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Supplementary Materials for

Aberrant Rac1-cofilin signaling mediates defects in dendritic spines, synaptic function, and sensory perception in fragile X syndrome

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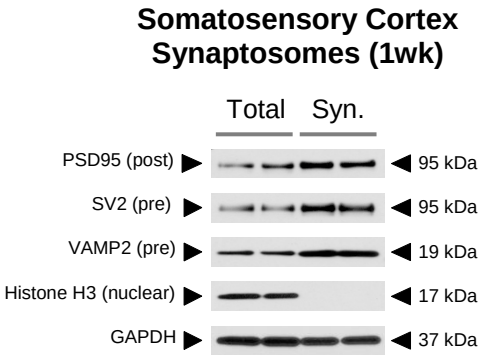

Figure S1. Validation of somatosensory cortex synapse enrichment. Representative western blot from a 1-week-old wild-type (WT) mouse showing enrichment of presynaptic (VAMP2 and SV2) and postsynaptic (PSD95) markers as well the absence of a nuclear marker (Histone H3) in somatosensory cortex synaptosome fractions versus total lysates.

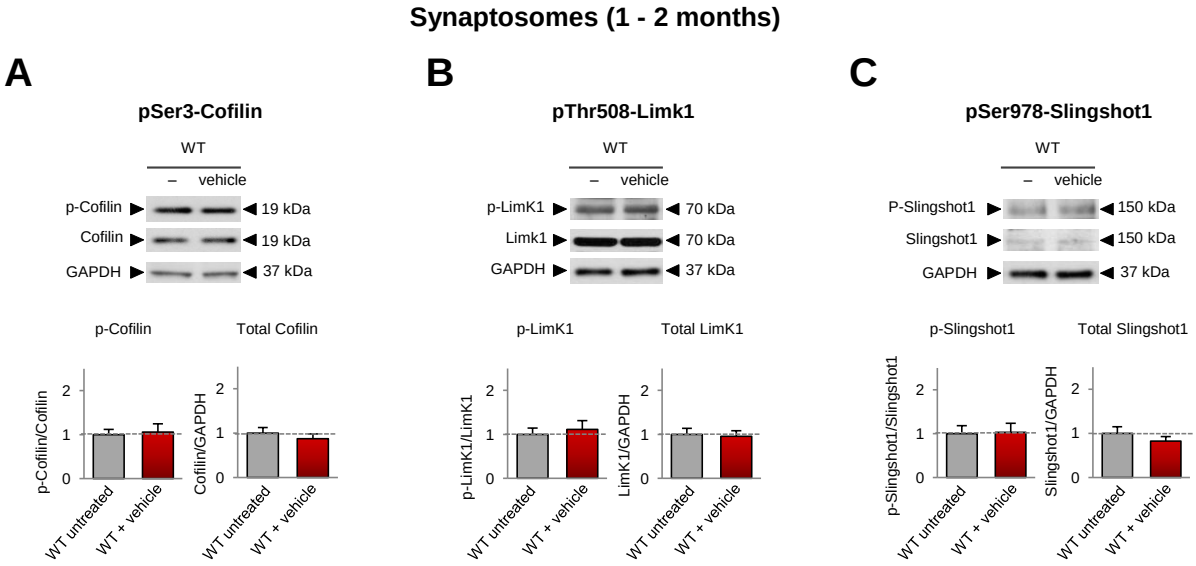

Figure S2. Subcutaneous administration of hydroxypropyl-β-cyclodextrin (vehicle) has little to no effect on cofilin signaling. (A to **C)** Representative Western blots (top) and summary data (bottom) from somatosensory synaptosomes of 1- to 2-month-old wild-type mice, either untreated (left) or treated with hydroxypropyl-β-cyclodextrin (vehicle; right). Synaptosomes from vehicle-treated animals exhibited no change in the abundance of phosphorylated (p-) cofilin (**A**), p-LIMK1 (**B**), and p-Slingshot1 (**C**) relative to those from untreated animals (n = 7 animals per group).

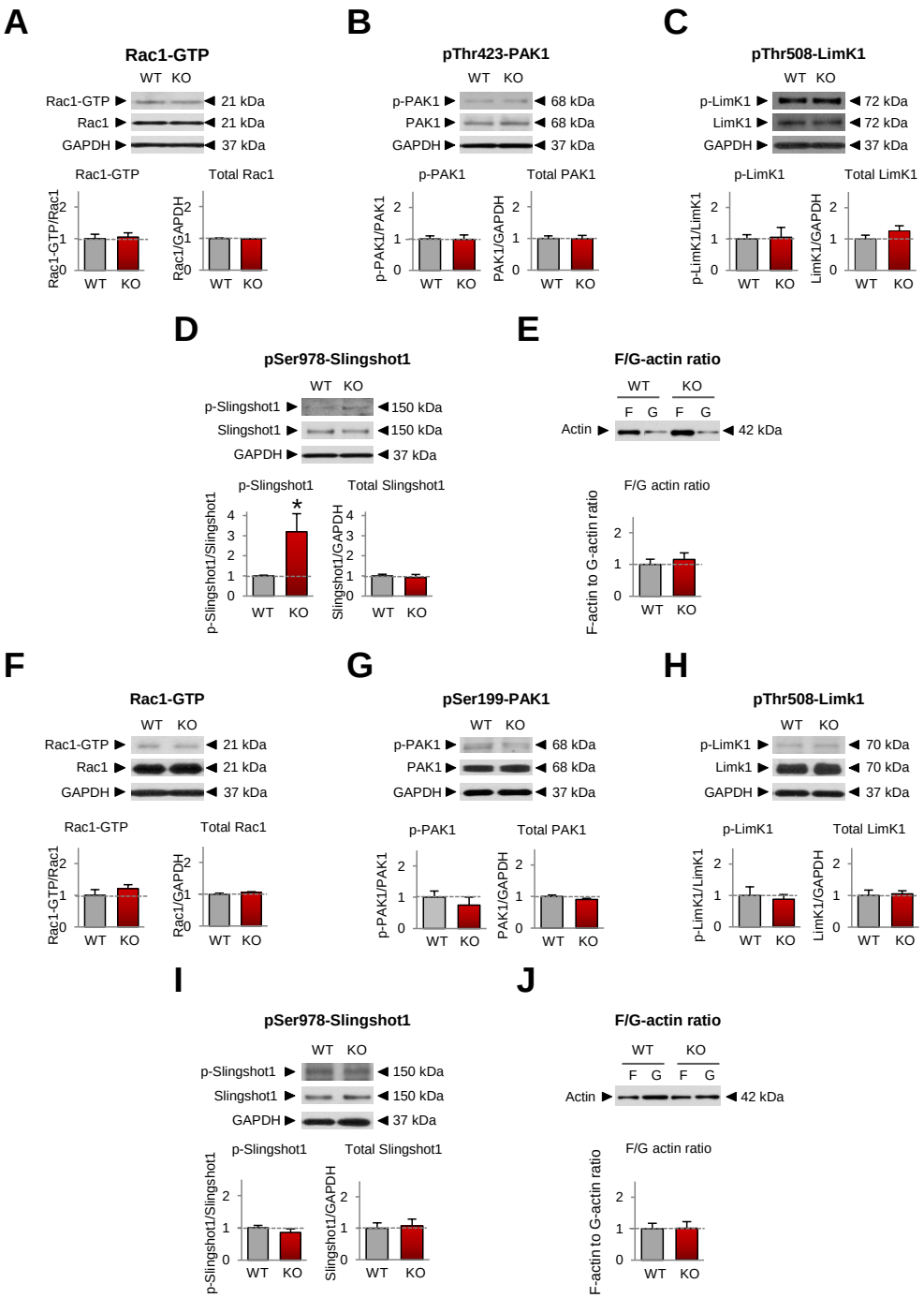

Figure S3. Mature *Fmr1* KO mice exhibit normal Rac1 signaling at somatosensory cortex synapses. (**A** to **E**) Representative Western blots (top) and summary data (bottom) from somatosensory lysates (**A**) or synaptosomes (**B** to **E**) of 4-week-old WT and *Fmr1* KO mice showing relative abundance of active, phosphorylated or total proteins in the Rac1 signaling pathway: (**A**) Rac1-GTP, (**B**) p-PAK1, (**C**) p-LIMK1, (**D**) p-Slingshot1, and (**E**) F-actin/G-actin ratio (n = 5 – 12 animals per group). (**F** to **J**) Representative western blots (top) and summary data (bottom) from somatosensory lysates (**F**) or synaptosomes (**G** to **J**) of 2- to 5-month-old WT and *Fmr1* KO mice showing relative abundance of active, phosphorylated or total proteins in the Rac1 signaling pathway, as in (**A** to **E**) (n = 4 – 10 animals per group). Data are mean ± SEM. *p < 0.05.

Synaptosomes (2 - 5 months)

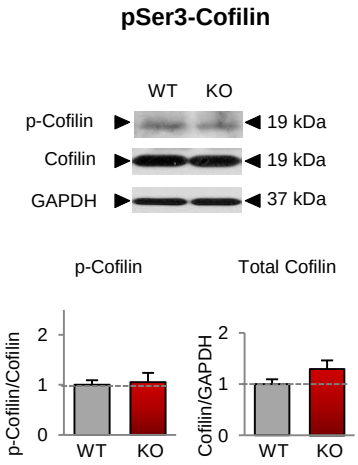

Figure S4. Mature *Fmr1* KO mice exhibit normal cofilin phosphorylation and activity at somatosensory cortex synapses. (**A**) Representative wWstern blots and summary data of cofilin phosphorylation in somatosensory synaptosomes from 2- to 5-month-old WT and *Fmr1* KO mice (9 – 14 mice per group). Membranes were probed with antibodies to p-cofilin and total cofilin.

Table S1. Summary of statistics for Fig. 1.

Figure	N	Mean ± SEM	p value	Statistical Test
1A	WT (p-Cofilin) = 12 animals KO (p-Cofilin) = 12 animals WT (Cofilin) = 12 animals KO (Cofilin) = 12 animals	1.000 ± 0.2277 1.764 ± 0.2814 1.000 ± 0.05896 1.080 ± 0.05266	– *, 0.046 – 0.327	Two-tailed unpaired *t*-test, WT vs KO
1B	WT (p-Cofilin) = 7 animals KO (p-Cofilin) = 7 animals WT (Cofilin) = 7 animals KO (Cofilin)= 7 animals	1.000 ± 0.1544 1.115 ± 0.2765 1.000 ± 0.1190 0.9104 ± 0.1131	– 0.729 – 0.5952	
1C	WT (p-Cofilin) = 12 animals KO (p-Cofilin) = 10 animals WT (Cofilin) = 12 animals KO (Cofilin)= 10 animals	1.000 ± 0.1458 1.958 ± 0.4158 1.000 ±0.0918 0.7626 ± 0.0823	– *, 0.030 – 0.073	
1D	WT (p-Cofilin) = 10 animals KO (p-Cofilin) = 9 animals WT (Cofilin) = 10 animals KO (Cofilin)= 9 animals	1.000 ± 0.1355 1.035 ± 0.2629 1.000 ± 0.1106 1.087 ± 0.1015	– 0.9038 – 0.5710	
1E	WT (F/G actin) = 5 animals KO (F/G actin) = 6 animals	1.000 ± 0.4336 3.495 ± 0.7284	– *, 0.021	
1G (% Filopodia)	WT = 3 animals / 12 dendrites KO = 3 animals / 13 dendrites	100.0 ± 4.957 126.2 ± 5.838	– ***, 0.0009	
1G (% Density)	WT = 3 animals / 12 dendrites, 154 segments, 660 spines KO = 3 animals / 13 dendrites, 182 segments, 933 spines	100.0 ± 7.941 131.1 ± 11.82	– *, 0.0429	

Table S2. Summary of statistics for Fig. 2.

Figure	N	Mean ± SEM	p value	Statistical Test
2A	WT (Rac1-GTP) = 4 animals KO (Rac1-GTP) = 4 animals WT (Rac1) = 4 animals KO (Rac1) = 4 animals	1.000 ± 0.0396 1.673 ± 0.0396 1.000 ± 0.0327 1.065 ± 0.0320	– ****,<0.0001 – 0.207	Two-tailed unpaired *t*-test, WT vs KO
2B	WT (p-PAK1) = 12 animals KO (p-PAK1) = 15 animals WT (PAK1) = 12 animals KO (PAK1) = 15 animals	1.000 ± 0.0904 1.791 ± 0.3295 1.000 ± 0.0865 1.109 ± 0.1615	– *, 0.047 – 0.327	
2C	WT (p-PAK4) = 8 animals KO (p-PAK4) = 7 animals WT (PAK4) = 8 animals KO (PAK4) = 7 animals	1.000 ± 0.1862 1.133 ± 0.3728 1.000 ± 0.1111 1.126 ± 0.1913	– 0.745 – 0.567	
2D	WT (p-LimK1) = 10 animals KO (p-LimK1) = 9 animals WT (LimK1) = 10 animals KO (LimK1) = 9 animals	1.000 ± 0.0929 2.047 ± 0.4434 1.000 ± 0.0730 0.9765 ± 0.1525	– *, 0.020 – 0.887	
2E	WT (p-SSH1) = 8 animals KO (p-SSH1) = 5 animals WT (SSH1) = 8 animals KO (SSH1) = 5 animals	1.000 ± 0.1109 1.764 ± 0.3759 1.000 ± 0.1424 1.148 ± 0.3567	– *, 0.037 – 0.673	

Table S3. Summary of statistics for Fig. 3.

Figure	N	Mean ± SEM	p value ($p < 0.05$)	Statistical Test
3A	WT vehicle (p-LimK1) = 13 animals	1.000 ± 0.2463	–	Two-way ANOVA with Tukey's post-test (WT vehicle vs KO vehicle, WT vehicle vs WT FRAX486 and KO vehicle vs KO FRAX486)
	KO vehicle (p- LimK1) = 12 animals	2.1853 ± 0.3478	*, 0.012	
	WT FRAX486 (p- LimK1) = 13 animals	0.9886 ± 0.2433	0.999	
	KO FRAX486 (p- LimK1) = 11 animals	0.9751 ± 0.1843	*, 0.016	
	WT vehicle (LimK1) = 13 animals	1.000 ± 0.1247	–	
	KO vehicle (LimK1) = 12 animals	0.9795 ± 0.1466	0.999	
	WT FRAX486 (LimK1) = 13 animals	1.0094 ± 0.1750	0.999	
	KO FRAX486 (LimK1) = 11 animals	1.1066 ± 0.2149	0.953	
3B	WT vehicle (p-SSH1) = 12 animals	1.000 ± 0.3312	–	
	KO vehicle (p-SSH1) = 10 animals	2.3299 ± 0.2033	**, 0.003	
	WT FRAX486 (p-SSH1) = 8 animals	1.3092 ± 0.2307	0.847	
	KO FRAX486 (p-SSH1) = 10 animals	1.1526 ± 0.2792	*, 0.013	
	WT vehicle (SSH1) = 12 animals	1.000 ± 0.3930	–	
	KO vehicle (SSH1) = 10 animals	0.8570 ± 0.3334	0.986	
	WT FRAX486 (SSH1) = 8 animals	0.7212 ± 0.1630	0.910	
	KO FRAX486 (SSH1) = 10 animals	0.7692 ± 0.1949	0.999	
3C	WT vehicle (p-Cofilin) = 14 animals	1.0000 ± 0.2270	–	
	KO vehicle (p-Cofilin) = 14 animals	2.0817 ± 0.2463	**, 0.006	
	WT FRAX486 (p-Cofilin) = 15 animals	0.9440 ± 0.2045	0.998	
	KO FRAX486 (p-Cofilin) = 9 animals	0.7251 ± 0.2293	**, 0.002	
	WT vehicle (Cofilin) = 14 animals	1.0000 ± 0.2013	–	
	KO vehicle (Cofilin) = 14 animals	1.0703 ± 0.2069	0.995	
	WT FRAX486 (Cofilin) = 15 animals	0.8696 ± 0.1850	0.966	
	KO FRAX486 (Cofilin) = 9 animals	1.0339 ± 0.2354	0.999	
3D	WT vehicle (F/G actin) = 10 animals	1.0000 ± 0.1430	–	
	KO vehicle (F/G actin) = 9 animals	1.9193 ± 0.3043	*, 0.012	
	WT FRAX486 (F/G actin) = 10 animals	1.0028 ± 0.1529	0.999	
	KO FRAX486 (F/G actin) = 7 animals	1.0919 ± 0.1543	*, 0.048	

Table S4. Summary of statistics for Fig. 4.

Figure	N	Mean ± SEM	p value (p < 0.05)	Statistical Test
4B (cultured neurons)	*3 independent experiments* No virus (F/G actin) = 14 wells GFP (F/G actin) = 13 wells WT-Cofilin (F/G actin) = 15 wells Cofilin S3A (F/G actin) = 13 wells Cofilin S3D (F/G actin) = 15 wells	 1.0000 ± 0.1266 1.1017 ± 0.1602 0.9950 ± 0.1625 0.4280 ± 0.0750 1.0938 ± 0.1421	 – 0.986 0.998 *, 0.042 0.988	One-way ANOVA with Tukey's post-test (No virus vs Cofilin S3A, No virus vs GFP, No virus vs Cofilin S3A, No virus vs Cofilin S3D)
4D (Spine length)	WT (GFP) = 10 animals, 21 neurons, 1286 spines, 196 segments of 10 μm KO (GFP) = 8 animals, 20 neurons, 1381 spines, 170 segments of 10 μm WT (WT. Cof.) = 7 animals, 15 neurons, 765 spines, 119 segments of 10 μm KO (WT. Cof.) = 9 animals, 15 neurons, 911 spines, 123 segments of 10 μm WT (S3A) = 6 animals, 16 neurons, 887 spines, 132 segments of 10 μm KO (S3A) = 7 animals, 17 neurons, 1119 spines, 151 segments of 10 μm	1.1442 ± 0.0532 1.3326 ± 0.0494 1.0087 ± 0.0403 1.2496 ± 0.0519 0.9462 ± 0.0364 0.9298 ± 0.0520	– *, 0.043 0.370 *, 0.019 *, 0.047 ****, <0.0001	Two-way ANOVA with Tukey's post-test (WT (GFP) vs KO (GFP), WT (GFP) vs WT (S3A), WT (GFP) vs WT (WT. Cof.), KO (GFP) vs KO (S3A), and WT (WT. Cof.) vs KO (WT. Cof.)
4E (Spine head width)	WT (GFP) – same as above KO (GFP) WT (WT. Cof.) KO (WT. Cof.) WT (S3A) KO (S3A)	0.7354 ± 0.0129 0.6509 ± 0.0221 0.6911 ± 0.0139 0.6046 ± 0.0146 0.7188 ± 0.0249 0.6356 ± 0.0191	– **, 0.009 0.541 *, 0.033 0.987 *, 0.031	
4F (Length-width ratio)	WT (GFP) – same as above KO (GFP) WT (WT. Cof.) KO (WT. Cof.) WT (S3A) KO (S3A)	1.6570 ± 0.0709 2.2756 ± 0.0868 1.6022 ± 0.0709 2.2876 ± 0.1141 1.4411 ± 0.0607 1.5550 ± 0.0773	– ****, <0.0001 0.899 ****, <0.0001 0.398 ****, <0.0001	
4G (%Stubby/ Mushroom)	WT (GFP) – same as above KO (GFP) WT (WT. Cof.) KO (WT. Cof.) WT (S3A) KO (S3A)	0.7823 ± 0.0204 0.6568 ± 0.0177 0.7892 ± 0.0193 0.6106 ± 0.0218 0.8405 ± 0.0136 0.8109 ± 0.0222	– ****, <0.0001 0.999 ****, <0.0001 0.269 ****, <0.0001	
4H (%Thin/ Filopodia)	WT (GFP) – same as above KO (GFP) WT (WT. Cof.) KO (WT. Cof.) WT (S3A) KO (S3A)	0.2127 ± 0.0201 0.3417 ± 0.0175 0.2108 ± 0.0193 0.3881 ± 0.0220 0.1595 ± 0.0136 0.1808 ± 0.0214	– ****, <0.0001 0.999 ****, <0.0001 0.355 ****, <0.0001	
4I (Spine density)	WT (GFP) – same as above KO (GFP) WT (WT. Cof.) KO (WT. Cof.) WT (S3A) KO (S3A)	6.8827 ± 0.3208 8.1235 ± 0.3485 6.1092 ± 0.3035 8.3008 ± 0.3801 6.7197 ± 0.3395 6.5762 ± 0.2974	– *, 0.046 0.583 ***, 0.0005 0.999 **, 0.009	

Table S5. Summary of statistics for Fig. 5.

Figure	N	Mean ± SEM	p value (p < 0.05)	Statistical Test
5B (NMDA/AMPA ratio)	WT vehicle = 13 animals KO vehicle = 11 animals WT FRAX486 = 11 animals KO FRAX486 = 6 animals	0.669 ± 0.054 1.531 ± 0.288 0.727 ± 0.042 0.503 ± 0.710	– **, 0.0014 – 0.8297	Two-way ANOVA with Tukey's post-test (WT vehicle vs. KO vehicle, KO vehicle vs KO FRAX486)
5D (mEPSC amplitude)	WT vehicle = 10 animals KO vehicle = 10 animals WT FRAX486 = 11 animals KO FRAX486 = 9 animals	25.65 ± 2.273 21.60 ± 1.317 26.28 ± 1.369 29.36 ± 2.521	– 0.4386 – *, 0.0365	
5D (mEPSC frequency)	WT vehicle = 10 animals KO vehicle = 10 animals WT FRAX486 = 11 animals KO FRAX486 = 9 animals	1.014 ± 0.131 0.650 ± 0.095 0.471 ± 0.111 0.655 ± 0.141	– 0.1577 *, 0.0119 0.9999	Two-way ANOVA with Tukey's post-test (WT vehicle vs KO vehicle, WT vehicle vs WT FRAX486, KO vehicle vs KO FRAX486)
5E (NMDA/AMPA ratio)	WT vehicle = 15 animals KO vehicle = 7 animals WT FRAX486 = 11 animals KO FRAX486 = 10 animals	0.606 ± 0.068 0.431 ± 0.065 0.571 ± 0.048 0.599 ± 0.089	– 0.3704 0.9821 0.4678	
5E (mEPSC amplitude)	WT vehicle = 18 animals KO vehicle = 7 animals WT FRAX486 = 16 animals KO FRAX486 = 12 animals	29.21 ± 1.273 27.79 ± 1.251 22.67 ± 1.379 22.14 ± 1.635	– 0.9301 **, 0.004 0.1254	
5E (mEPSC frequency)	WT vehicle = 18 animals KO vehicle = 7 animals WT FRAX486 = 16 animals KO FRAX486 = 12 animals	2.472 ± 0.426 2.454 ± 0.212 2.873 ± 0.761 1.920 ± 0.391	– 0.9999 0.9452 0.9505	

Table S6. Summary of statistics for Fig. 6.

Figure	N	Mean ± SEM	p value (p < 0.05)	Statistical Test
6B	WT (familiar) = 13 animals WT (novel) = 13 animals KO (familiar) = 7 animals KO (novel) = 7 animals	42.05 ± 3.276 58.96 ± 2.663 48.54 ± 4.097 51.46 ± 4.097	– ***, 0.0005 – 0.6259	Two-tailed unpaired *t* test for WT (familiar) vs WT (novel) and KO (familiar) vs KO (novel)
6C	WT (familiar) = 10 animals WT (novel) = 10 animals	47.50 ± 2.435 52.60 ± 2.449	– 0.1569	
6D	WT (familiar) = 7 animals WT (novel) = 7 animals	52.54 ± 4.100 47.46 ± 4.100	– 0.3989	
6E	WT (familiar) = 7 animals WT (novel) = 7 animals KO (familiar) = 8 animals KO (novel) = 8 animals	38.13 ± 2.872 61.87 ± 2.872 41.08 ± 3.631 58.92 ± 3.631	– ****, <0.0001 – **, 0.003	
6F	WT-vehicle (familiar) = 15 animals WT-vehicle (novel) = 15 animals KO-vehicle (familiar) = 10 animals KO-vehicle (novel) = 10 animals WT-FRAX486 (familiar) = 9 animals WT- FRAX486 (novel) = 9 animals KO- FRAX486 (familiar) = 10 animals KO- FRAX486 (novel) = 10 animals	43.57 ± 2.705 56.43 ± 2.705 51.79 ± 3.446 48.71 ± 2.997 41.44 ± 4.219 58.83 ± 4.344 40.19 ± 3.188 59.81 ± 3.188	– **, 0.0022 – 0.5089 – *, 0.0111 – ***, 0.0003	

Table S7. Summary of statistics for fig. S2.

Figure	N	Mean ± SEM	p value ($p < 0.05$)	Statistical Test
Supp. 2A	WT untreated (p-Cofilin) = 7 animals WT vehicle (p-Cofilin) = 7 animals WT untreated (Cofilin) = 7 animals WT vehicle (Cofilin) = 7 animals	1.000 ± 0.1259 1.060 ± 0.2010 1.000 ± 0.1360 0.8786 ± 0.1109	– 0.8031 – 0.5023	Two-tailed unpaired *t* test for WT untreated vs WT vehicle treated animals
Supp. 2B	WT untreated (p-LimK1) = 7 animals WT vehicle (p-LimK1) = 7 animals WT untreated (LimK1) = 7 animals WT vehicle (LimK1) = 7 animals	1.000 ± 0.1525 1.116 ± 0.2125 1.000 ± 0.1458 0.9594 ± 0.1327	– 0.6659 – 0.8402	
Supp. 2C	WT untreated (p-SSH1) = 7 animals WT vehicle (p-SSH1) = 7 animals WT untreated (SSH1) = 7 animals WT vehicle (SSH1) = 7 animals	1.000 ± 0.1953 1.028 ± 0.2255 1.000 ± 0.1571 0.8207 ± 0.1127	– 0.9270 – 0.3718	

Table S8. Summary of statistics for figs. S3 and S4.

Figure	N	Mean ± SEM	p value	Statistical Test
3A	WT (Rac1-GTP) = 8 animals KO (Rac-GTP) = 7 animals WT (Rac1) = 8 animals KO (Rac1) = 7 animals	1.000 ± 0.1437 1.052 ± 0.1341 1.000 ± 0.01174 0.9846 ± 0.0082	– 0.7984 – 0.3136	Two-tailed unpaired *t* test for WT vs KO
3B	WT (p-PAK1) = 8 animals KO (p-PAK1) = 8 animals WT (PAK1) = 8 animals KO (PAK1) = 8 animals	1.000 ± 0.0937 0.9798 ± 0.1460 1.000 ± 0.0786 0.9904 ± 0.1071	– 0.9089 – 0.9435	
3C	WT (p-LIMK1) = 10 animals KO (p-LIMK1) = 9 animals WT (LIMK1) = 10 animals KO (LIMK1) = 9 animals	1.000 ± 0.1349 1.050 ± 0.3280 1.000 ± 0.1205 1.253 ± 0.1664	– 0.8849 – 0.2291	
3D	WT (p-SSH1) = 8 animals KO (p-SSH1) = 7 animals WT (SSH1) = 8 animals KO (SSH1) = 7 animals	1.000 ± 0.0716 3.127 ± 0.9302 1.000 ± 0.0795 0.9207 ± 0.1603	– *, 0.0293 – 0.6537	
3E	WT (F/G actin) = 5 animals KO (F/G actin) = 5 animals	1.000 ± 0.0543 1.156 ± 0.1955	– 0.4592	
3F	WT (Rac1-GTP) = 7 animals KO (Rac-GTP) = 5 animals WT (Rac1) = 7 animals KO (Rac1) = 5 animals	1.000 ± 0.1767 1.213 ± 0.1235 1.000 ± 0.3615 1.054 ± 0.0246	– 0.3944 – 0.2852	
3G	WT (p-PAK1) = 4 animals KO (p-PAK1) = 6 animals WT (PAK1) = 4 animals KO (PAK1) = 6 animals	1.000 ± 0.2757 0.7542 ± 0.2850 1.000 ± 0.0708 0.9089 ± 0.0372	– 0.5723 – 0.2461	
3H	WT (p-LIMK1) = 8 animals KO (p-LIMK1) = 5 animals WT (LIMK1) = 8 animals KO (LIMK1) = 5 animals	1.000 ± 0.2764 0.8735 ± 0.1535 1.000 ± 0.1722 1.055 ± 0.0918	– 0.7419 – 0.8034	
3I	WT (p-SSH1) = 9 animals KO (p-SSH1) = 7 animals WT (SSH1) = 9 animals KO (SSH1) = 7 animals	1.000 ± 0.0783 0.8557 ± 0.1082 1.000 ± 0.1692 1.079 ± 0.2167	– 0.2863 – 0.7750	

3J	WT (F/G actin) = 9 animals KO (F/G actin) = 10 animals	1.000 ± 0.1697 1.054 ± 0.2098	– 0.9599	
4A	WT (p-Cofilin) = 13 animals KO (p-Cofilin) = 9 animals WT (Cofilin) = 13 animals KO (Cofilin)= 9 animals	1.000 ± 0.0925 1.052 ± 0.1864 1.000 ± 0.0933 1.295 ± 0.1668	– 0.7860 – 0.1097	