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Supporting Material

Characterization of the Functional Domains of a Mammalian Voltage-Sensitive Phosphatase

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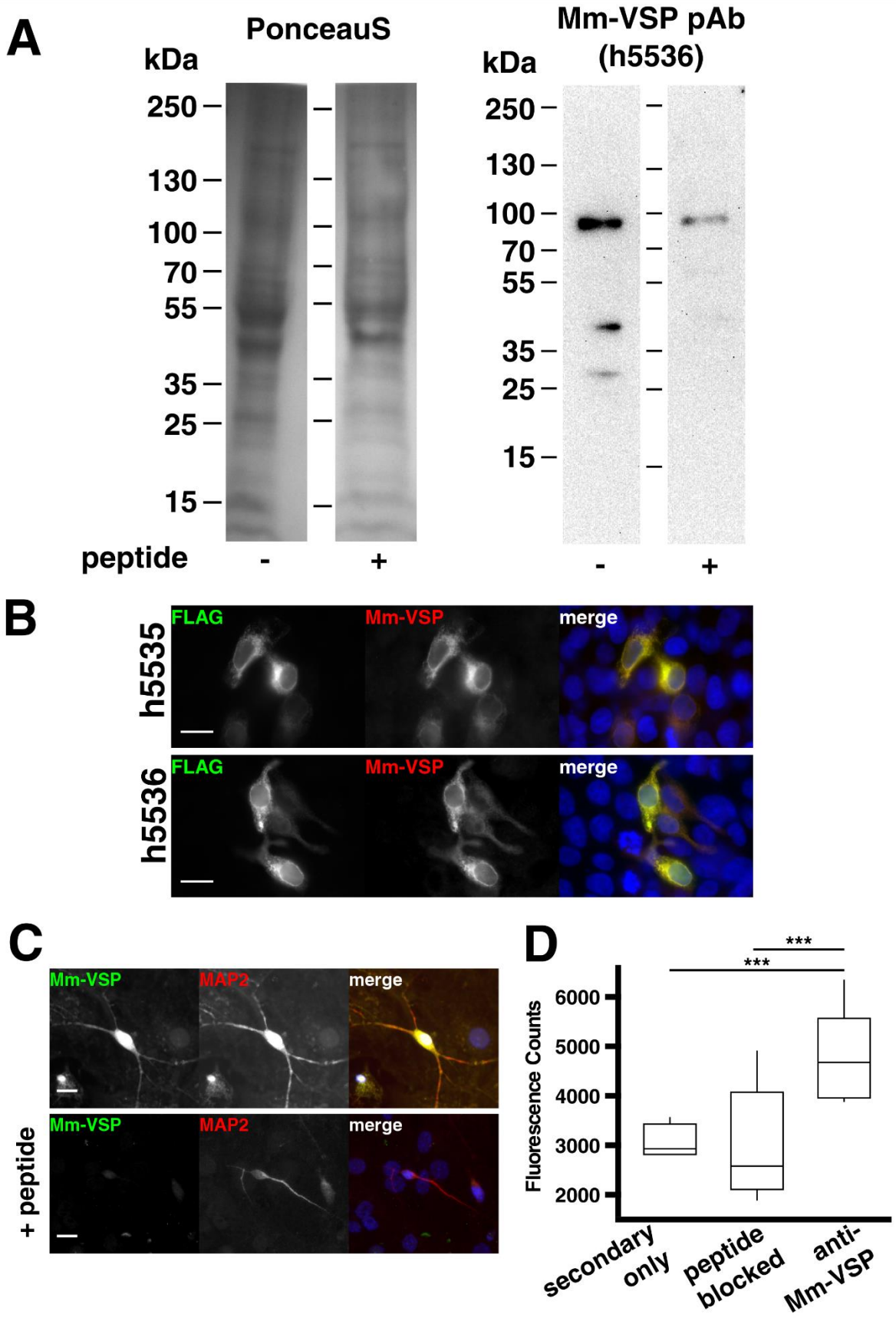
A
PonceauS
kDa
250
130
100
70
55
35
25
15
peptide
-
+
Mm-VSP pAb
(h5536)
kDa
250
130
100
70
55
35
25
15
-
+
B
h5535
FLAG
Mm-VSP
merge
h5536
FLAG
Mm-VSP
merge
C
Mm-VSP
MAP2
merge
+ peptide
Mm-VSP
MAP2
merge
D
***
***
Fluorescence Counts
6000
5000
4000
3000
2000
secondary only
peptide blocked
anti-Mm-VSP

Figure S1: Development of an antibody against Mm-VSP.
(A) Western blot results for anti-Mm-VSP antibody h5536. Shown is a blot of whole-brain homogenate from a 1 year old mouse. Images on the left show the total protein in each lane, labeled with Ponceau S. Ponceau S was rinsed away and the blot was probed with anti-Mm-VSP antibody h5536 (right), with or without pre-incubation with the immunizing peptide (Mm-VSP a.a 1-113).
(B) Anti-Mm-VSP antisera labels Mm-VSP in fixed cells. Shown are fixed HEK293T/17 cells that were transfected with a construct encoding a FLAG-Mm-VSP fusion protein. Cells were co-labeled with an anti-FLAG monoclonal antibody (left panels, green in merged image) and anti-Mm-VSP sera (center panels, red in merged image). Cell nuclei were labeled with Hoechst dye (blue in merged image). Sera from two rabbits, h5535 (top) and h5536 (bottom), were tested. The strong co-localization of the anti-FLAG and anti-Mm-VSP labeling demonstrates that the anti-Mm-VSP antibody recognizes Mm-VSP. The lack of anti-Mm-VSP labeling in cells that don't express the FLAG-Mm-VSP protein demonstrates that the anti-Mm-VSP antibody specifically binds to Mm-VSP. Scale bars indicate 15 μm.
(C) Anti-Mm-VSP labeling is blocked by the immunizing peptide. Shown are fixed 15 DIV cortical neurons, co-labeled with anti-Mm-VSP sera (left, green in merged image) and an anti-MAP2 antibody (center, red in merged image). Cell nuclei were labeled with Hoechst dye (blue in merged image). When the antibodies were incubated with the immunizing peptide before labeling the sample (bottom row), we observed a decrease in the anti-Mm-VSP labeling, indicating the specificity of the antisera in recognizing Mm-VSP. Image acquisition settings and display look up tables are identical between the two sets of images. Scale bars indicate 15 μm.
(D) Quantification of immunolabeling block by the immunizing peptide. Shown are box and whisker plots quantifying the fluorescence counts observed for fixed 15 DIV cortical neurons labeled with anti-Mm-VSP serum (n=20). To test the specificity of the anti-Mm-VSP serum, the immunizing peptide was incubated with the antiserum before labeling (n=20). Cells were co-labeled with an anti-MAP2 antibody, which was used to identify neurons. As a negative control to measure the background fluorescence, cells were incubated with only secondary antibodies, and no primary antibodies (n=4). Neurons in this condition were identified by morphology. The ends of the box represent the 25^{th} and 75^{th} percentiles, and the ends of the whiskers represent the 10^{th} and 90^{th} percentiles. The resulting data were compared using a Wilcoxon rank-sum test. *** indicates $P < 0.0005$.

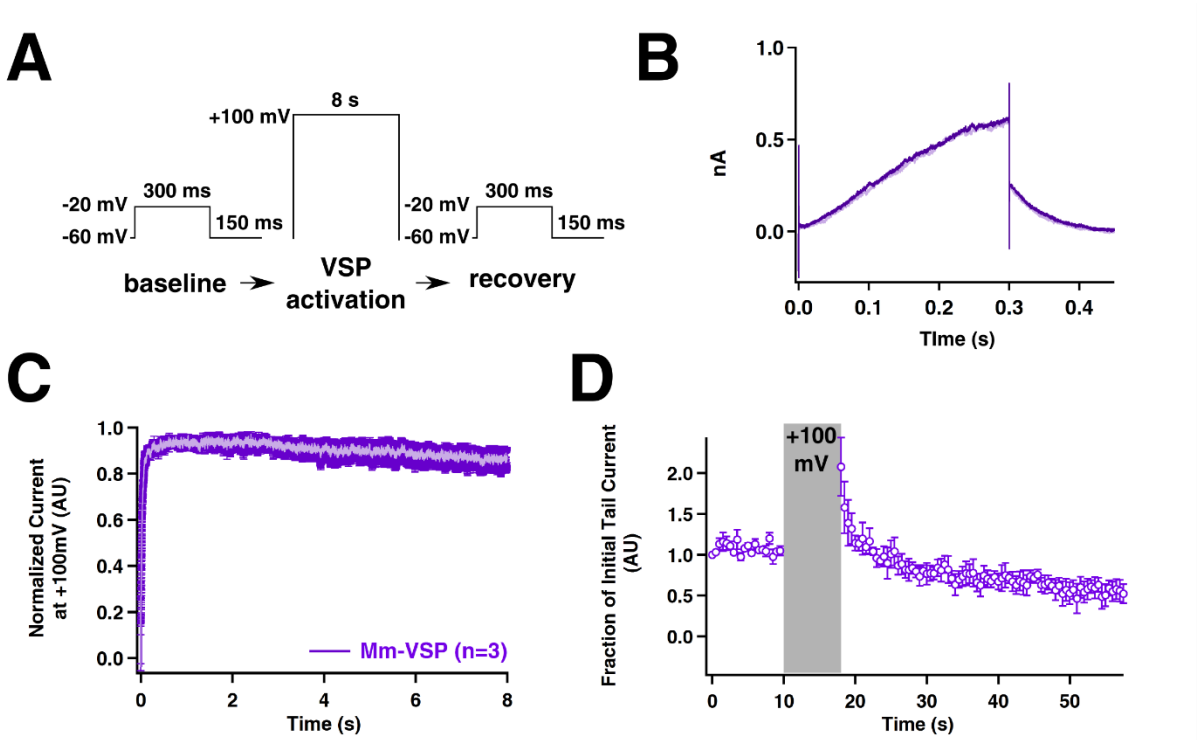

Figure S2: Full-length Mm-VSP does not inhibit KCNQ2/3 currents when co-expressed in HEK293T/17 cells.

Full-length Mm-VSP does not deplete PI(4,5)P_2 at the plasma membrane. All recordings performed as those in Fig. 3, with the exception that the intracellular solution was pH 6.6 to keep Mm-VSP deactivated at -60 mV, based on the data shown in Figs. 3 and 4.

(A) The voltage protocol used to probe VSP-based inhibition of PI(4,5)P_2-dependent M currents. Identical to the protocol used in Fig 3. Baseline tail current amplitude was established by activating M currents at -20 mV for 300 ms and then measuring instantaneous currents when the cell was repolarized to -60 mV. Measurements were taken once every 500 ms for 10 s. Cells were then depolarized to +100 mV for 8 s to activate VSP activity. Following VSP activation, tail currents were again recorded once every 500 ms for 40 s.

(B) Representative currents recorded from HEK293T/17 cells co-transfected with KCNQ2, KCNQ3, and Mm-VSP. Trace recorded immediately before depolarizing cells to +100 mV is shown in light purple, and trace recorded 1 s after depolarization is shown in dark purple.

(C) Average normalized M current (I/I_{max}) of cells held at +100 mV. The lighter center of the trace represents the average I/I_{max}, and darker bars represent per-timepoint standard error of the mean. n=3 cells.

(D) Normalized tail currents amplitudes plotted over time. Amplitudes were normalized to the initial tail current in each cell. Error bars are standard error of the mean for the same 3 cells shown in panel C. Gray bar indicates time when cell was depolarized to +100 mV. The brief potentiation following the +100 mV depolarization is due to the voltage sensitivity of the KCNQ2/3 channels, and is seen in the absence of VSP co-expression as well (Fig 5G).

Dr-VSP	M Current Inhibition ($y = y_0+A(exp[(-x-x_0)/\tau])$				M Current Recovery ($y = y_0+A(exp[(-x-x_0)/\tau])$					
	y_0	A	τ	x_0	y_0	A		τ		x_0
	0.36 ± 0.00	0.54 ± 0.00	0.30 ± 0.00	0.05	0.90 ± 0.00	-0.87 ± 0.01		4.45 ± 0.11		18
Chimera Containing the Mm-VSP Phosphatase	M Current Inhibition ($y = y_0+A(exp[(-x-x_0)/\tau])$				M Current Recovery ($y = y_0+A_1(exp[(-x-x_0)/\tau_1]+A_2 (exp[(-x-x_0)/\tau_2])$					
	y_0	A	τ	x_0	y_0	A_1	A_2	τ_1	τ_2	x_0
	0.60 ± 0.00	0.30 ± 0.00	4.48 ± 0.02	0.05	0.67 ± 0.03	-0.26 ± 0.05	-0.26 ± 0.03	2.54 ± 0.65	18.58 ± 7.31	18

Table S1: The average time courses of M current inhibition and recovery shown in Figs 5F and 5G were fitted with either a single or double exponential as noted in the table, generating the listed values for the fit parameters. Values are reported ± one standard deviation, as derived from the fit.

mRNA target region	**Forward Primer (5’ to 3’ on forward strand)**	**Reverse Primer (5’ to 3’ on reverse strand)**
Mm-VSP bp -83 to 178	CGACTTCTGAGCCCAAGCAGCC	TGTCGTAGCCAGTGCTGCCATTTA
Mm-VSP bp 308 to 635	GGGGAGCTTCGAGCAGCACAAC	ACCGAGGACACAAGGATGCGCA
Mm-VSP bp 795 to 1260	GTCTGTAGAAGGGAGACGGCGC	GCGGACCCTGTAGTGGAAGTGC
MAP2	TCAAACATTCTGCTGGGGGCGGA	CCCTGCTTAGCAAGCGCCGC

Table S2: Shown are the primer pairs used to screen for the expression of Mm-VSP using RT-PCR. Numbering indicates base pairs relative to the start of the open reading frame. Primers targeting the MAP2 transcript were used as a positive loading control.

	Construct	Forward Primer	Reverse Primer	Cloning sites
1	**FLAG-MmVSP.pRRL**	AAACCCGGGATGGACT ACAAGGACGATGACG ACAAGTATGGAGAAAA GAAGAGCCATTTG	GGATCCCTGCAGCTAG TTCTCACCAAAATCCAC	PstI/BamHI
2	**KvSynth1-GFP.pcDNA3**	AAGCTTGGATCCATGG AGGGATTCGACGGT	AAACGGCGCGCCCCTA AAGTTAGAACGATGAA	BamHI/AscI
3	**KvSynthM-GFP.pcDNA3 (Kcv pore)**	AAATTAATTAAAATGTT AGTGTTTAGTAAA	AAACGGCGCGCCCCTA AAGTTAGAACGATGAA	HindIII/PacI
4	**KvSynthM-GFP.pcDNA3 (Mm-VSP VSD)**	AAAAAGCTTATGGAAA TCAAAATCCCA	AAATTAATTAATTGTCT CTTTTGATGAGC	PacI/AscI
5	**DrVSP-MmVSP-Chimera.pIRES2-EGFP (Dr-VSP VSD)**	AAAAAGCTTATGACGT CTGTGCATTTT	AAAGGTACCCTCTCTCT TCTGCGAGGC	SacI/KpnI
6	**DrVSP-MmVSP-Chimera.pIRES2-EGFP (Mm-VSP phosphatase)**	AAAGGTACCCTCGAAA GGCTGACCAGG	AAATTAATTAATTGTCT CTTTTGATGAGC	KpnI/PstI
7	**Removal of KpnI site in Mm-VSP-Dr-VSP-Chimera.pIRES2-EGFP by QuikChange**	CTGGCCTCGCAGAAGA GAGAGCTCGAAAGGCT GACCAGGAAG	CTTCCTGGTCAGCCTTT CGAGCTCTCTCTTCTGC GAGGCCAG	N/A
8	**His-MBP-MmVSP-1-113.pRSF**	GGGGGATCCGAATTCA TGTATGGAGAAAAGAA GAGCCAT	GGGGAATTCAAGCTTC TGCAGCTACAGTTCAT ACAGGGTTGTGCTGCT	HindIII/EcoRI

Table S3: Constructs were generated using PCR cloning. The constructs generated are listed on the left, and the plasmid backbone is listed following the period. Details for each construct are listed below.

(1) Base pairs encoding the FLAG epitope were added to the 5' end of the Mm-VSP cDNA, by PCR.

(2) The original Kv_{Synth1} construct was subcloned using PCR from the psGEM oocyte expression vector into pcDNA3 for expression in mammalian cells. The pcDNA3 vector was engineered to contain an in-frame GFP coding sequence fused to the 3' end of the construct.

(3,4) Kv_{SynthM}, was generated by fusing the VSD from Mm-VSP to the Kcv pore, then inserted into pcDNA3. The pcDNA3 vector was engineered to contain an in-frame GFP coding sequence fused to the 3' end of the construct.

(5,6,7) The Dr-VSP/Mm-VSP chimera was generated by fusing the VSD from Dr-VSP to the Mm-VSP phosphatase using an engineered KpnI restriction site, then inserted into pIRES2-EGFP. The base pairs encoding the KpnI site were then removed by QuikChange mutagenesis. All DNA constructs were verified by sequencing.

(8) The cDNA encoding the first 113 amino acids of Mm-VSP was amplified by PCR and inserted into the pRSF vector, encoding an amino-terminal 6xHis-MBP dual affinity purification tag.