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Supplemental Information

Characterization of a Mouse Model of Börjeson-Forssman-Lehmann Syndrome

Cheng Cheng, Pan-Yue Deng, Yoshiho Ikeuchi, Carla Yuede, Daofeng Li, Nicholas Rensing, Ju Huang, Dustin Baldridge, Susan E. Maloney, Joseph D. Dougherty, John Constantino, Arezu Jahani-Asl, Michael Wong, David F. Wozniak, Ting Wang, Vitaly A. Klyachko, and Azad Bonni

Supplemental information

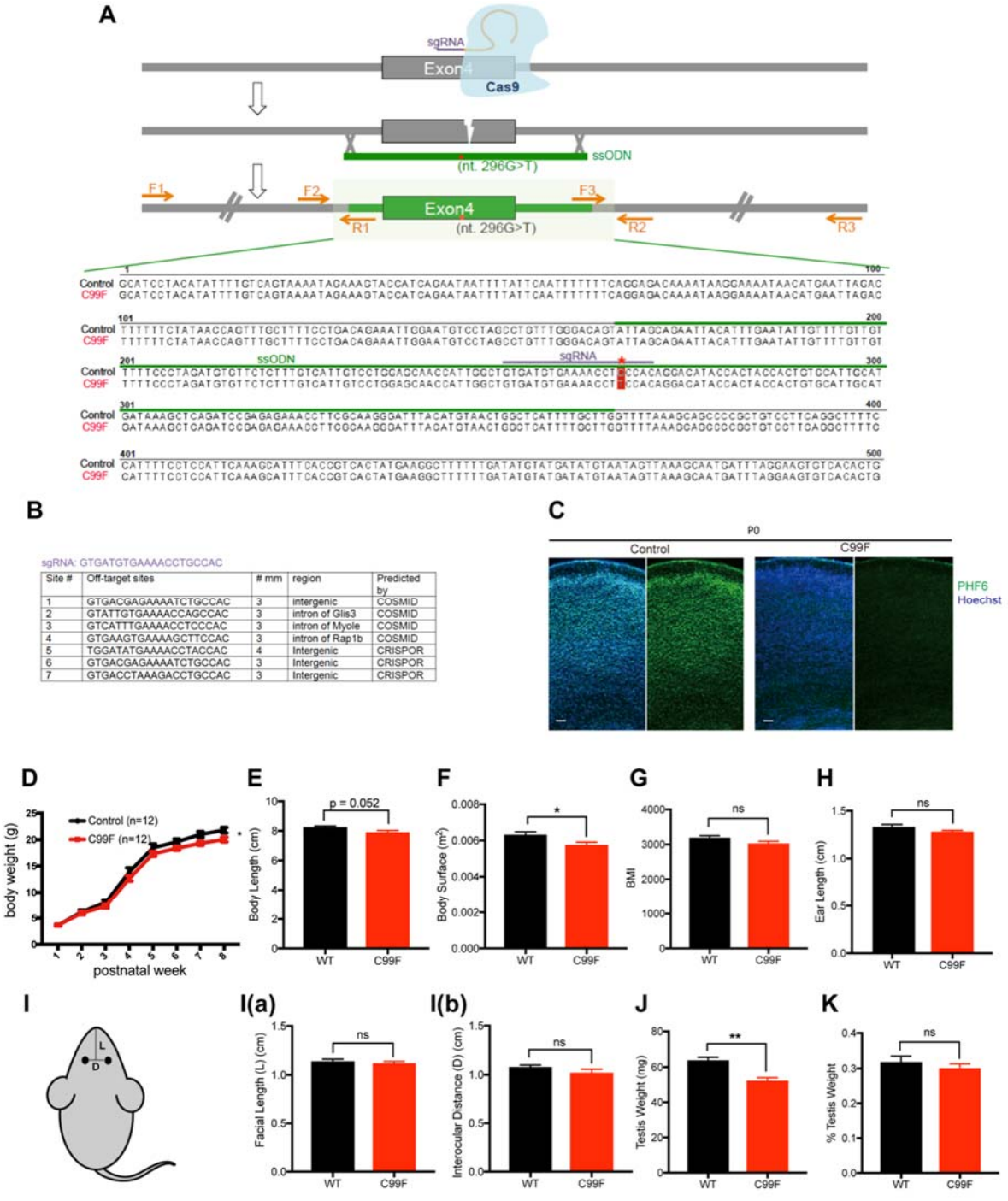

Figure S1. Validation of PHF6 C99F knock-in mice. Related to Figure 1.

(A) Schematic of PHF6 C99F knock-in mouse generation using CRISPR-Cas9 technology. Primers labeled in orange were used for Sanger sequencing to detect potential off-target mutations. No off-target mutations were detected.

(B) Potential off-target sites in the genome predicted by COSMID and CRISPOR were subjected to Sanger Sequencing and no mutations were detected.

(C) Sections of the cerebral cortex from control and C99F knock-in mice at postnatal day 0 (P0) were subjected to immunohistochemistry using PHF6 antibody and the DNA dye bisbenzimide (Hoechst). PHF6 expression was reduced in the cortical plate in C99F mice. Scale bar = 50um.
(D) Body weight of PHF6 C99F mice (n = 12) was slightly reduced compared to control littermate mice (n = 12). Two way repeated measure ANOVA, *$p<0.05$.
(E-F) PHF6 C99F knock-in mice (n = 5) displayed trend in body length reduction (E) and reduced body surface area (F) compared to control mice (n = 5). Body surface area was calculated using Du Bois Methods.
(G-H) No differences in BMI (G) and ear length (H) between C99F and control mice (n = 5). BMI was calculated as ratio of body weight and square surface area.
(I) Schematic of facial length (L) and interocular distance (D). No differences in facial length (Ia) and interocular distance (Ib) between C99F and control mice (n = 5).
(J-K) No difference in normalized testicular weight between C99F and control mice (n = 5).
* $p<0.05$, **$p<0.01$. Unless specified, unpaired t-test. Data are presented as mean ± SEM.

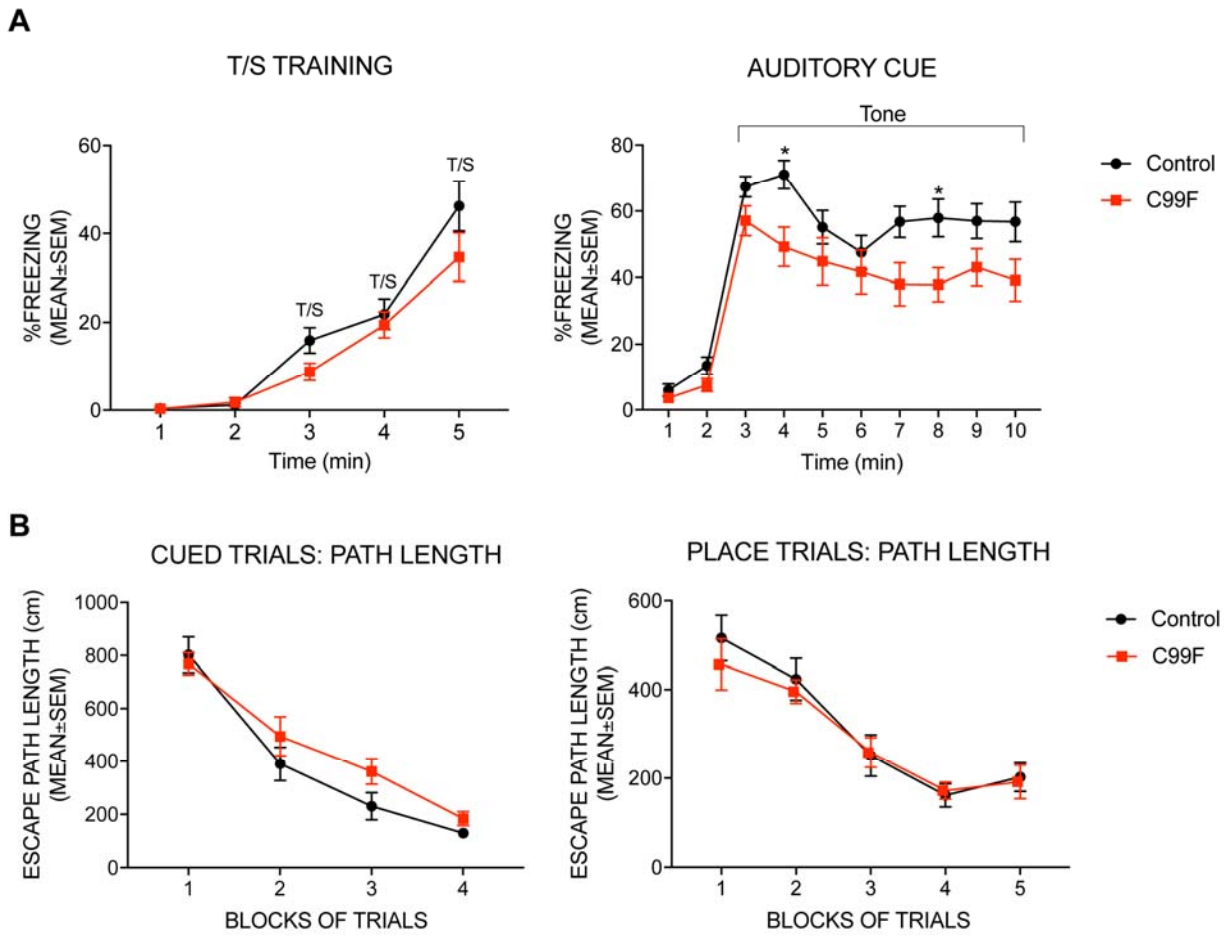

Figure S2. Behavior phenotyping of PHF6 C99F knock-in mice. Related to Figure 2.
(A) Control (n = 12) and PHF6 C99F knock-in mice (n = 12) were tested on the conditioned fear procedure. (Left panel): Freezing levels were not found to be different during either the 2-min baseline period or during tone-shock (CS-US) training on day 1. (Right panel): Freezing levels were also observed to be similar during the 2-min altered context baseline on day 3, although the C99F knock-in mice displayed reduced freezing response compared to controls during the auditory cue test.
(B) No performance differences were observed between the C99F knock-in (n = 12) and control (n = 12) groups for the cued or place (spatial learning) trials in the Morris water maze.
Two-way repeated measure ANOVA, followed by pair-wise comparisons that were Bonferroni adjusted. *p <0.05. Data are presented as mean ± SEM.

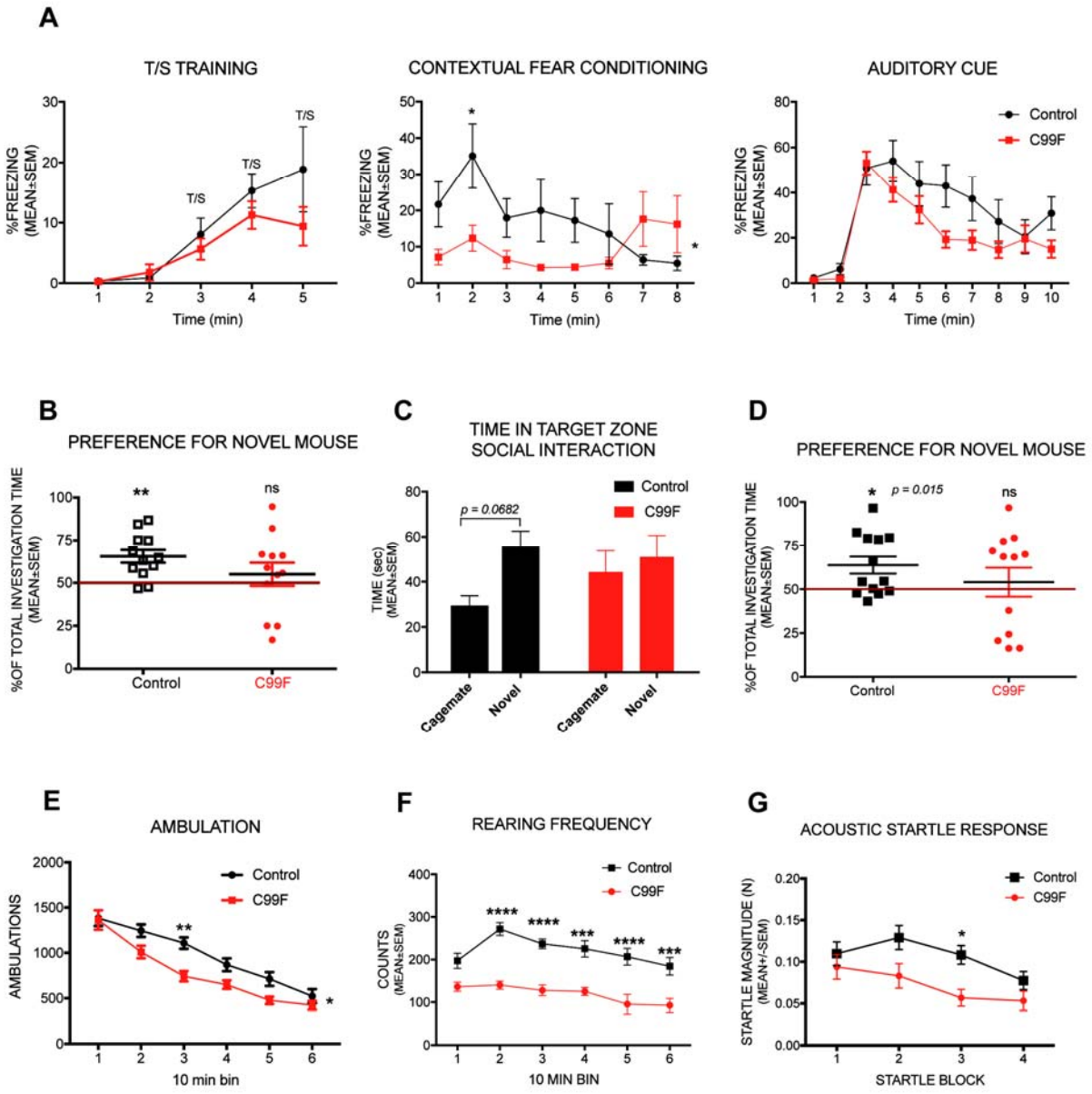

Figure S3. Additional cohort of C99F knock-in mice show deficits in cognition, social interaction and emotionality. Related to Figure 2.

(A) (Left panel) testing the control (n = 11) and PHF6 C99F knock-in mice (n = 11) on the conditioned fear procedure revealed that the two groups displayed similar freezing levels during the 2-min baseline and tone-shock training periods on day 1. (Middle panel) The C99F knock-in mice exhibited reduced freezing levels during the contextual fear test (genotype x minutes interaction: $F(7,140) = 4.34$, $p = 0.014$), particularly during the first two minutes of the test session. (Right panel) Although the C99F knock-in mice generally displayed lower freezing levels compared to controls, no significant overall effects involving genotypes were found for the auditory cue data.

(B) The percentage of time control mice (n = 12), but not *PHF6* C99F knock-in mice (n = 12) from the first cohort, interacted with the novel mice was significantly above chance (**p<0.01, one sample t-test).

(C) Control mice (n = 13), but not C99F knock-in mice (n = 12) from the second cohort displayed an increasing trend in time spent for investigating a novel mouse than a cagemate.

(D) The percentage of time control mice, but not *PHF6* C99F knock-in mice from the second cohort, interacted with the novel mice was significantly above chance (*p<0.05, one sample t-test).

(E) PHF6 C99F knock-in mice (n = 12) displayed reduced ambulatory activities compared to control (n = 13) in the

open field test. *p<0.05 for genotype x time (10 min bins) interaction.
(F) PHF6 C99F knock-in mice (n = 12) displayed reduced rearing frequency compared to control (n = 13) in the open field test.
(G) C99F knock-in mice (n = 12) displayed reduced acoustic startle response compared to control littermate animals (n = 13).
*p<0.05, ***p<0.001, ****p<0.0001, unless specified, two-way repeated measures ANOVA, followed by pair-wise comparisons that were Bonferroni adjusted were used for statistical analysis. Data are presented as mean ± SEM.

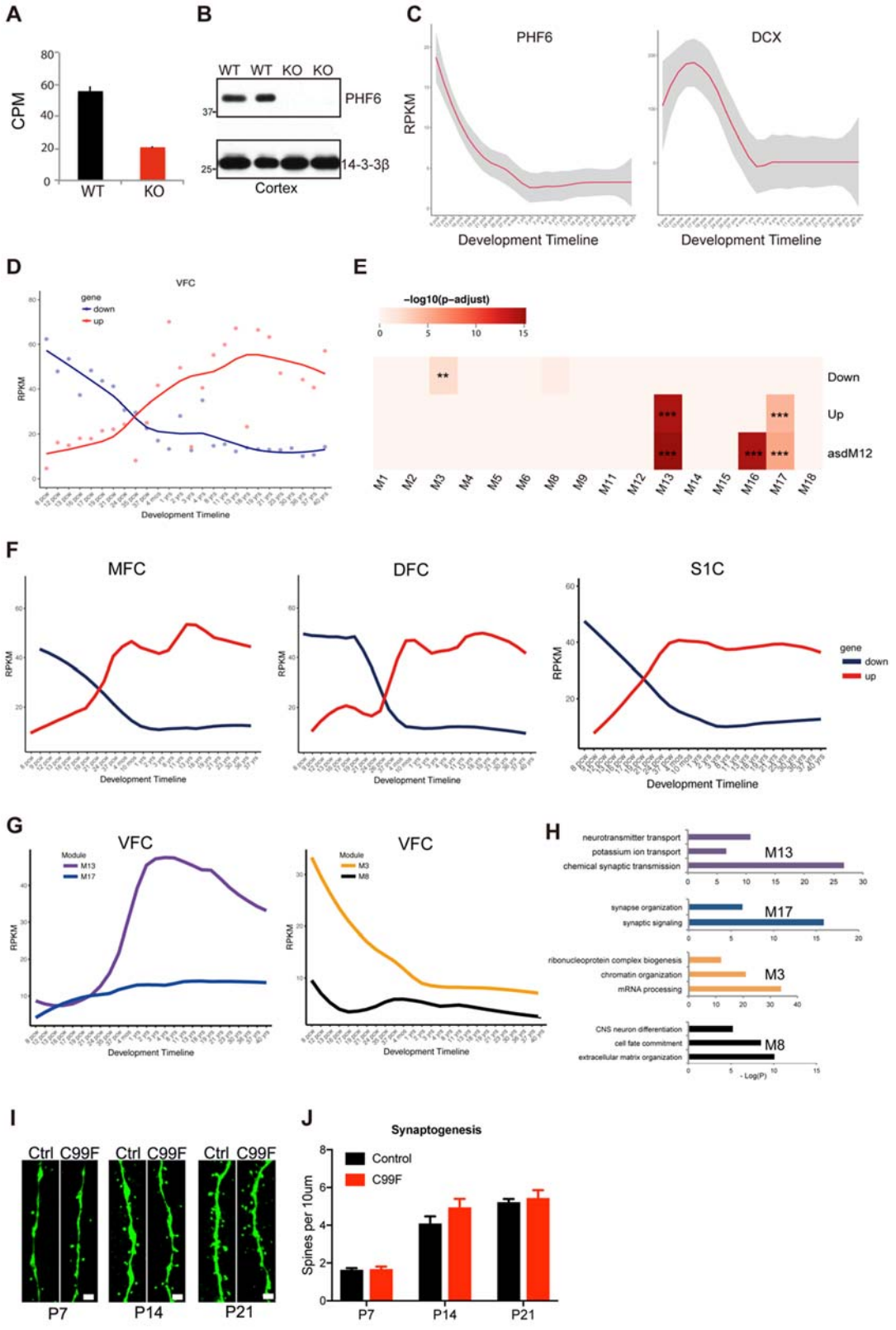

Figure S4. Gene expression in the PHF6 knockout mouse. Related to Figure 4.

(A) The level of PHF6 mRNA in the cerebral cortex of PHF6 knockout mice in RNA-seq analysis (n = 4) was reduced compared to control mice (n = 4). Data are presented as mean ± SEM.

(B) Lysates of the cerebral cortex at P0 from control male and PHF6 knockout male mice were subjected to immunoblotting with the PHF6 and 14-3-3β antibodies. Levels of PHF6 protein were diminished in the cortex of PHF6 knockout mice.
(C) Expression of PHF6 (left) and DCX (right) during development in ventral frontal cortex (VFC) using BrainSpan dataset. PHF6 was developmentally downregulated, and DCX was transiently expressed from post conceptual week 8 (pcw8) to pcw24.
(D) Average expression of downregulated and upregulated genes upon PHF6 knockout discovered by DEseq2 was plotted across developmental time in the VFC based on human BrainSpan data. Downregulated genes upon PHF6 loss were also developmentally downregulated, whereas upregulated genes upon PHF6 loss were developmentally upregulated.
(E) Enrichment analysis of misregulated genes upon PHF6 knockout discovered by DEseq2 and the asdM12 gene module with previously described developmental gene coexpression network modules (Parikshak et al., 2013). Upregulated genes upon PHF6 knockout coalesced with autism gene modules. Enrichment analysis was tested by hypergeometric-test, followed by Benjamini-Hochberg multiple testing corrections. **p.adjusted <0.01, ***p.adjusted <0.001.
(F) Average expression of up- and down-regulated genes upon PHF6 knockout in medial frontal cortex (MFC), dorsal frontal cortex (DFC) and primary somatosensory cortex (S1C). Downregulated genes upon PHF6 knockout were developmentally downregulated, and upregulated genes upon PHF6 knockout were upregulated throughout development in these brain regions.
(G) Average expression of genes in developmental modules M3, M8, M13 and M17 was plotted across developmental time in the VFC based on human BrainSpan dataset.
(H) Genes from developmental modules including modules M3, M8, M13 and M17 were subjected to gene ontology analysis using Metascape.
(I) Layer II stellate neurons in entorhinal cortex were subjected to biocytin injection. Spine density from P7-9, P14-16 and P21-25 were analyzed. C99F neurons had little or no effect in synaptogenesis. Scale bar = 2um.
(J) Quantification of spine density at P7-9, P14-16 and P21-25 from C99F and control stellate neurons.

Table S1. Primers for potential CRISPR off-target sites. Related to Figure 1.

*Site #	F primer	R primer
1	TTGGGAGCTTGTGGGACCTG	CTTGGCTCCTGATGACGGCA
2	TTTGGAAACTTTCCGGTTGGGGG	GGCACTTGCTGTCCATAATGCTG
3	GGAAGTGGTCCATGCCACTG	GCCAAAGCTCGTTCTGAAGTGATG
4	CTTTAACCAGGTACTAAGTGTGACAG TGTG	GGAATGGACGTGGCCTTTAAAGC
5	GTTGGCAACCCAGAGAGACA	ACCACCAGTGTTGTGTCCAG
6	ACCTGAGTAACCAAGTCCACAC	TTACAGCAGACCTCTAGGGATT
7	CCACTGTGGTTAGACTCCCTC	AGACATGCCTGTAGCAGACT
Up F1/R1	GAACAAGGCGTTTGGCAACCAGG	GGAGCTGAGCTAGATACAACTG
Mid F2/R2	GTATCCAGCAAGTTCTGGATTGC	GGAAAAACTTAGTAGAGGACTAC
Down F3/R3	CTGCACATAACTCGGAAGGTATG	GGTACACAGACATACCCGCAGG

*Site #1-7 were the corresponding sites from Figure S1b. Up F1/R1, Mid F2/R2 and Down F3/R3, labeled in orange in figure S1a were primers used for sequencing ~1kb flanking C99F mutation.