Supplemental Methods & Figures

Temporal manipulation of *Cdkl5* reveals essential post-developmental functions and reversible CDKL5 deficiency disorder-related deficits

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Behavioral Assessments

Elevated zeromaze. The elevated zeromaze (San Diego Instruments; California, USA) consists of a circular-shaped platform elevated 3 feet above the floor. Two opposite quadrants of the maze are enclosed (wall height, 12 inches), whereas the other two are open (wall height, 0.5 inches). Mice were placed in one of the closed quadrants and their movement traced over the course of 5 min. Analysis, including the quantification of percent of time spent in open arms and the number of entries, was performed manually using a stopwatch. An entry was defined as a transition from a closed to open arm, or vice versa, that involves all four paws. Total distance traveled over the entire assay was measured by a ceiling-mounted camera coupled to a video-tracking software (SmartScan 3.0), allowing for real-time analysis of all movements.

Open-field test. Locomotor activity was measured via an open-field test where mice were individually placed into, and allowed to explore, a 15" x 15" arena for a total of 60 min. A ceiling-mounted camera allowed for a video-tracking software (SmartScan 3.0) to real-time analyze the total distance traveled as well as the percent time spent in the center of the arena (defined as the central 25% of the total area).

Y-maze. Spontaneous alternation behavior was measured on a Y-maze apparatus (San Diego Instruments; California, USA), composed of three arms (Arm A: 8in. x 5in. x 3in.; Arms B and C: 6in. x 5in. x 3in.). For testing, the mouse was placed in Arm C, facing the center, and allowed to freely explore the maze for 5 min. A spontaneous alternation was defined an entry into the arm less recently explored. Percent spontaneous alternation was calculated as the number of spontaneous alternations over the total number of entries. For example, the sequence C,B,A,B,C,B,A,C (starting in arm C) resulted in a percent spontaneous alternation of 4/6 = 67%.

Three-chambered social approach assay. The social choice test was carried out in a three-chambered apparatus that consisted of a center chamber and two end chambers. Before the start of the test and in a counter-balanced sequence, one end chamber was designated the social chamber, into which a stimulus mouse would be introduced, and the other end chamber was designed the nonsocial chamber. Two identical, clear Plexiglas cylinders with multiple holes to allow for air exchange were placed in each end chamber. In the habituation phase of the test (Phase I), the test mouse was placed in the center chamber and allowed to explore all three chambers for 10 min. During this acclimation period, baseline measurements of how much time the mouse spent in each of the three chambers and the distance traveled by the test mouse were collected. In the social choice phase of the test (Phase II), an age-matched stimulus mouse (adult, gonadectomized A/J mice) was placed in the cylinder in the social chamber while a novel object was simultaneously placed into the other cylinder in the nonsocial chamber. During the subsequent 10 min social choice period, chamber times and numbers of transitions among chambers were again recorded as well as the percent time spent sniffing the social cylinder. In the direct social interaction test, the cylinders were removed simultaneously following the social choice test, and the amount of time test and stimulus mice spent in direct contact (sniffing, allogrooming) was measured for a total of 5 min. If fighting persisted for more than several seconds, the mice were removed from the apparatus and excluded from the study.

Accelerating rotarod assay. Mice were placed on an accelerating rotarod apparatus (Harvard Apparatus) for 16 trials (four trials a day for four consecutive days) with at least 15 min of rest between the trials. Each trial lasted for a maximum of 5 min, during which the rod accelerated linearly from 4 to 40 rpm. The amount of time for each mouse to fall from the rod was recorded for each trial.

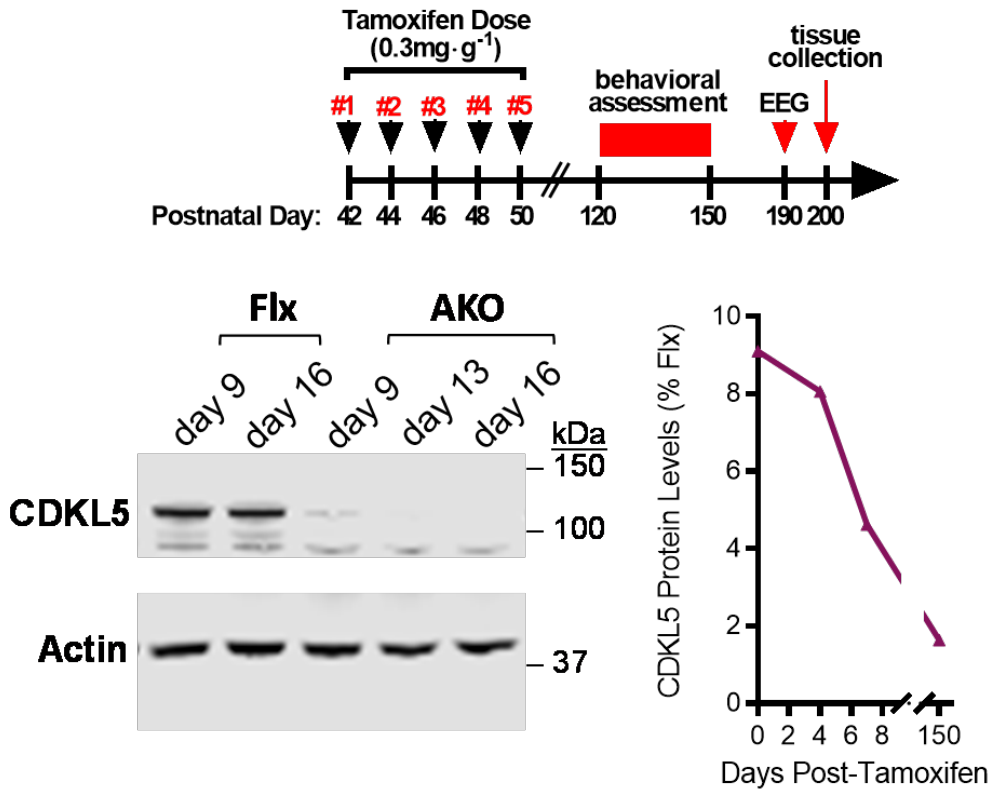
Context- and cue-dependent fear conditioning. For the training day, mice were placed in individual chambers (Med Associates) for 2 min followed by a loud tone (85 dB, 2 kHz) lasting 30 s that co-terminated with a 2-s, 1.25-mA foot shock. Mice were left undisturbed for an additional 30 s in the chamber and then immediately placed back into their home cage. Freezing behavior, defined as no movement except for respiration, was determined before and after the tone-shock pairings and scored by FreezeScan NI version 2.00. To test for

context-dependent learning, we placed mice back into the same testing boxes 24 hr later for a total of 5 min without any tone or shock, and again measured the total time spent freezing. After 4 hrs, we tested for cue-dependent fear memory by placing the mice into a novel chamber consisting of altered flooring, wall-panel inserts, and vanilla scent. After 2 min in the chamber, the cue tone (85 dB, 2 kHz) was played for a total of 3 min, and the total time spent freezing during the presentation of this cue tone was recorded.

Olfaction. Mice were tested for whether they could detect and differentiate odors in a habituation-dishabituation protocol modified from Yang and Crawley[50]. Mice were presented with cotton-tipped wooden applicators dipped in either water, vanilla, or swiped across the bottom of an unfamiliar social cage. Each stimulus was presented for 2 min with a 1-min inter-trial interval. Time spent sniffing was defined as when the animal was oriented with its nose 2 cm or closer toward the cotton tip.

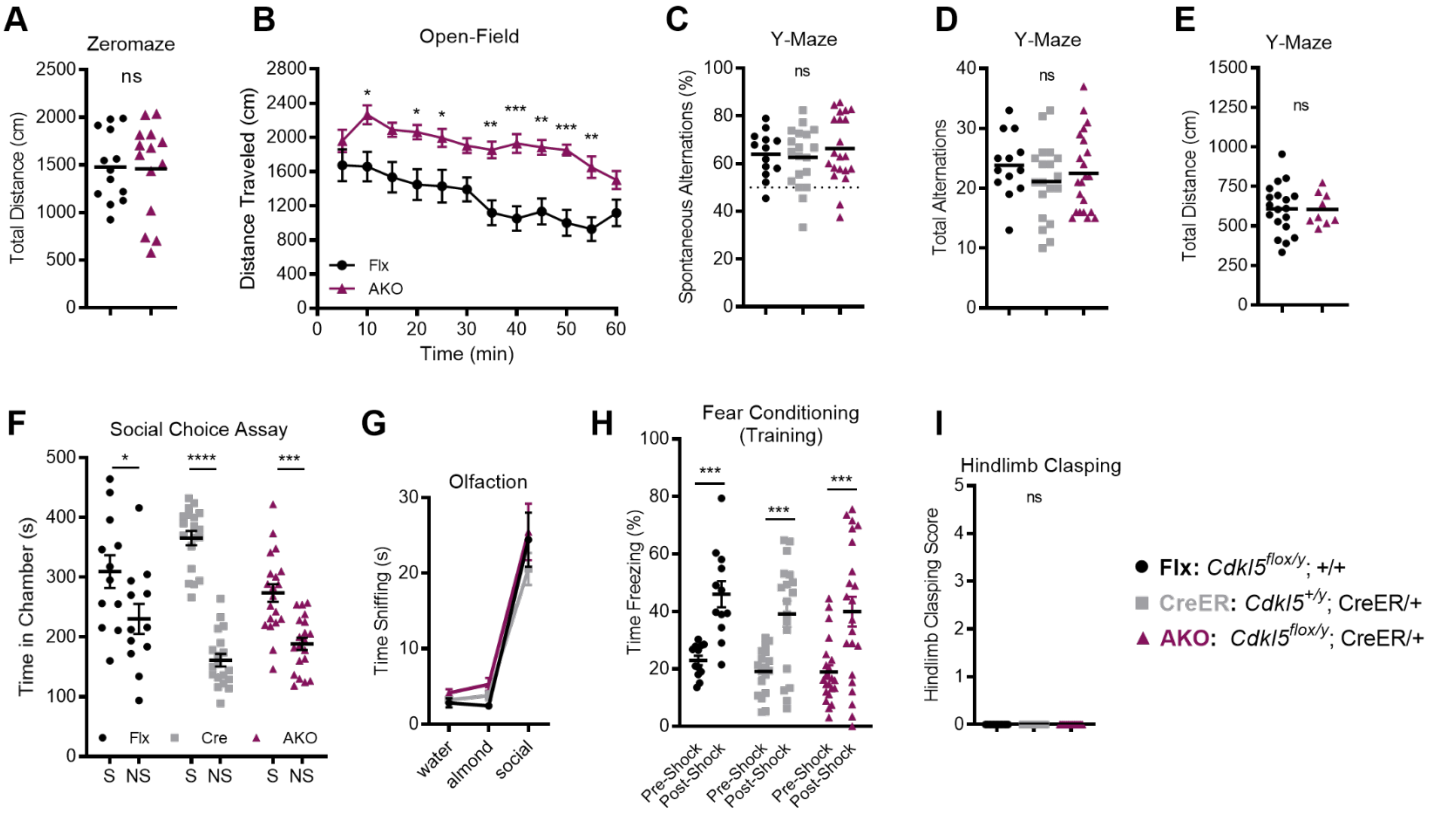
Repetitive behavior. Mice were individually placed into a clean, home-cage like environment lined with bedding. After allowing 5 min for habituation, 10 min of activity was videotaped for each mouse. The duration of repetitive behavior, defined as grooming or digging, was scored manually using a stopwatch.

Hindlimb clasping. Mice were suspended by the base of their tail at least 6 inches above a flat surface for up to 1 min. If the hindlimbs were consistently splayed outward, away from the abdomen, the mouse was assigned a score of 0. If one hindlimb was retracted towards the abdomen for more than 50% of the time, the score was 1. If both hindlimbs were partially retracted for greater than 50% of the time, the score was 2. Finally, if both hindlimbs were entirely retracted and touching the abdomen for more than 50% of the time suspended, the mouse received a score of 3.



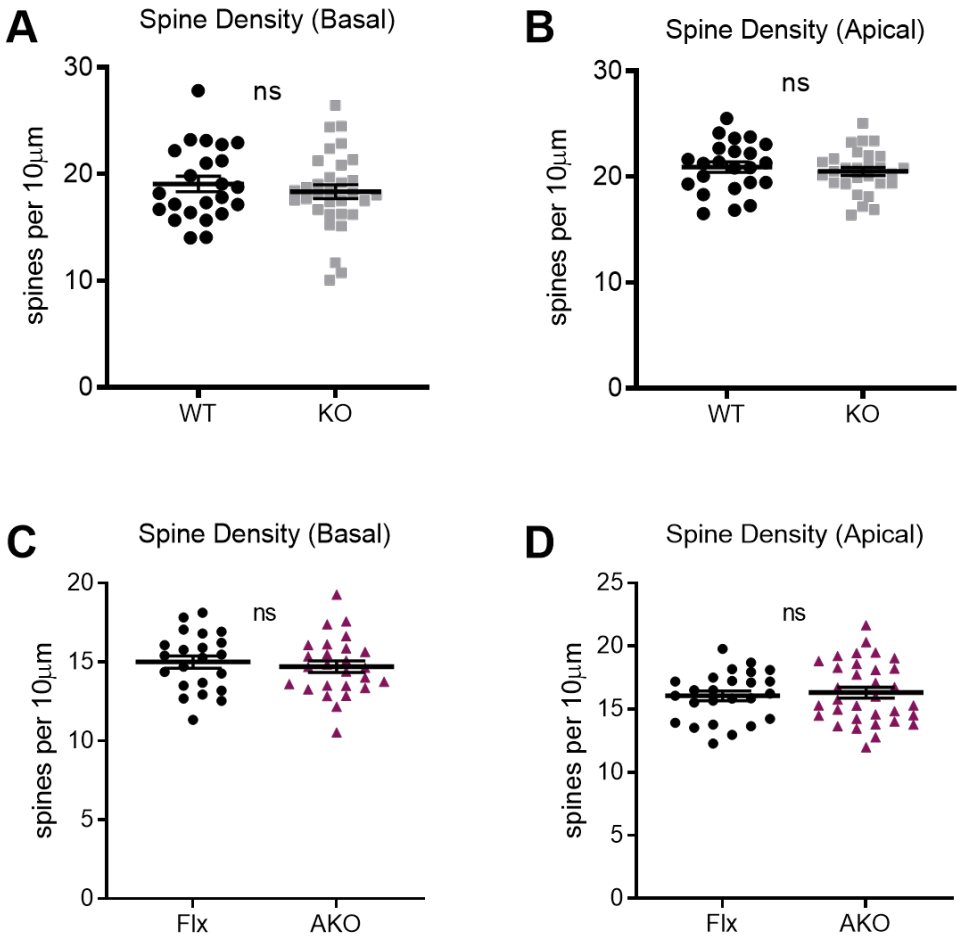
Supplemental Figure 1. Time course of CDKL5 protein knockdown with tamoxifen

Top: tamoxifen administration scheme and experimental schedule; Bottom: representative western blot results and quantification demonstrating significant reduction in CDKL5 protein in forebrain tissues of *Cdkl5*$^{flox/y}$; CreER/+ (AKO) mice compared to *Cdkl5*$^{flox/y}$; +/+ (Flx) littermate controls after tamoxifen administration. Forebrain tissues were collected and analyzed for CDKL5 protein 9, 13 and 16 days after the first tamoxifen dose, and all experimental mice were sacrificed after behavioral assessment for post-hoc protein quantification. Actin protein was a loading control, and AKO CDKL5 protein was normalized to Flx levels. Full-scan western blots of all samples are available in Supplemental Materials.



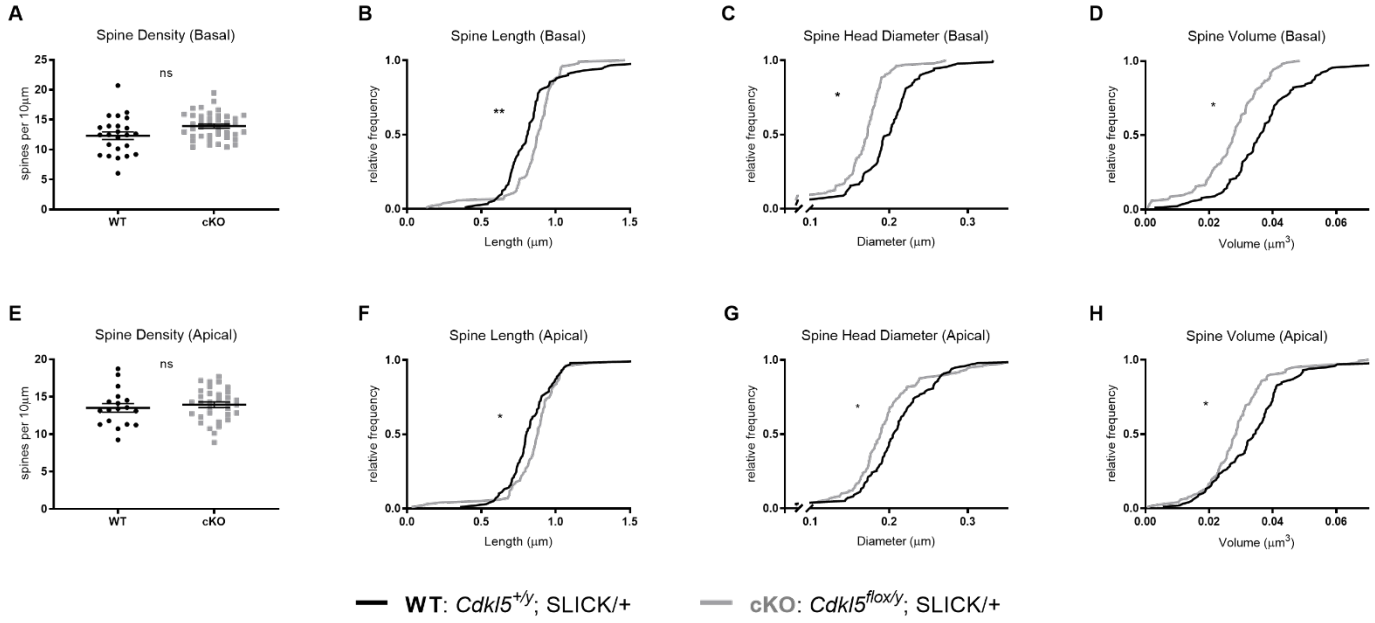
Supplemental Figure 2. Additional behavioral domains analyzed upon post-developmental deletion of *Cdkl5*

(A) Total distance traveled in the elevated Zeromaze assay was similar between AKO and Flx mice (unpaired *t* test). **(B)** AKO mice traveled significantly more in an open-field assay relative to Flx littermates over the course of one hour (distance traveled binned in 5min intervals; two-way, repeated measures ANOVA with Sidak's multiple comparisons test). **(C)** AKO mice showed no difference in percentage of spontaneous alternations in a Y-Maze assay, and no difference in the **(D)** total number of alternations or **(E)** total distance traveled when compared to Flx and CreER littermate controls (One-way ANOVA). **(F)** Flx, CreER, and AKO mice all spent significantly more time in the chamber containing a social stimulus (S; novel mouse) over a non-social stimulus (NS; rock) during the 3-chambered social choice test (paired t test). **(G)** Flx, CreER, and AKO mice all spent significantly more time sniffing a social scent over either water or almond scents, with no significant difference detected between genotypes (two-way ANOVA with Dunnett's multiple comparisons test). **(H)** Flx, CreER, and AKO mice all significantly increase the percent time they spend freezing in respond to a mild footshock during a Pavlovian fear conditioning training paradigm (paired t test). **(I)** No Flx, CreER, or AKO animals displayed any hindlimb clasping phenotypes (One-way ANOVA). For all panels: Flx, n=13; CreER, n=19; AKO, n=23 where all genotypes received tamoxifen; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.



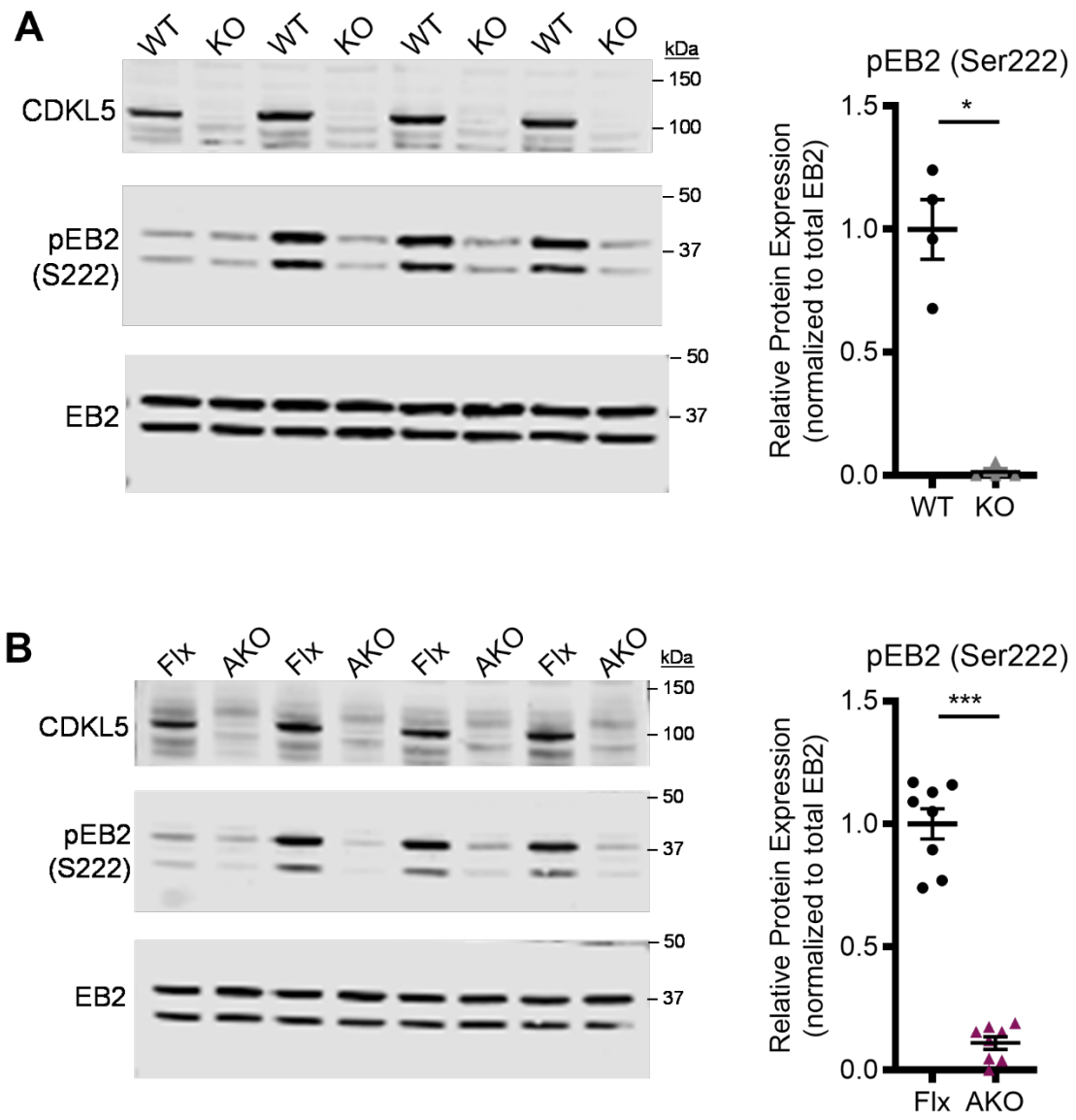
Supplemental Figure 3. Germline or post-developmental deletion of *Cdkl5* does not alter dendritic spine density on hippocampal CA1 neurons

(A) *Cdkl5* germline knock-in mice (*Cdkl5*$^{R59X/y}$; Thy1-GFPm/+), labelled as KO here, show no significant difference in spine density compared to wild-type littermate controls (*Cdkl5*$^{+/y}$; Thy1-GFPm/+), WT, on either (A) basal or **(B)** apical dendritic arbors of hippocampal CA1 pyramidal neurons (Basal: n=23 cells/5 mice for WT; n=32 cells/5 mice for KO. Apical: n=23 cells/5 mice for WT; n=28 cells/5 mice for KO). **(C)** *Cdkl5*$^{flox/y}$; CreER/+; Thy1-GFPm/+ (AKO) mice similarly show no significant change in spine density compared to *Cdkl5*$^{flox/y}$; +/+; Thy1-GFPm/+ (Flx) littermate controls on either (C) basal or **(D)** apical dendritic arbors of hippocampal CA1 pyramidal neurons (Basal: n= 22 cells/8 mice for Flx, n= 30 cells/7 mice for AKO. Apical: n=25 cells/8 mice for Flx; n=36 cells/7 mice for AKO). Linear mixed effects analysis.



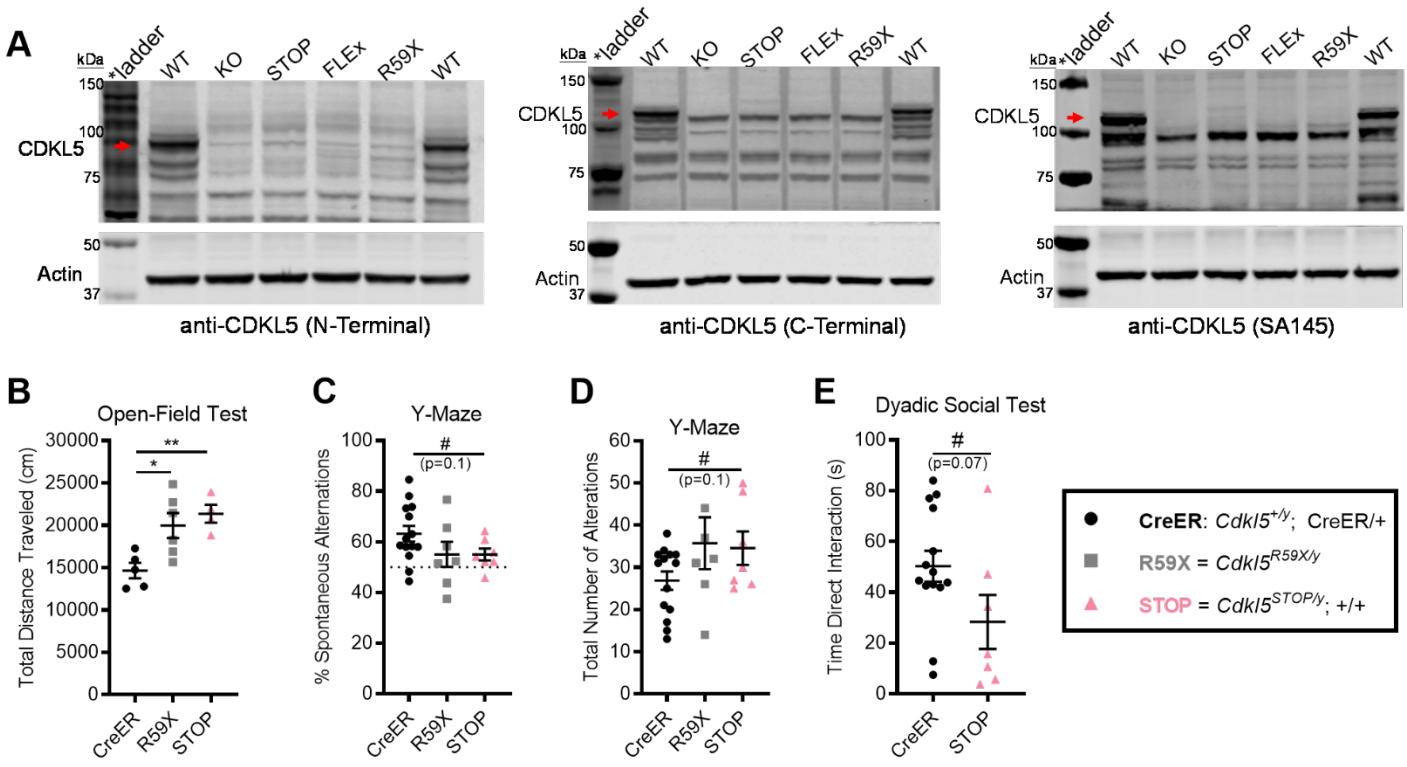
Supplemental Figure 4. Loss of CDKL5 disrupts hippocampal CA1 dendritic spine morphology in a cell-autonomous manner

(A) *Cdkl5*$^{flox/y}$; SLICK/+ (cKO) mice show no significant change in spine density on (A) basal or **(E)** apical dendritic arbors of CA1 pyramidal neurons compared to *Cdkl5*$^{+/y}$; SLICK/+ (WT) littermate control neurons. cKO neurons have increased spine length compared to WT controls on both **(B)** basal and **(F)** apical dendritic arbors as well as significantly reduced head diameter [**(C)** basal; **(G)** apical] and reduced volume [**(D)** basal; **(H)** apical]. Basal: n= 24 cells/6 mice for WT, n= 40 cells/8 mice for cKO; Apical: n=19 cells/6 mice for WT, n=33 cells/8 mice for cKO. Linear mixed effects analysis. *p<0.05, **p<0.01.



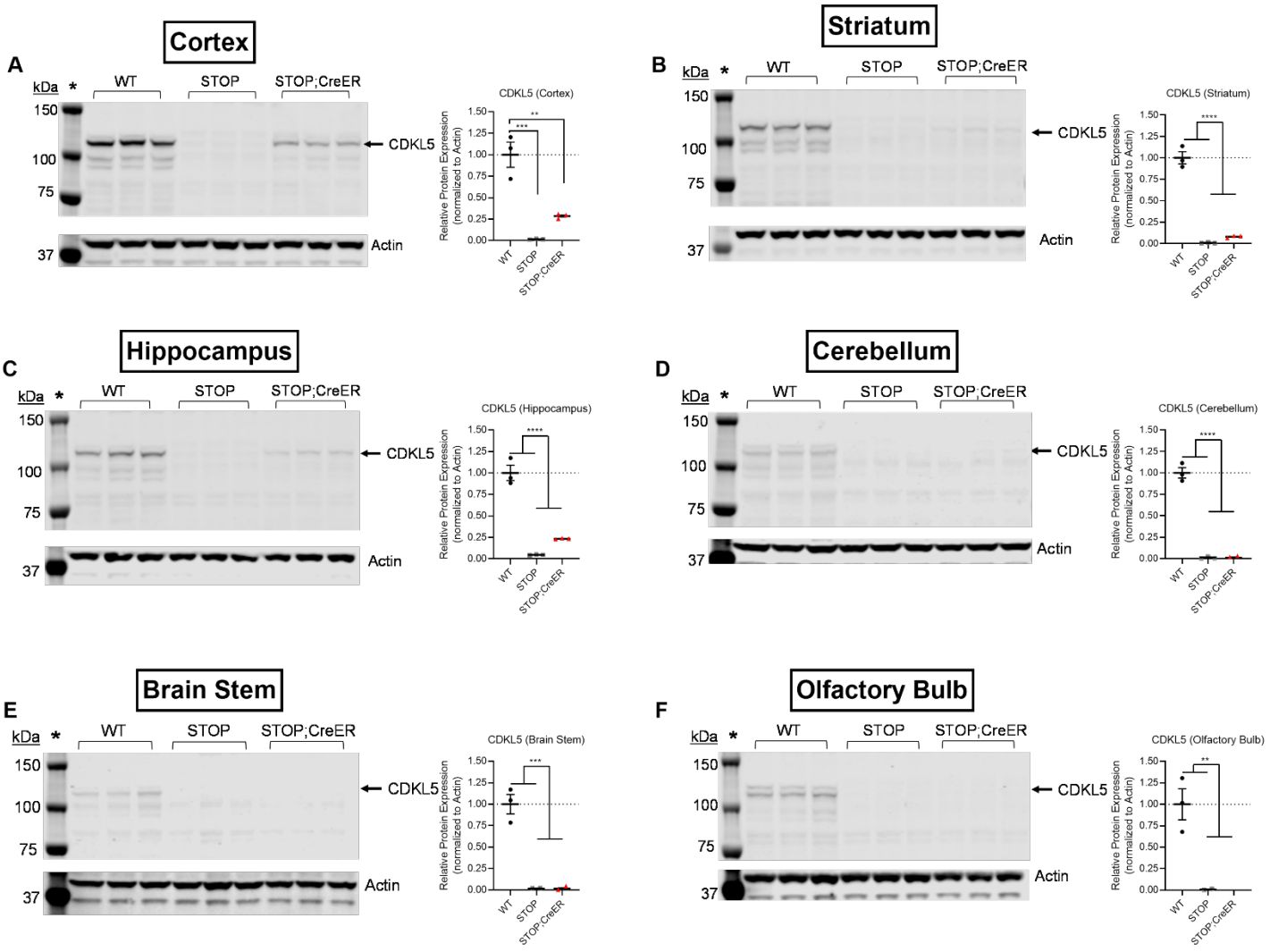
Supplemental Figure 5. Germline and post-developmental deletion of *Cdkl5* abrogate EB2 phosphorylation

(A) Left: representative western blot demonstrating CDKL5, phospho-EB2 (S222), and total EB2 protein levels in wild-type (WT) versus CDKL5-R59X knock-in (*Cdkl5*$^{R59X/y}$; KO) forebrain tissues; Right: quantification of phospho-EB2 (S222) western blot results demonstrates a nearly total loss of EB2 phosphorylation at Ser222 in KO forebrains compared to WT, normalized to total EB2 protein levels (n=4 per genotype). **(B)** Left: representative western blot demonstrating CDKL5, phospho-EB2 (S222), and total EB2 protein levels in *Cdkl5*$^{flox/y}$; +/+ (Flx) versus *Cdkl5*$^{flox/y}$; CreER/+ (AKO) forebrain tissues (both genotypes receiving tamoxifen); Right: quantification of phospho-EB2 (S222) western blot results demonstrates a nearly total loss of EB2 phosphorylation at Ser222 in AKO forebrains compared to Flx, normalized to total EB2 protein levels (n=8 per genotype). For all panels: Mann-Whitney test; *p<0.05, **p<0.01, ***p<0.001. Full-scan western blots of all samples are available in Supplemental Materials.



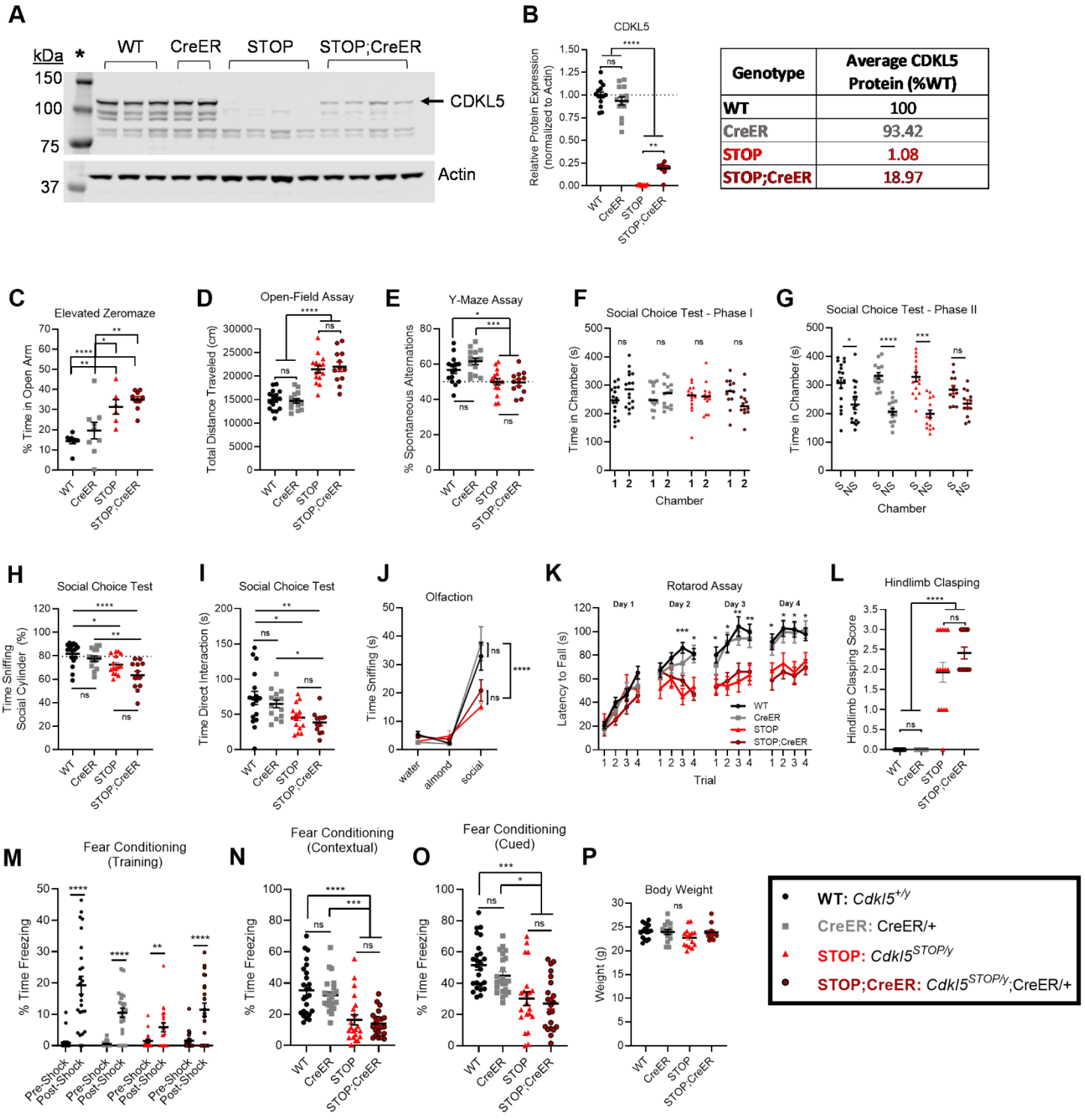
Supplemental Figure 6. STOP mice are behaviorally similar to CDKL5-R59X knock-in mice by postnatal day 42

(A) Representative, overly-exposed western blots demonstrating no detectable CDKL5 protein in cortical tissues of *Cdkl5*^{STOP} (STOP) animals compared to wild-type (WT) and other CDKL5 mutant animals. Cortical lysates from *Cdkl5* knockout (*Cdkl5Δ6*; KO), *Cdkl5*^{FLEx} (*loxP*-flanked, and inverted endogenous *Cdkl5* exon 4, resulting in exon 4 skipping equivalent to exon 4 deletion; FLEx), and CDKL5 R59X knock-in (R59X) mice were used as a reference control for antibody specificity, and Actin was a loading control. All gels were run for an extended period of time (3hrs at 100V) to allow for specific examination of cross-reacting bands at the position of full-length CDKL5 protein. Each blot was probed with a distinct antibody, left: N-terminal anti-CDKL5 antibody (in house); middle: C-terminal anti-CDKL5 antibody (Millipore, ABS402); right: anti-CDKL5 antibody (MRC Dundee, SA145). **(B)** STOP and R59X mice move significantly more in the open-field test compared to CreER controls suggestive of hyperactivity. **(C)** STOP and R59X mice show a trending decrease in percentage of spontaneous alternations performed during the Y-Maze assay compared to CreER controls suggestive of impaired working memory. **(D)** STOP and R59X mice make more total alternations compared to CreER controls in the Y-Maze assay. **(E)** STOP mice spend less time directly interacting with a novel stimulus mouse during a dyadic social test, demonstrating decreased sociability as reported for other *Cdkl5* knockout and knock-in lines. For all panels: n=14 *Cdkl5*$^{+/y}$; CreER/+ (CreER); n=7 *Cdkl5*$^{STOP/y}$; +/+ (STOP); n=7 *Cdkl5*$^{R59X/y}$ (R59X) all at postnatal day 42. One-way ANOVA with Holm-Šidák's *post-hoc* test (except dyadic social assay: Mann-Whitney test); *p<0.05, **p<0.01. Bars represent mean ± SEM.



Supplemental Figure 7. CDKL5 expression and knockout validation across brain regions.

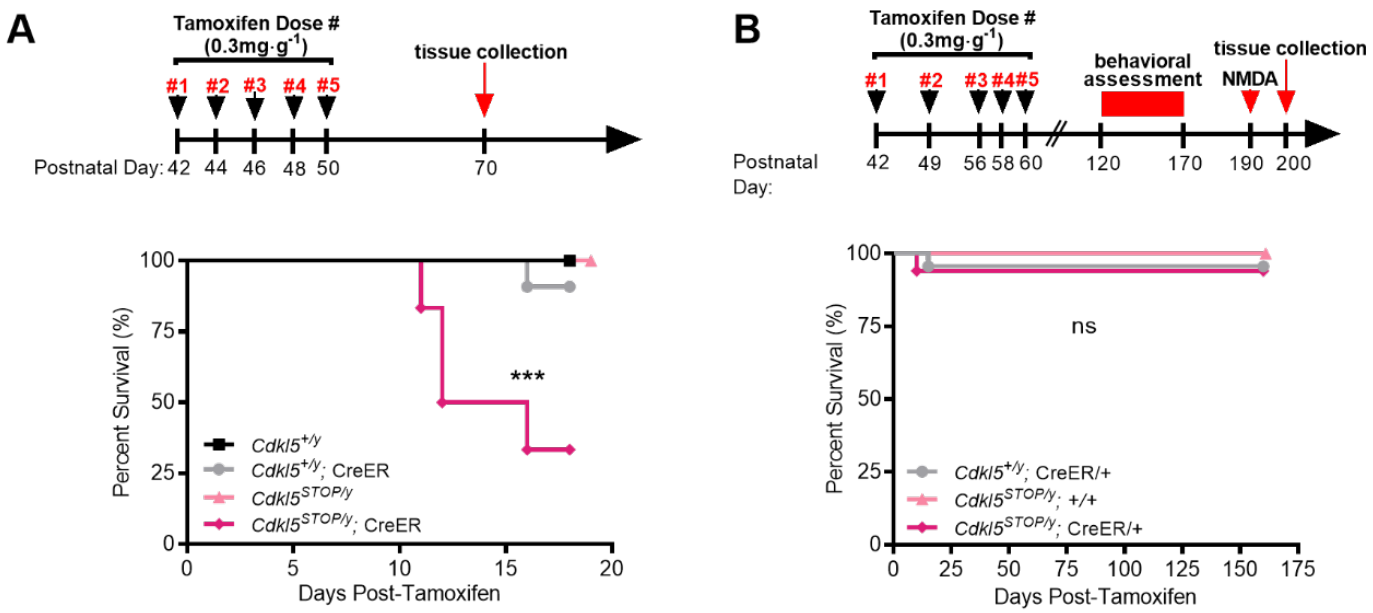
Left (each panel): Western blot results demonstrating CDKL5 protein levels across **(A)** cortex **(B)** striatum **(C)** hippocampus **(D)** cerebellum **(E)** brain stem and **(F)** olfactory bulb regions in wild-type *Cdkl5*$^{+/y}$; +/+ (WT), *Cdkl5*$^{STOP/y}$;+/+ (STOP), and *Cdkl5*^{STOP};CreER/+ (STOP;CreER) animals where Actin was referenced as a loading control; Right (each panel): quantification of CDKL5 western blot results demonstrates a nearly total loss of CDKL5 protein from STOP animals across multiple tissues with variable amounts of leaky CDKL5 expression in STOP;CreER (highest levels in cortex), normalized to Actin protein levels (n=3 per genotype). For all panels: One-way ANOVA with Tukey's multiple comparisons test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Full-scan western blots of all samples are available in Supplemental Materials.



Supplemental Figure 8. STOP and STOP;CreER animals are behaviorally indistinguishable prior to tamoxifen administration

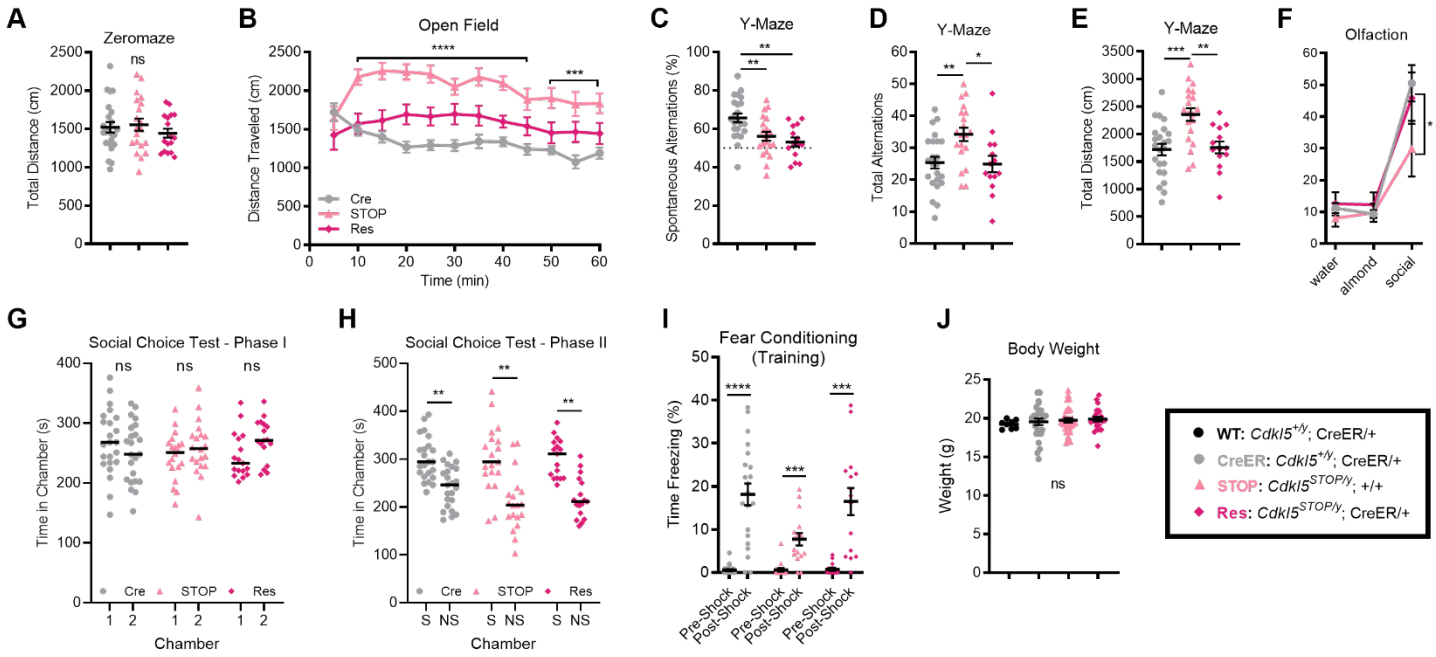
(A) Representative western blot demonstrating significant loss of CDKL5 protein from forebrains of *Cdkl5*$^{STOP/y}$ (STOP) mice compared to wild-type, *Cdkl5*$^{+/y}$; +/+ (WT) and *Cdkl5*$^{+/y}$; CreER/+ (CreER) animals, as well as detectable levels of CDKL5 protein in *Cdkl5*$^{STOP/y}$; CreER/+ (STOP;CreER) animals in the absence of tamoxifen **(B)** Left: quantification of CDKL5 protein levels in forebrain tissues of all genotypes demonstrates significant loss of CDKL5 protein in STOP and STOP;CreER mice compared to WT and CreER animals, but

also a significant increase in CDKL5 protein in STOP;CreER versus STOP-only animals without tamoxifen administration; Right: table summarizing average CDKL5 protein expression levels (as percentage of WT) across all genotypes prior to tamoxifen administration. **(C)** STOP and STOP;CreER mice spend significantly more time than WT and CreER littermates in the open arm of the elevated zeromaze assay suggestive of decreased anxiety. **(D)** STOP and STOP;CreER mice travel significantly more distance in the open-field assay compared to WT and CreER littermates suggestive of hyperactivity. **(E)** STOP and STOP;CreER mice perform significantly fewer spontaneous alternations (percent) in a Y-Maze assay. **(F)** WT, CreER, STOP, and STOP;CreER mice spend similar amounts of time exploring chambers 1 & 2 of the social-choice apparatus during the acclimation stage (Phase I) of the 3-chambered social choice test (paired t-test). **(G)** WT, CreER, STOP, and STOP;CreER mice spend more time in the chamber containing a social stimulus (S; novel mouse) over a non-social stimulus (NS; rock) during Phase II of the 3-chambered social choice test (paired t-test). However, STOP and STOP;CreER mice spend significantly less time **(H)** sniffing and **(I)** directly interacting with a stimulus mouse during the 3-chambered social choice test compared to WT and CreER littermates, highlighting decreased sociability. **(J)** WT, CreER, STOP, and STOP;CreER mice all spend significantly more time sniffing a social scent compared to water or almond scents, with STOP and STOP;CreER mice both spending significantly less time sniffing the social scent compared to WT and CreER animals (Two-way ANOVA with Tukey's multiple comparisons test) **(K)** STOP and STOP;CreER mice take significantly less time to fall from an accelerating, rotating rod, compared to WT and CreER animals, suggestive of impaired motor coordination (Two-way, repeated measures ANOVA with Sidak's multiple comparisons test). **(L)** STOP and STOP;CreER mice present hindlimb clasping behaviors, whereas no WT or CreER mice were observed to carry hindlimb clasping phenotypes. **(M)** WT, CreER, STOP, and STOP;CreER mice all significantly increase their baseline freezing behavior in response to a mild footshock (paired t test), but **(N)** STOP and STOP;CreER mice show decreased percent time freezing compared to WT and CreER littermates 24-hours later when returned to the testing chamber (contextual) and **(O)** upon hearing the testing tone (cue), demonstrating impaired memory selectively in STOP and STOP;CreER mice. **(P)** There were no significant differences in body weight between WT, CreER, STOP, and STOP;CreER animals. For all panels: WT, n=17; Cre, n=14; STOP, n=15; STOP;CreER, n=12 (for elevated Zeromaze: WT, n=8; CreER, n=9; STOP, n=6; STOP;CreER, n=10) where no animals received tamoxifen; One-way ANOVA with Holm-Šidák's *post-hoc* test (except where otherwise noted). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM. Full-scan western blots of all samples are available in Supplemental Materials.



Supplemental Figure 9. Rapid, but not gradual, reversal of *Cdkl5* expression is associated with significant lethality

(A) Top: tamoxifen administration schematic (rapid); Bottom: survival curve demonstrating significant lethality in *Cdkl5*$^{STOP/y}$; CreER/+ mice, exclusively, with tamoxifen delivery suggesting rapid *Cdkl5* reintroduction could be detrimental (n=8 *Cdkl5*$^{+/y}$; +/+; n=11 *Cdkl5*$^{+/y}$; CreER/+; n=8 *Cdkl5*$^{STOP/y}$; +/+; n=6 *Cdkl5*$^{STOP/y}$; CreER/+). **(B)** Top: tamoxifen administration schematic (gradual); Bottom: survival curve demonstrating no significant difference in lethality between *Cdkl5*$^{STOP/y}$; CreER/+ mice and other genotypes with tamoxifen delivery, suggesting that gradual *Cdkl5* reintroduction mitigates lethality associated with its re-expression (n=23 *Cdkl5*$^{+/y}$; CreER/+; n=20 *Cdkl5*$^{STOP/y}$; +/+; n=17 *Cdkl5*$^{STOP/y}$; CreER/+). For all panels: all genotypes received tamoxifen; Mantel-Cox (log-rank) test; *p<0.05, **p<0.01, ***p<0.001.



Supplemental Figure 10. Additional behavioral domains analyzed upon adult rescue of *Cdkl5*

(A) Total distance traveled during the elevated Zeromaze assay was similar between CreER, STOP, and Res mice. **(B)** STOP, but not Res, mice traveled significantly more in an open-field assay relative to CreER littermates over the course of one hour (distance traveled binned in 5min intervals) suggestive of rescued hyperactivity in Res mice. **(C)** STOP and Res mice show a significant decrease in percentage of spontaneous alternations in a Y-Maze assay compared to CreER littermates, suggestive of impaired working memory. **(D)** STOP, but not Res, mice make significantly more total number of alternations and **(E)** travel more distance during the Y-Maze when compared to CreER littermate controls further highlighting rescued hyperactivity in Res animals. **(F)** CreER, STOP, and Res mice all spent significantly more time sniffing a social scent over either water or almond scents, with STOP mice spending significantly less time than CreER or Res animals sniffing the social scent. **(G)** CreER, STOP, and Res mice all spent equal time exploring chamber 1 and chamber 2 during Phase I of the 3-chambered social choice test (prior to any social/non-social stimulus presentation). **(H)** CreER, STOP, and Res mice all spent significantly more time in the chamber containing a social stimulus (S; novel mouse) over a non-social stimulus (NS; rock) during Phase II of the 3-chambered social choice test. **(I)** CreER, STOP, and Res mice all significantly increase the percent time they spend freezing in respond to a mild footshock during a Pavlovian fear conditioning training paradigm. **(J)** There were no significant differences in body weight across WT, CreER, STOP, and STOP;CreER animals (WT, n=8; CreER, n=27; STOP, n=31; Res, n=23). For all other panels: CreER, n=23; STOP, n=20; Res, n=17 where all genotypes received tamoxifen; One-way ANOVA test with Holm-Šidák's *post-hoc* test (except open field & olfaction: two-way, repeated measures ANOVA with Dunnett's multiple comparisons test; social choice test & fear conditioning: paired t test); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.

Figure 1A

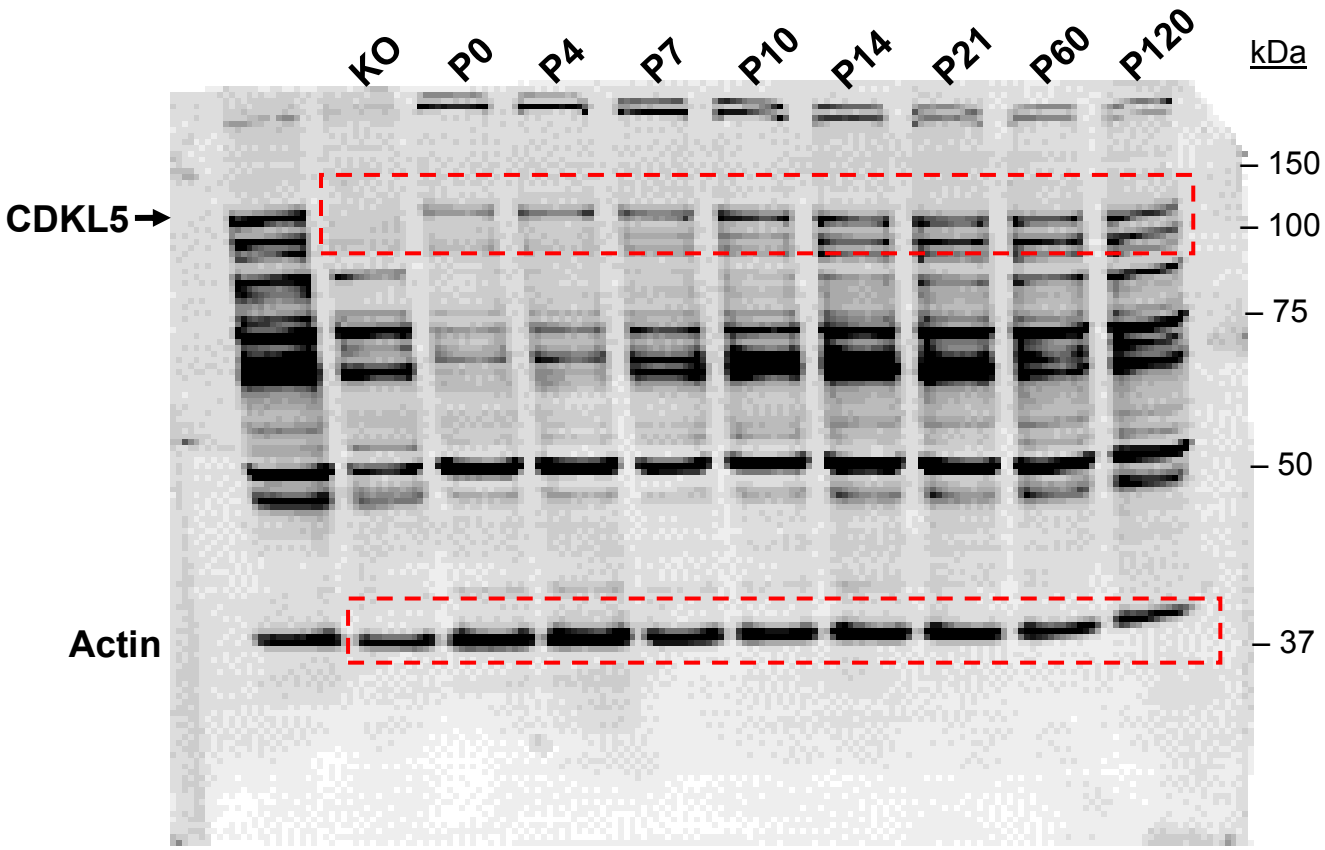


Figure 1B

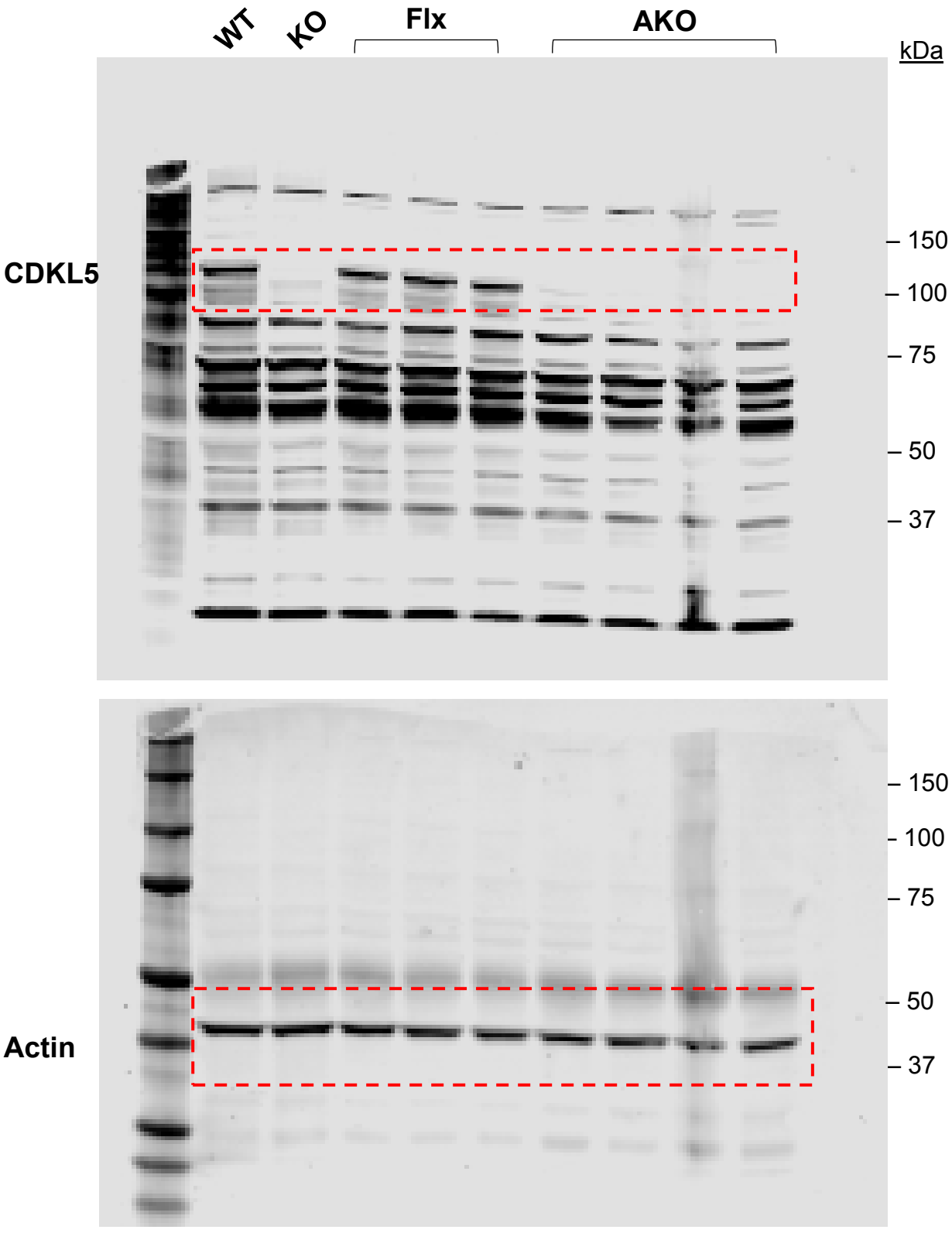


Figure 3B (bottom half of cut blot re-probed with Actin)

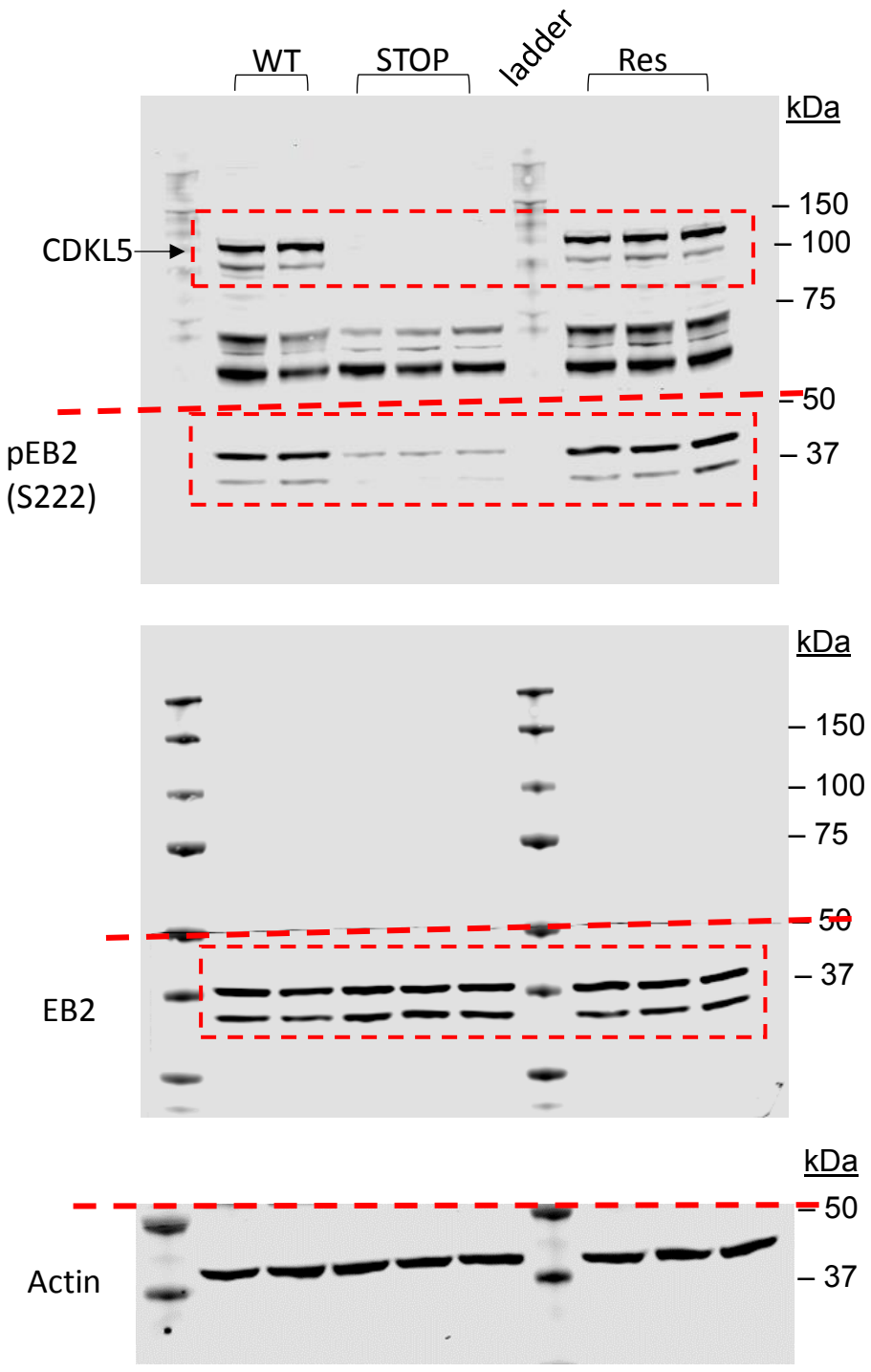
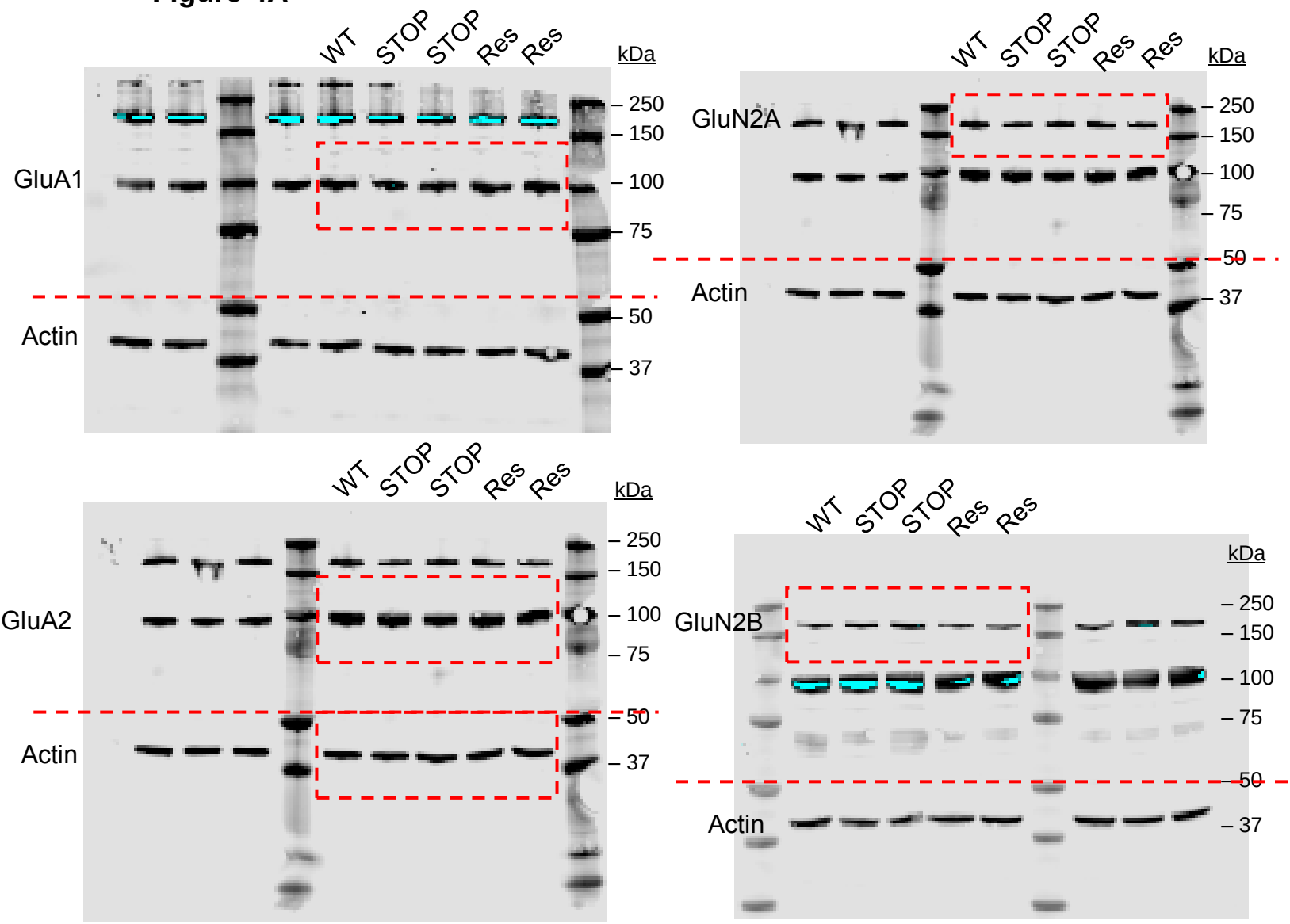


Figure 4A


WT
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STOP
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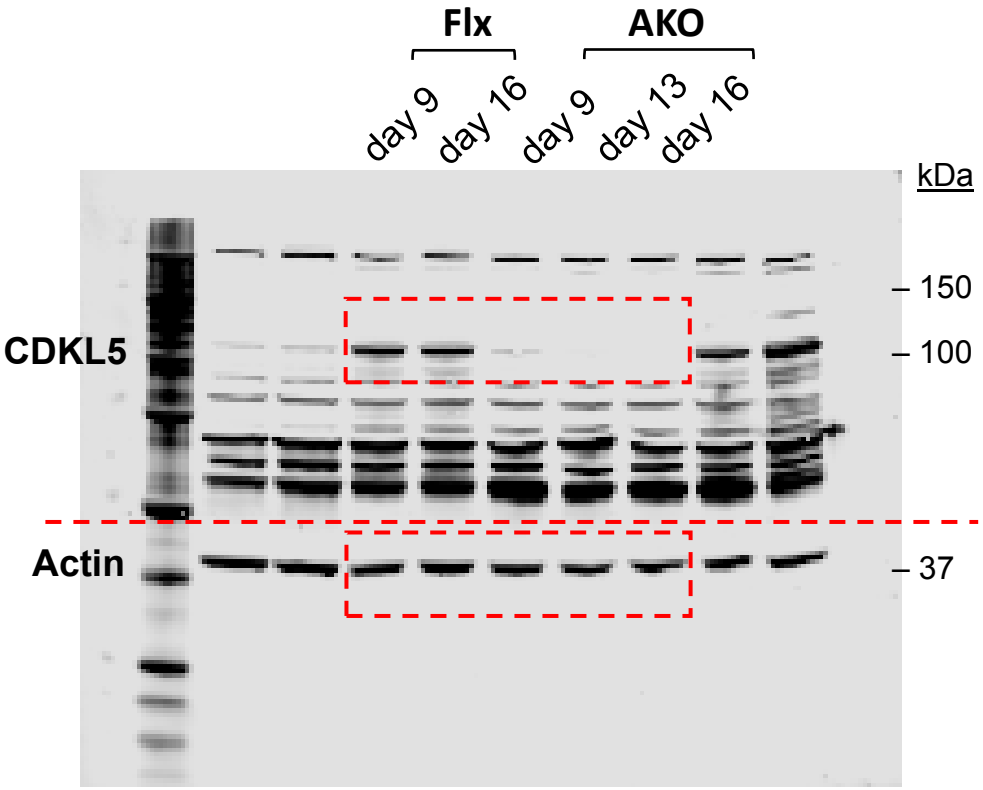
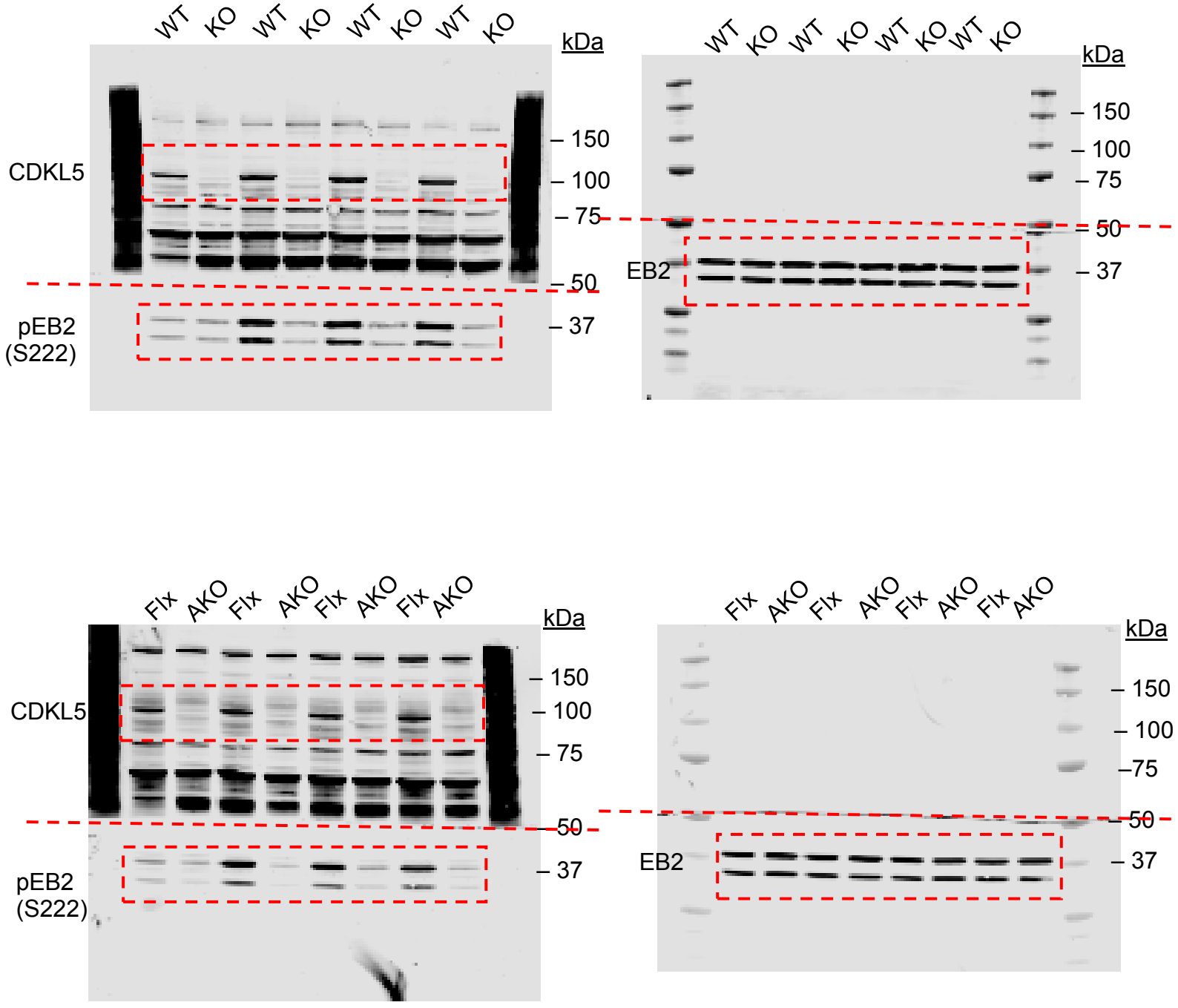
GluA2
GluN2B

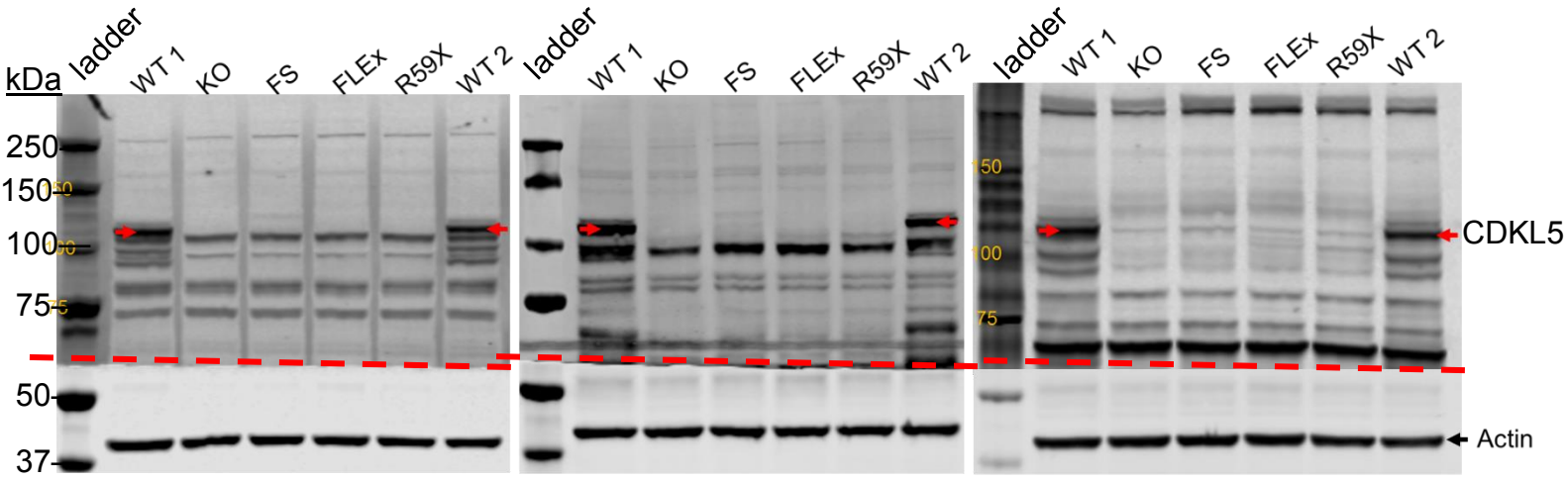
Supplemental Figure 1



Supplemental Figure 5



Supplemental Figure 6A

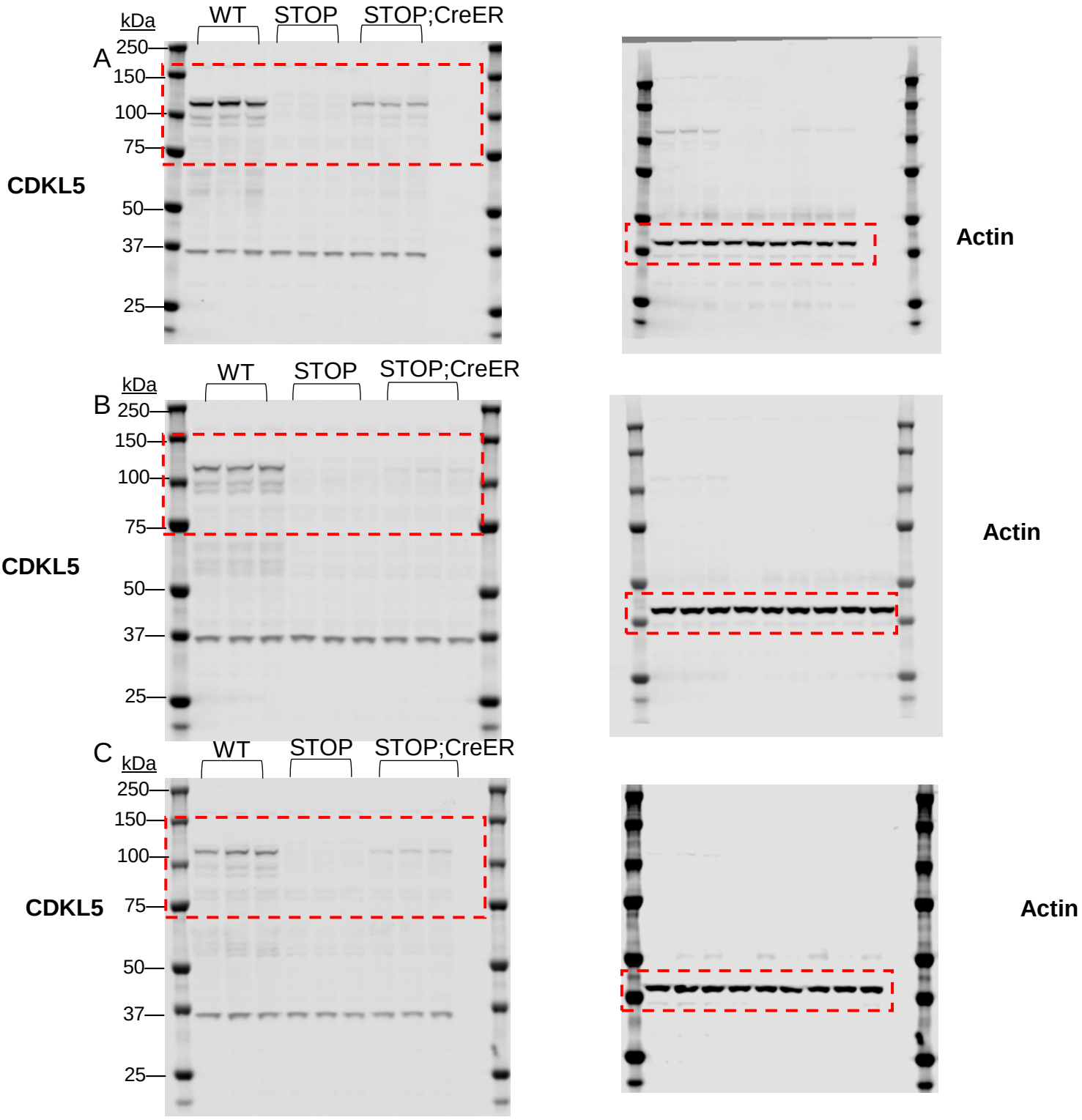


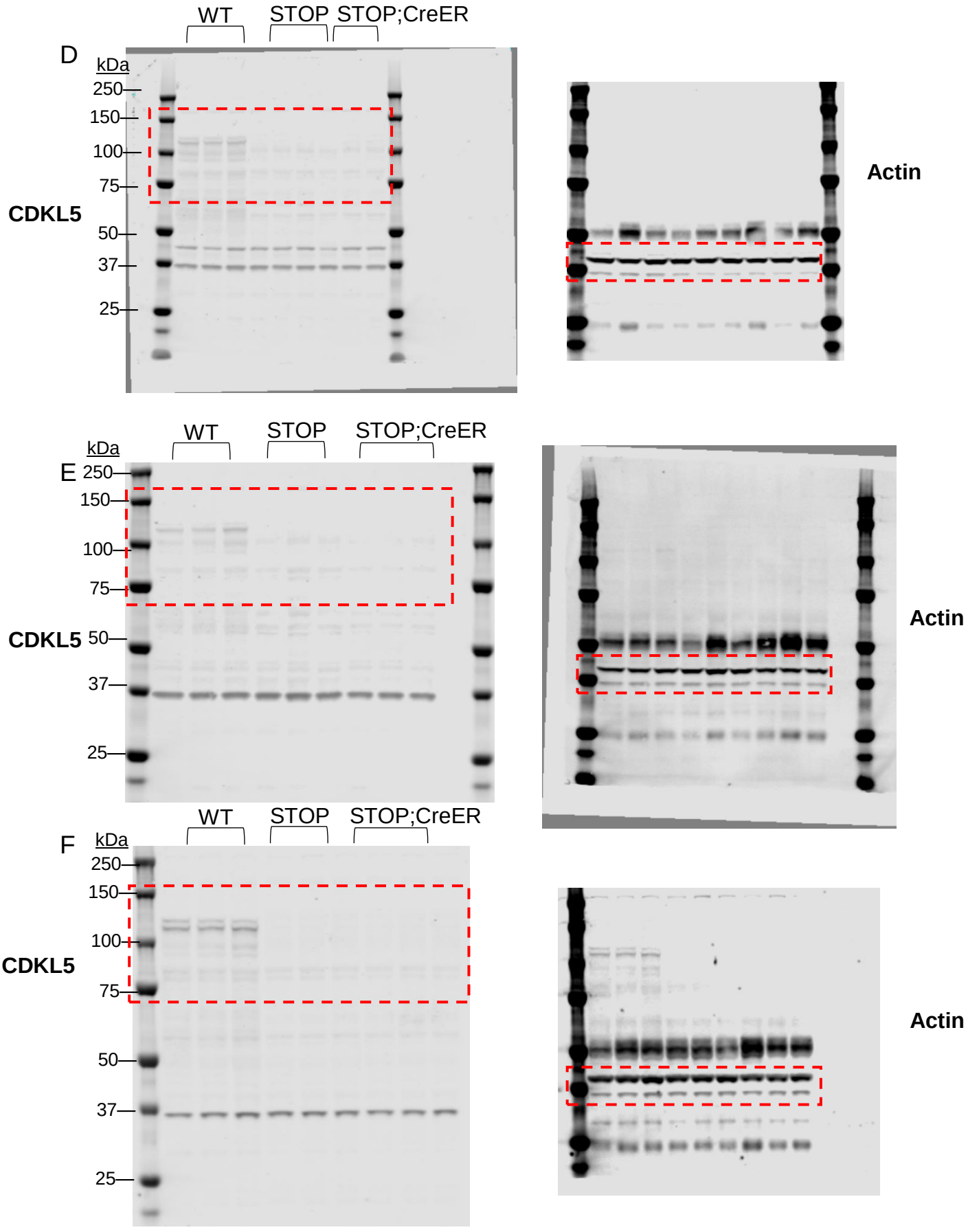
anti-CDKL5 C-terminal ab
(Wang et al 2012; 1:500)

anti-CDKL5 ab from UK
(SA145; 1:1000)

anti-CDKL5 N-terminal ab
(Millipore ABS402; 1:250)

Supplemental Figure 7 (blots re-probed with Actin)

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Supplemental Figure 8A (blot re-probed for Actin)