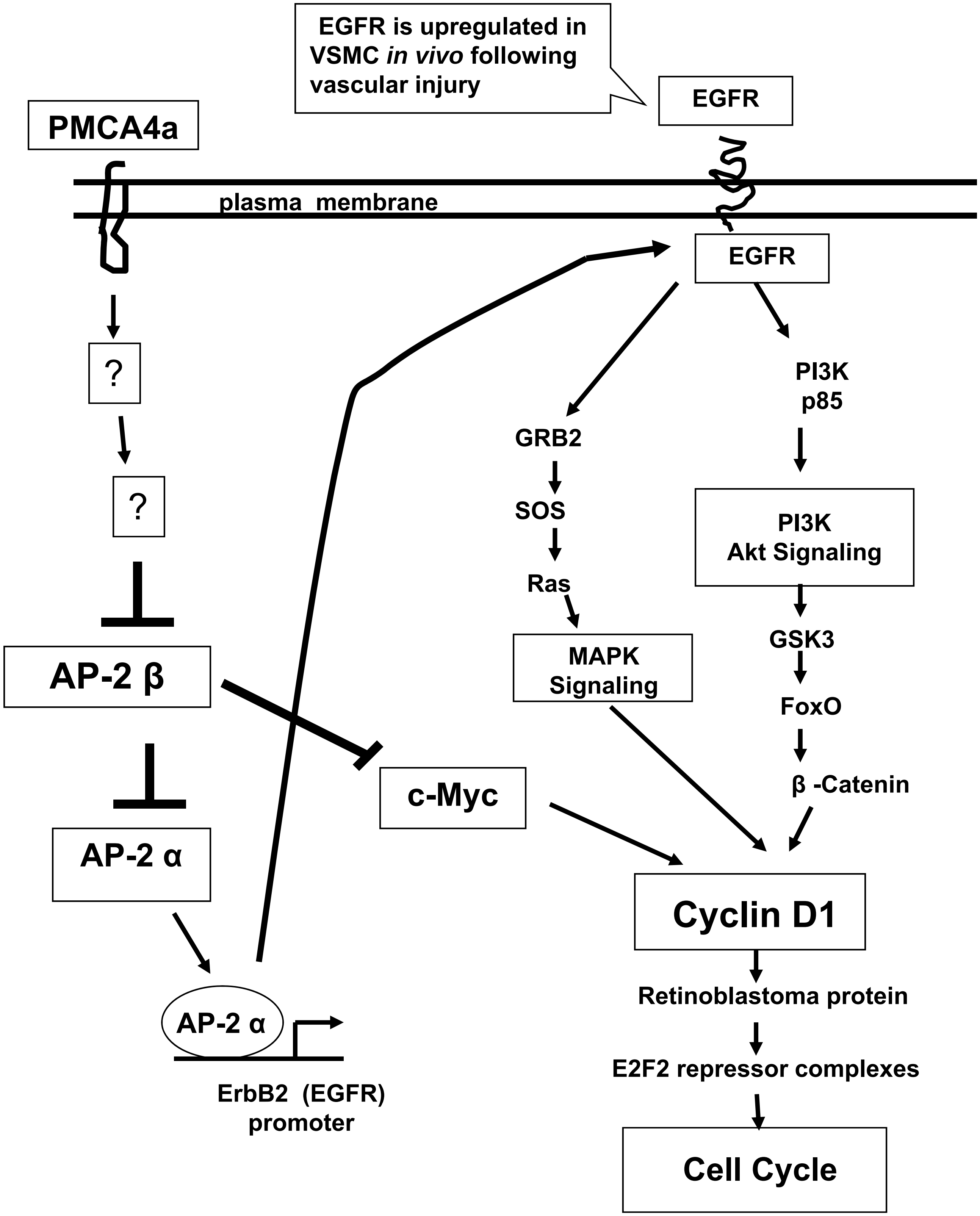


Figure S-VIII. Schematic showing role of proteins modulated by PMCA4a overexpression in PMCA4 knockout cells. AP-2β represses Myc transactivation and also inhibits AP-2 α from binding to the EGFR promoter and enhancing proliferation. PMCA4a overexpression in PMCA4 knockout VSMC de-represses Myc activity and allows AP-2 α to induce EGFR expression which leads to enhanced proliferation. PMCA4a effects on Decorin and Rgs16 omitted for simplicity.

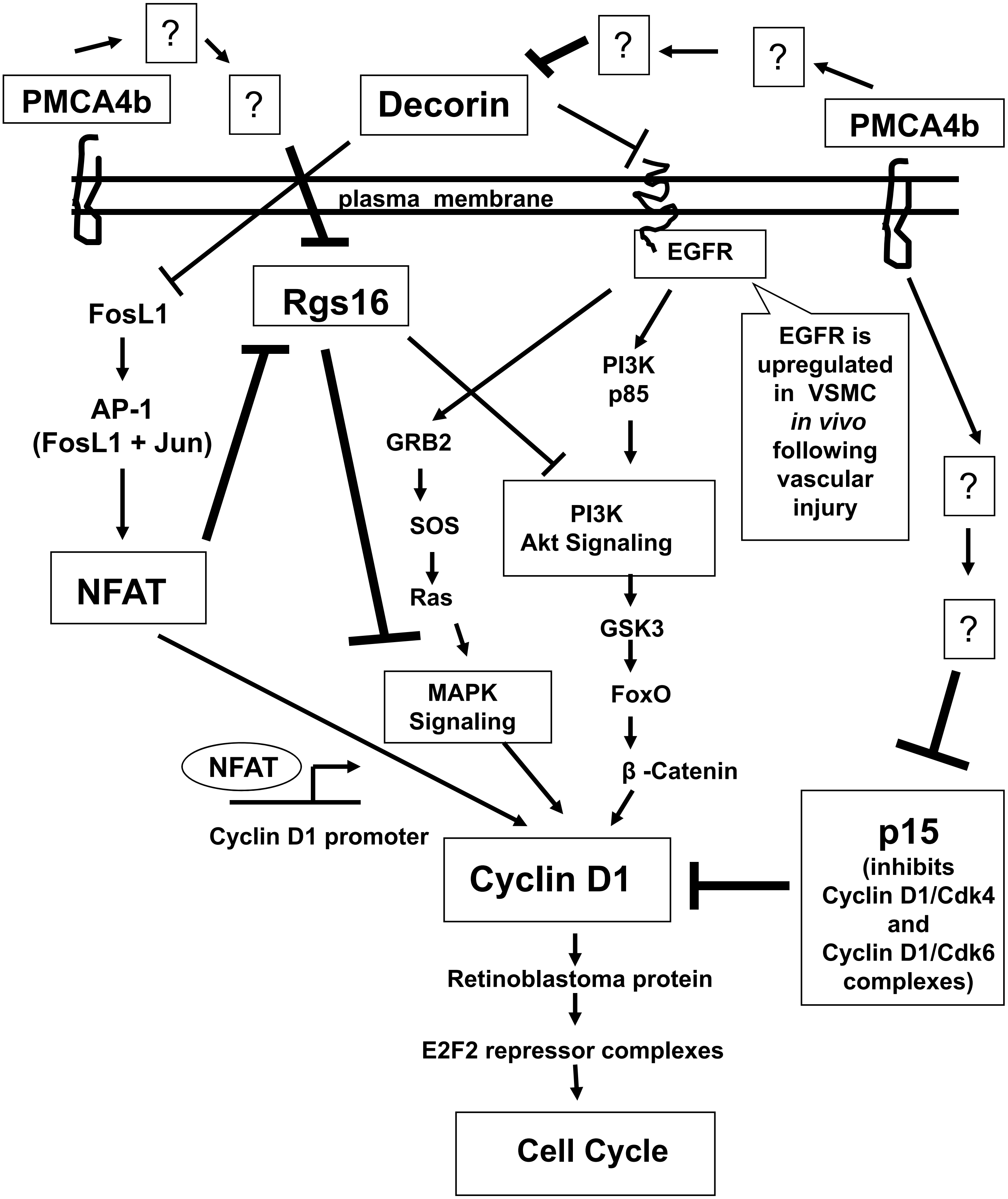


Figure S-IX. Schematic showing role of proteins modulated by PMCA4b overexpression in PMCA4 knockout cells. PMCA4b overexpression in PMCA4 knockout VSMC represses Rgs16 and Decorin, which in turn regulates NFAT and Cyclin D1 expression in the early G1 phase of the cell cycle.