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Supplemental Information

Phenotypic Screen with TSC-Deficient Neurons Reveals Heat-Shock Machinery as a Druggable Pathway for mTORC1 and Reduced Cilia

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Figure S1

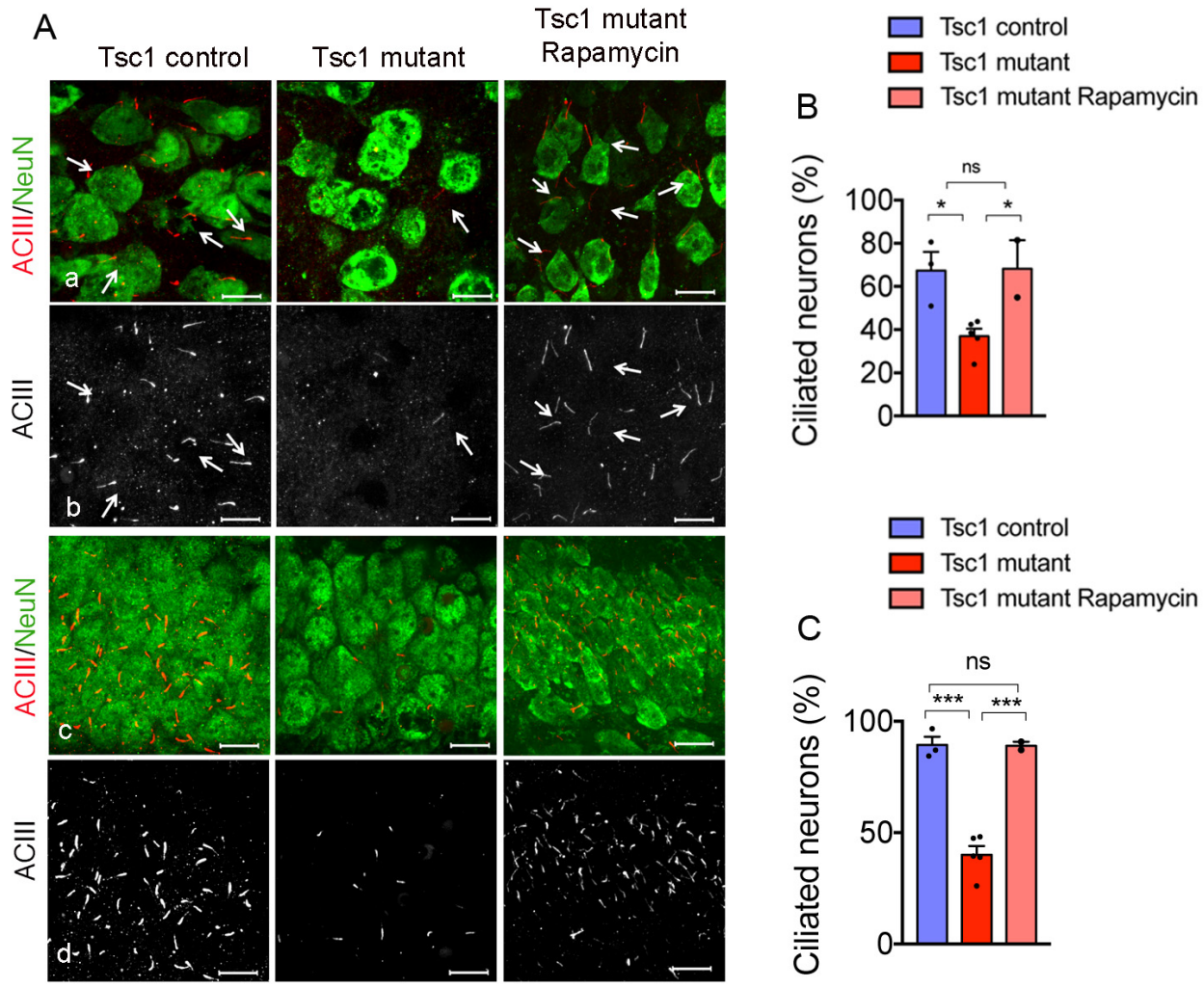

Figure S1. *Tsc1* mutant mice have reduced ciliation in the cortex and in the dentate gyrus. Related to Figure 1. (A) Representative confocal images from the cortex (a-b) and the dentate gyrus (c-d) of vehicle-treated *Tsc1* control *(n=3),* vehicle-treated *Tsc1* mutant *(n=5),* and rapamycin-treated *Tsc1* mutant *(n=2),* stained with the neuronal marker, NeuN (in green in *a* and *c*) and with the cilia marker ACIII (in red in a and c; in greyscale in b and d). White arrows indicate cilia. Quantification of the percentage of ciliated neurons in the cortex (B) and in the dentate gyrus (C) (700 neurons/mouse, one-way ANOVA with Tukey's *post hoc* test, *$p<0.05$). Bars represent mean ± SEM. Scale bar is 10μm.

Figure S2

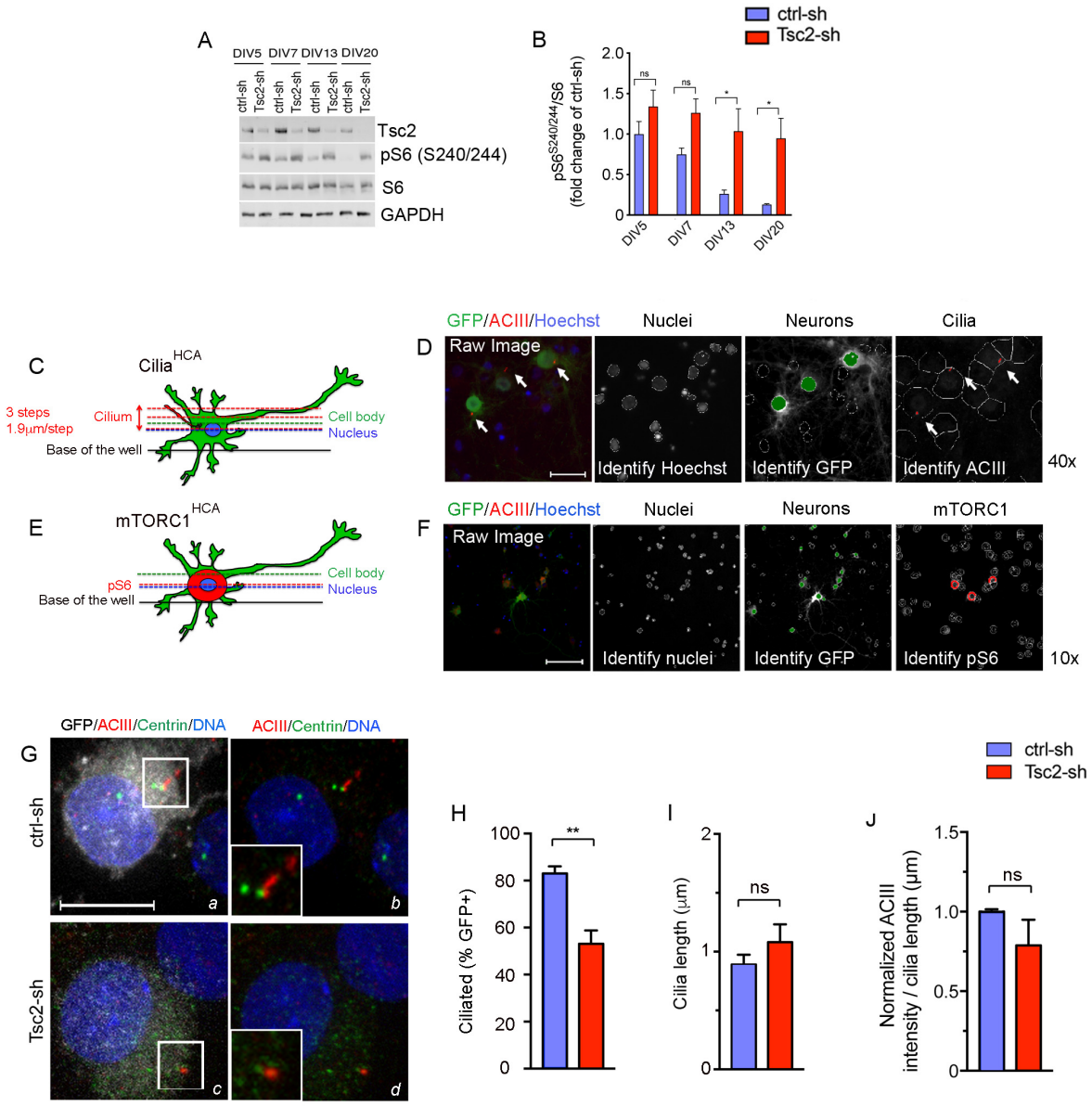

Figure S2. *Tsc2* gene knockdown in hippocampal neurons results in reduced ciliation by high content imaging and manual confocal microscopy. Related to Figure 2.

(A) Representative western blot of protein lysates from ctrl-sh, and Tsc2-sh neurons to monitor Tsc2 and S6 phosphorylation in a time course experiment at DIV5, 7, 13, 20. (B) Quantification of pS6 (n=4). Western blot data were normalized using GAPDH as loading control. Data are expressed as fold changes of vehicle-treated ctrl-sh neurons (Mann-Whitney Test, *$p<0.05$). Error bars indicate ± SEM. (C-F) Image-based high-content assays for cilia and mTORC1 using the Arrayscan XTi. Schematic representation of focal planes and object identification used for the cilia^{HCA} (C-D) and the mTORC1^{HCA} (E-F). Raw images of hippocampal neurons transduced with GFP-tagged lentivirus show nuclei (Hoechst staining in blue), infected neurons (GFP staining in green), cilia and mTORC1 activity in red [ACIII staining (D) and pS6 staining (F) respectively]. Image acquisition for the cilia^{HCA} and the mTORC1^{HCA} was performed with respectively a 40x (scale bar is 25µm) and a 10x (scale bar is 100µm) objective. White masks show the region of interest (ROI) for object identification. (G) Representative images of cilia imaging in ctrl-sh and Tsc2-sh neurons using confocal microscopy. Neurons at DIV13 were stained with GFP (in grey in *a* and *c*), ACIII (in red in *a-d*), centrin (in green in *a-d*) and DAPI (in blue in *a-d*). Images on the left (*a* and *c*) include GFP staining to show the LV-transduced neurons and a white box indicating the regions enlarged for cilia and centrin staining magnified in the zoom (in *b* and *d*). Scale bar is 10µm. (H) Quantification of the percentage of GFP$^+$ neurons with cilia. Bars represent mean ± SEM (n=3, 88-100 GFP$^+$ neurons/exp; unpaired t-test, **$p<0.01$). (I) Cilia length. Bars represent mean ± SEM (unpaired t-test, ns, non-significant). (J) ACIII intensity. Bars represent mean ± SEM (unpaired t-test, ns, non-significant).

Figure S3

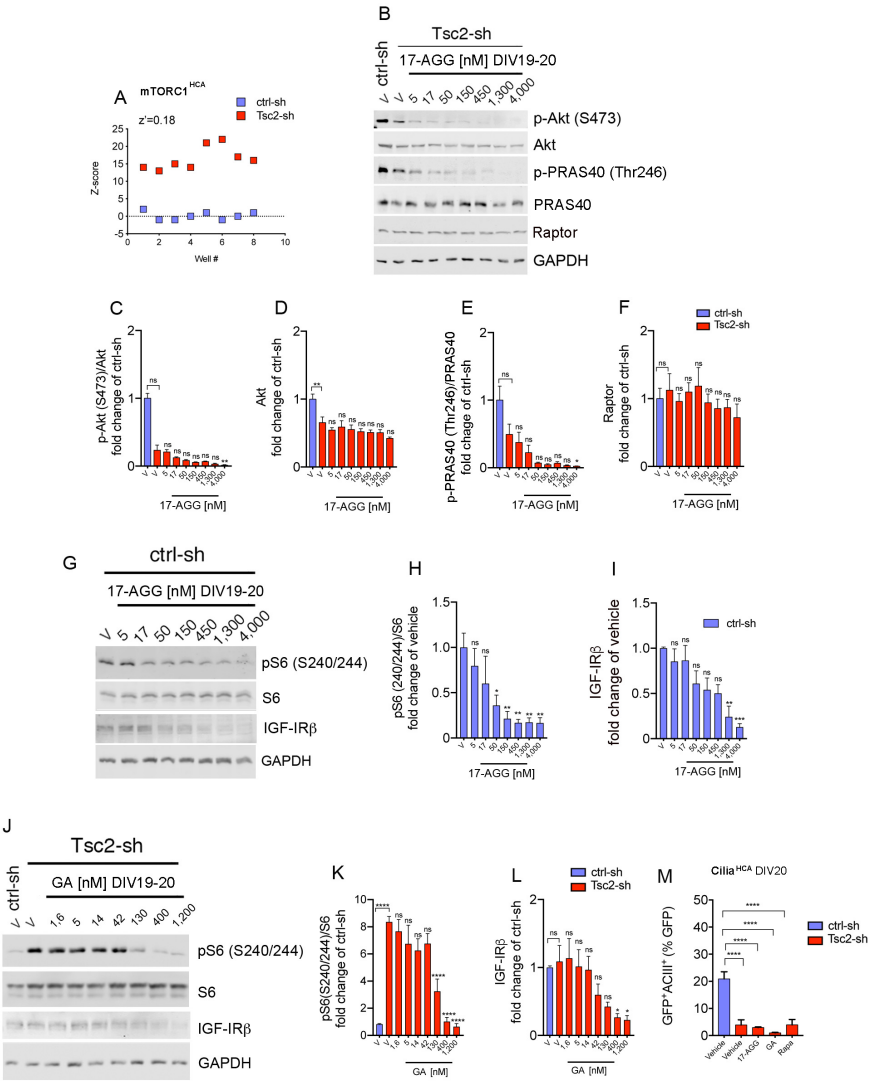

Figure S3. Robustness of mTORC1^{HCA} and effect of the hits identified in the screen on the mTORC1 pathway and on ciliation between DIV19-20. Related to Figure 3.

(A) mTORC1^{HCA} quality control by Z-scores calculated as the absolute deviation of GFP^{+} pS6^{+} neurons between positive (ctrl-sh neurons) and negative (Tsc2-sh) controls at DIV20 (n=8 wells/condition, Z'=0.18). (B) Western blot of protein lysates from ctrl-sh and Tsc2-sh treated with vehicle or with 17-AGG dose curve for 24hrs between DIV19-20. Quantification of p-Akt (Ser473) (C), Akt (D), p-PRAS40 (Thr246) (E), and Raptor (F). Western blot data were normalized using GAPDH as loading control. Data are fold changes of ctrl-sh neurons and are quantified relative to vehicle-treated Tsc2-sh neurons (n=4 experiment/condition). Kruskal-Wallis test followed by the Dunn's multiple comparison test *p<0.05, **p<0.01 (C, E), and one-way ANOVA with Dunnett's multiple comparison test **p<0.01 ns=not significant (D, F). (G) Western blot of protein lysates from ctrl-sh treated with vehicle or with 17-AGG dose curve. Quantification of pS6 (H), and IGF-IRβ (I). Western blot data were normalized using GAPDH as loading control. Data are fold changes of vehicle-treated ctrl-sh neurons (n=3-4 experiment/condition, one-way ANOVA with Dunnett's multiple comparison test, *p<0.05, **p<0.01, ***p<0.001). (J) Western blot of protein lysates from ctrl-sh and Tsc2-sh treated with vehicle or with GA dose curve. Quantification of pS6 (K, n=3), and IGF-IRβ (L, n=4). Western blot data were normalized using GAPDH as loading control. Data are fold changes of ctrl-sh neurons and are quantified relative to vehicle-treated Tsc2-sh neurons (One-way ANOVA with Dunnett's multiple comparison test, *p<0.05, ****p<0.0001). Error bars indicate ± SEM. (M) Quantification of ciliation using the cilia^{HCA} in ctrl-sh and in Tsc2-sh neurons treated with vehicle, 8.5μM 17-AGG, 9μM geldanamycin (GA) and 5.5μM rapamycin for 24hrs at DIV20 (n=2-4 wells/condition, one-way ANOVA with Sidak's multiple comparison test, ****p<0.0005).

Figure S4

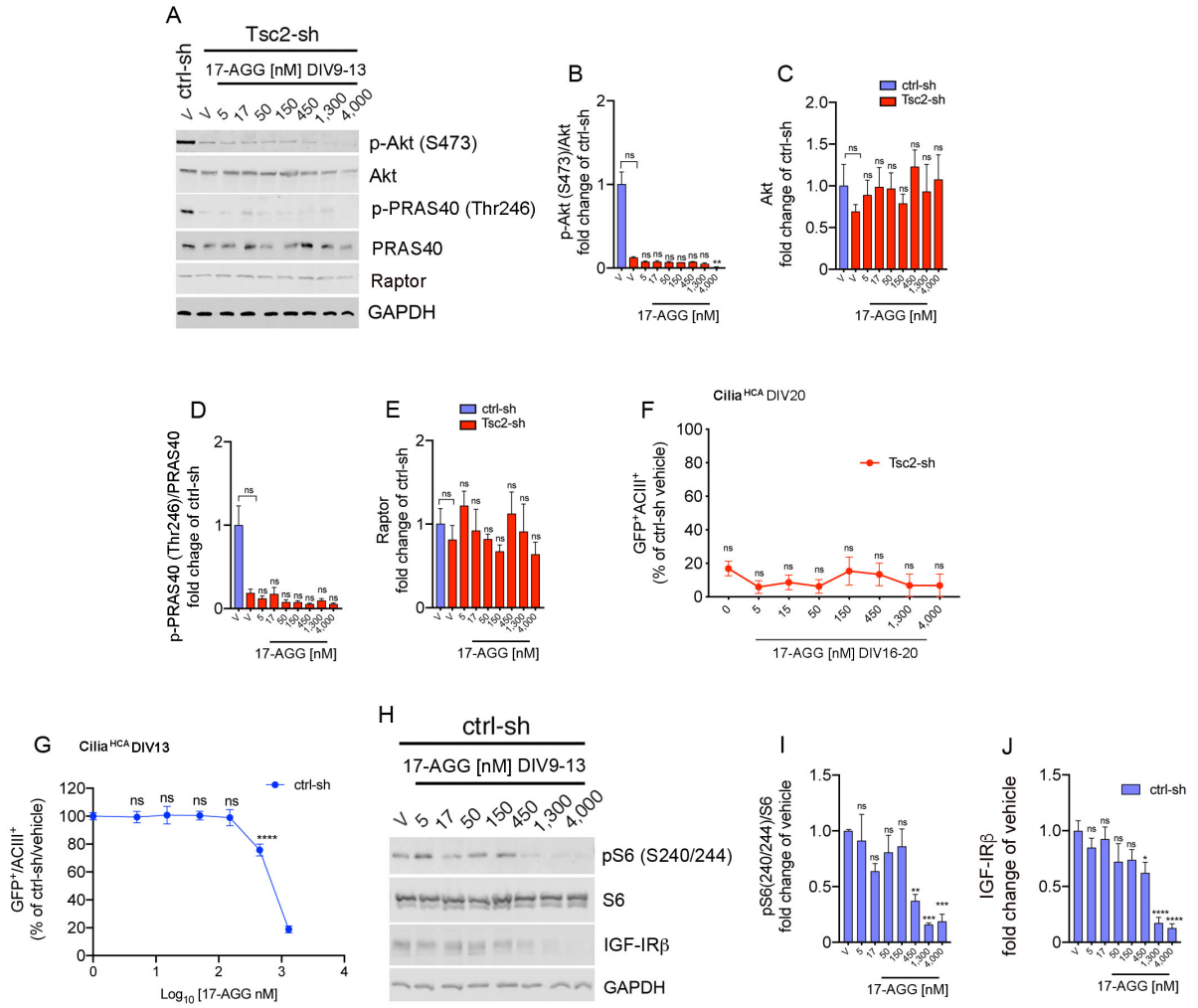

Figure S4. Effect of Hsp90 inhibition with 17-AGG on PI3K/Akt signaling in Tsc2-knockdown neuronal cultures at DIV13. Related to Figure 4.

(A) Western blot of protein lysates from ctrl-sh and Tsc2-sh treated with vehicle or with 17-AGG dose curve. Quantification of p-Akt at S473 (B), Akt (C), p-PRAS at Thr246 (D), and Raptor (E). Western blot data were normalized using GAPDH as loading control. Data are fold changes of ctrl-sh neurons and are quantified relative to vehicle-treated Tsc2-sh neurons (n=4 experiment/condition, Kruskal-Wallis test followed by Dunn's multiple comparison test **p<0.01 (B, D) and one-way ANOVA with Dunnett's multiple comparison test (C, E), ns=not significant. Error bars indicate ± SEM. (F) Quantification of ciliation using the cilia^{HCA} in Tsc2-sh neurons treated with a four-day 17-AGG dose curve between DIV16-20 with assay endpoint at DIV20. Data quantification was done relative to vehicle-treated Tsc2-sh neurons (n=8-16 biological replica/condition, one-way ANOVA with Dunnett's multiple comparison test, not significant). (G) Quantification of ciliation using the cilia^{HCA} in ctrl-sh neurons treated with a four-day 17-AGG dose curve between DIV9-13 with assay endpoint at DIV13. Data were quantified relative to vehicle-treated ctrl-sh neurons (n=16-32 biological replica/condition, one-way ANOVA with Dunnett's multiple-comparison test, ****p<0.0001) and are expressed as average ± SEM. (H) Western blot of protein lysates from ctrl-sh treated with vehicle or with 17-AGG dose curve. Quantification of pS6 (I), and IGF-IRβ (J). Western blot data were normalized using GAPDH as loading control. Data are fold changes of vehicle-treated ctrl-sh neurons (n=4-8 experiment/condition, one-way ANOVA with Dunnett's multiple comparison test, ***p<0.001, ****p<0.0001). Error bars indicate ± SEM.