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RESEARCH ARTICLE

Figures and figure supplements

The adipocyte hormone leptin sets the emergence of hippocampal inhibition in mice

Camille Dumon *et al*

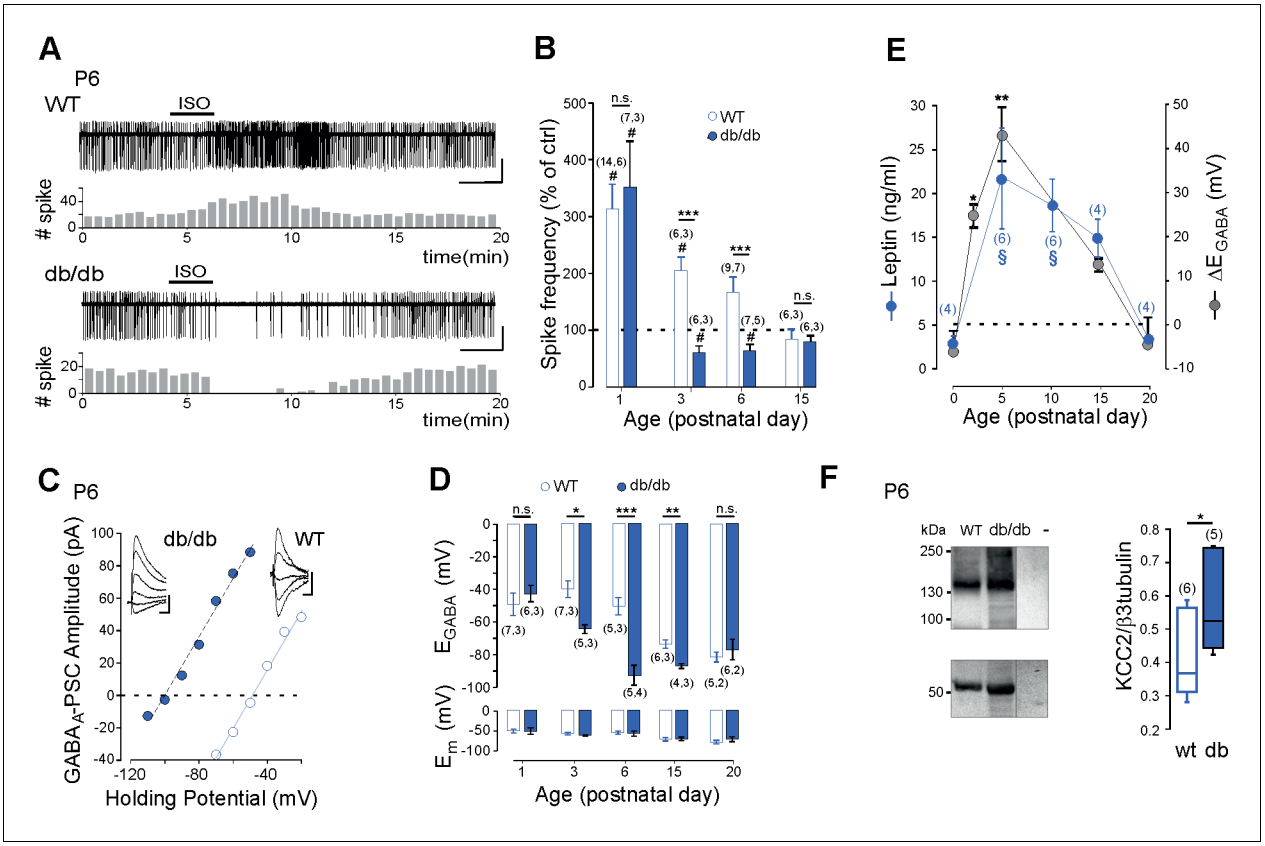

Figure 1. Early GABA developmental sequence in leptin-receptor deficient mice. (**A**) Cell attached recordings of CA3 pyramidal neurons on acute hippocampal slices. Scale bar, 2 min, 50 pA. Corresponding time course of spike frequency changes are shown under each trace. (**B**) Developmental changes of isoguvacine action on spike activity. Mean ± SEM. (**C**) Current-voltage relationships for evoked GABAergic synaptic currents. Insets: examples of GABAergic synaptic current evoked at holding potentials ranging from −110 to −60 mV (10 mV increment) in *db/db* and from −70 to −30 (10 mV increment) in wt CA3 pyramidal neuron. Scale bar, 10 ms, 20 pA. (**D**) Developmental changes in E_{GABA} and Em at zero current. Mean ± SEM. In *B and* D, the number of cells recorded and number of mice used are indicated in parenthesis; #p<0.05 when compared to pre-isoguvacine values, two-tailed paired Student's *t*-test, *p<0.05, **p<0.01 and ***p<0.001 when compared to age-matched wt, two-tailed unpaired Student's *t*-test. (**E**) Developmental profile of plasma leptin levels in the wt (blue circle) and developmental profile of ΔE_{GABA} (gray circle). ΔE_{GABA} was calculated as the difference in E_{GABA} values between the wt and *db/db* at each developmental stage depicted in *D*. Numbers in parenthesis indicate the number of mice used. Mean +SEM. §p<0.05 when compared to P0 plasma leptin values, *p<0.05 and **p<0.01 when compared to P0 ΔE_{GABA} values, one way ANOVA followed by a Tukey's *post hoc* test. (**F**) Left: representative immuno-blots for hippocampal panKCC2 and β3-tubulin in wt and *db/db* mice (first two lanes). The third lane (-) illustrates background (empty well). Right: box plots of normalized pan KCC2 in P6 wt and *db/db* hippocampi. Numbers in parenthesis indicate the number of mice used. *p<0.05, two-tailed unpaired Student's *t*-test.

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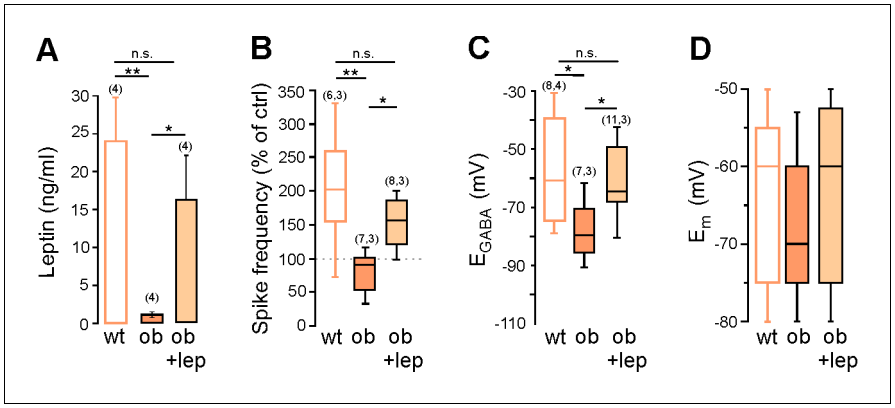

Figure 1—figure supplement 1. Leptin controls GABA signaling in the mice hippocampus in vivo. (**A**) Plasma leptin levels in P8 wt, *ob/ob* and leptin-treated *ob/ob* mice (*ob* +lep, 5 mg/kg twice a day sub-cutaneous from P3 to P8, n = 4, blood samples were collected 30 min after the last injection). (**B**) Box plot of isoguvacine action on spike frequency in wt mice, *ob/* and leptin-treated *ob/ob* mice at P8. (**C,D**) Box plots of E_{GABA} and Em at zero current in 4 wt mice, *ob/ob* and leptin-treated *ob/ob* CA3 pyramidal neurons. The cells recorded and number of animal used are indicated in parenthesis *p<0.05, **p<0.01, one way ANOVA followed by a Tukey's *post hoc* test.
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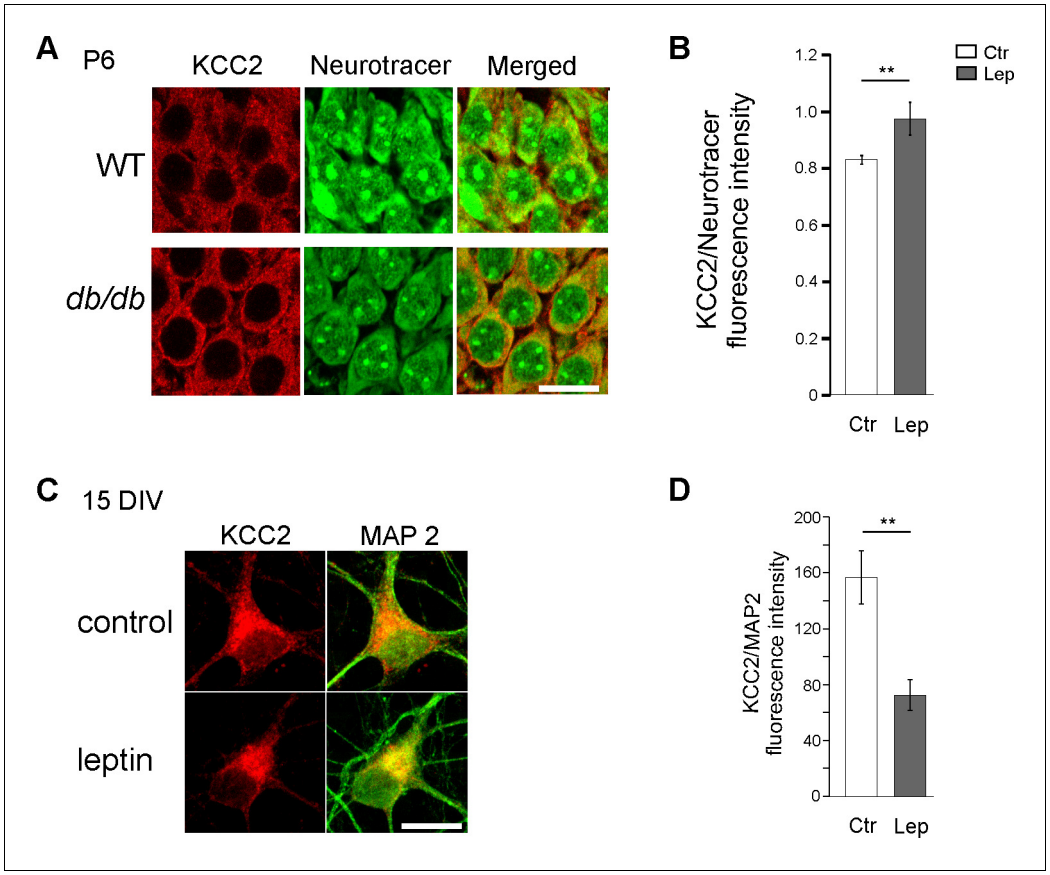

Figure 1—figure supplement 2. KCC2 expression in *db/db* hippocampal neurons in vivo and leptin-treated hippocampal neuronal cultures in vitro. (**A**) Confocal images showing KCC2 immuno-labeling in the CA3 pyramidal layer of wt and *db/db* hippocampi at P6. (**B**) Summary plot of the normalized KCC2 fluorescence intensity ratio in P6 wt and *db/db* hippocampal CA3 pyramidal layer. Calibration bar 20 µm. 3 wt and *db/db* littermate animals. mean ±sem. **p<0.01, two-tailed unpaired Student's *t*-test. (**C**) Examples of KCC2 and MAP2 immunostaining in control and leptin-treated (100 nM, 24 hr) cultured hippocampal neurons (15 DIV). Scale bar 20 µM. (**C**) Summary plots of the normalized KCC2 fluorescence intensity in control (n = 30) and leptin-treated (n = 45) hippocampal neurons. Pooled data from 3 different cultures.

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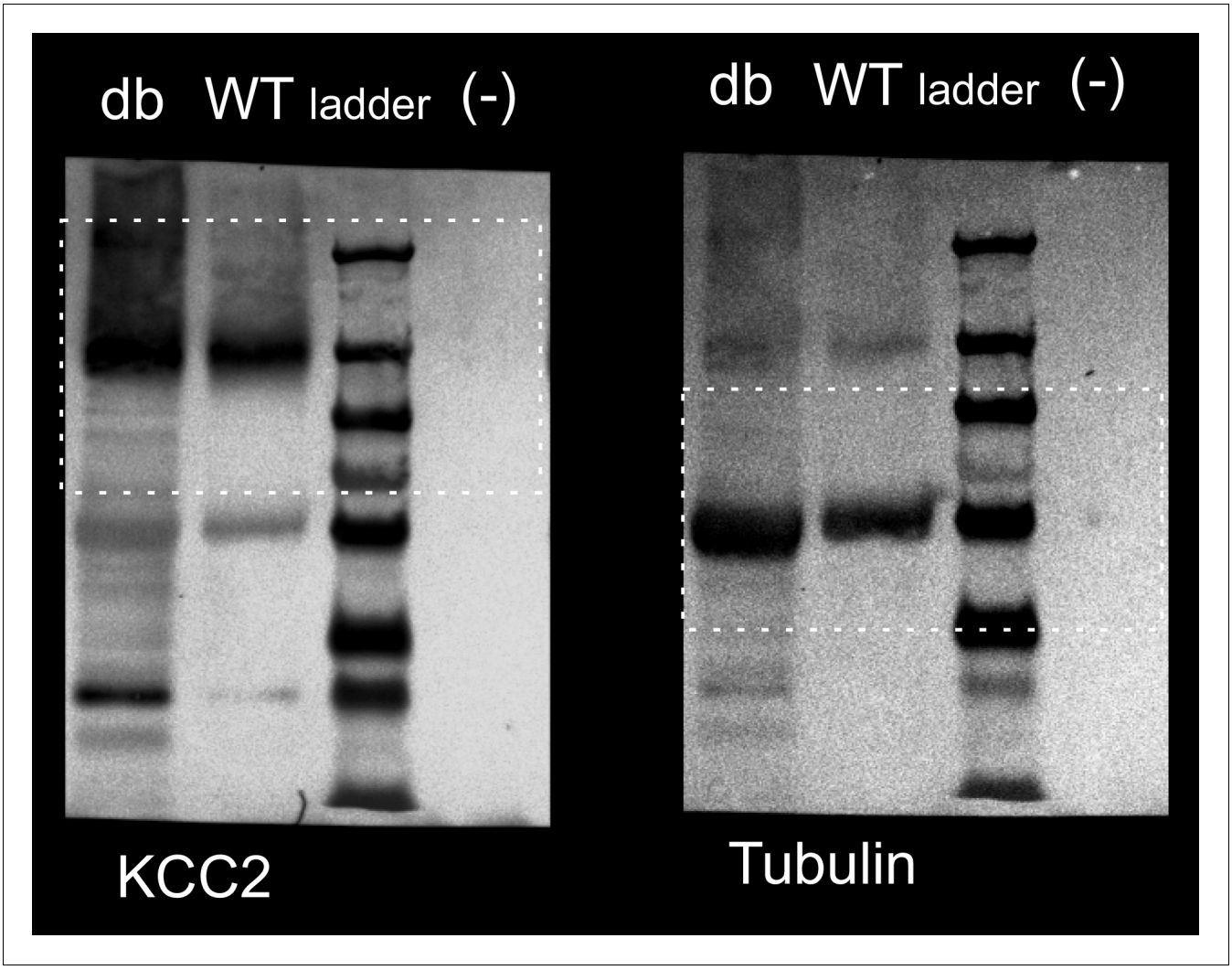

Figure 1—figure supplement 3. Raw blots for panel F (WT and db).
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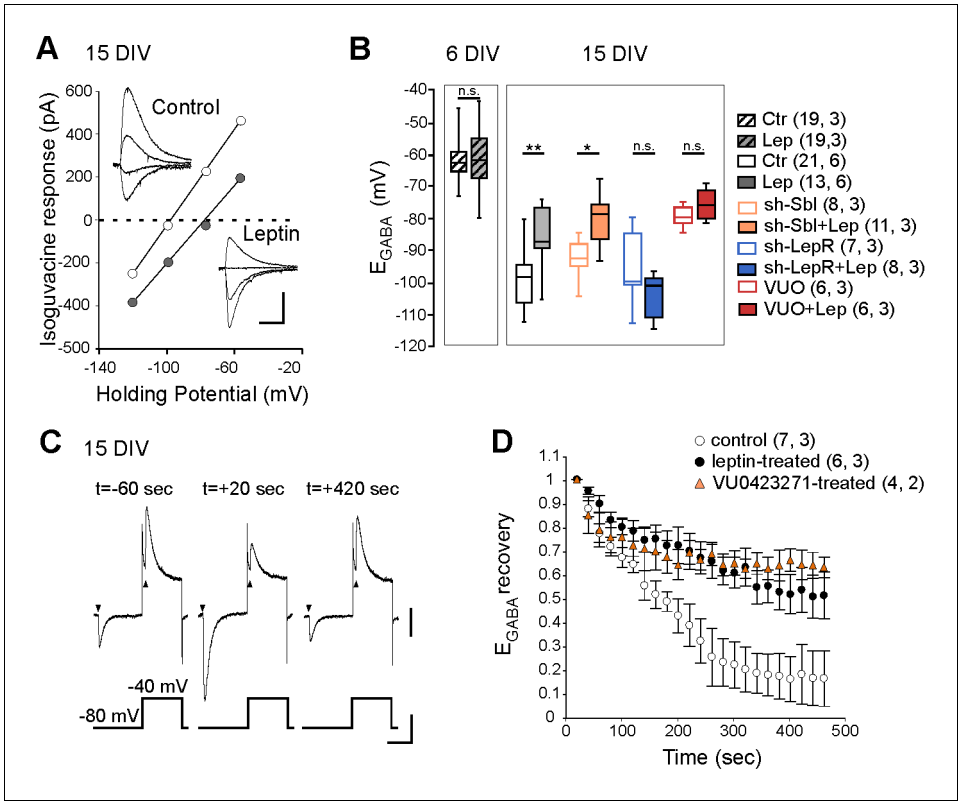

Figure 2. Leptin down-regulates KCC2 activity in cultured hippocampal neurons. (**A**) I-V relationships for isoguvacine currents in vehicle (control) and leptin-treated (100 nM, 24 hr) hippocampal (15 DIV) cultures. Gramicidin perforated patch clamp recordings. Insets depict the isoguvacine currents. Scale bar, 500 ms, 100 pA. (**B**) Box plots of E_{GABA} in the indicated conditions. *p<0.05, **p<0.01, ANOVA followed by a Tukey's *post hoc* test. (**C**) Examples of isoguvacine currents (arrow heads) recorded at −80 and −40 mV before (t=-60 sec) and after (t = +20 and +420 s) neuronal chloride loading in control neuronal culture (15 DIV). Gramicidin perforated patch clamp recordings. Scale bar, 100 pA, 40 mV, 1 s. (**D**) Summary plots of normalized E_{GABA} recovery after neuronal chloride loading in the indicated conditions. Mean ± SEM. In *B* and *D*, the number of cells recorded and number of cultures used are indicated in parenthesis.

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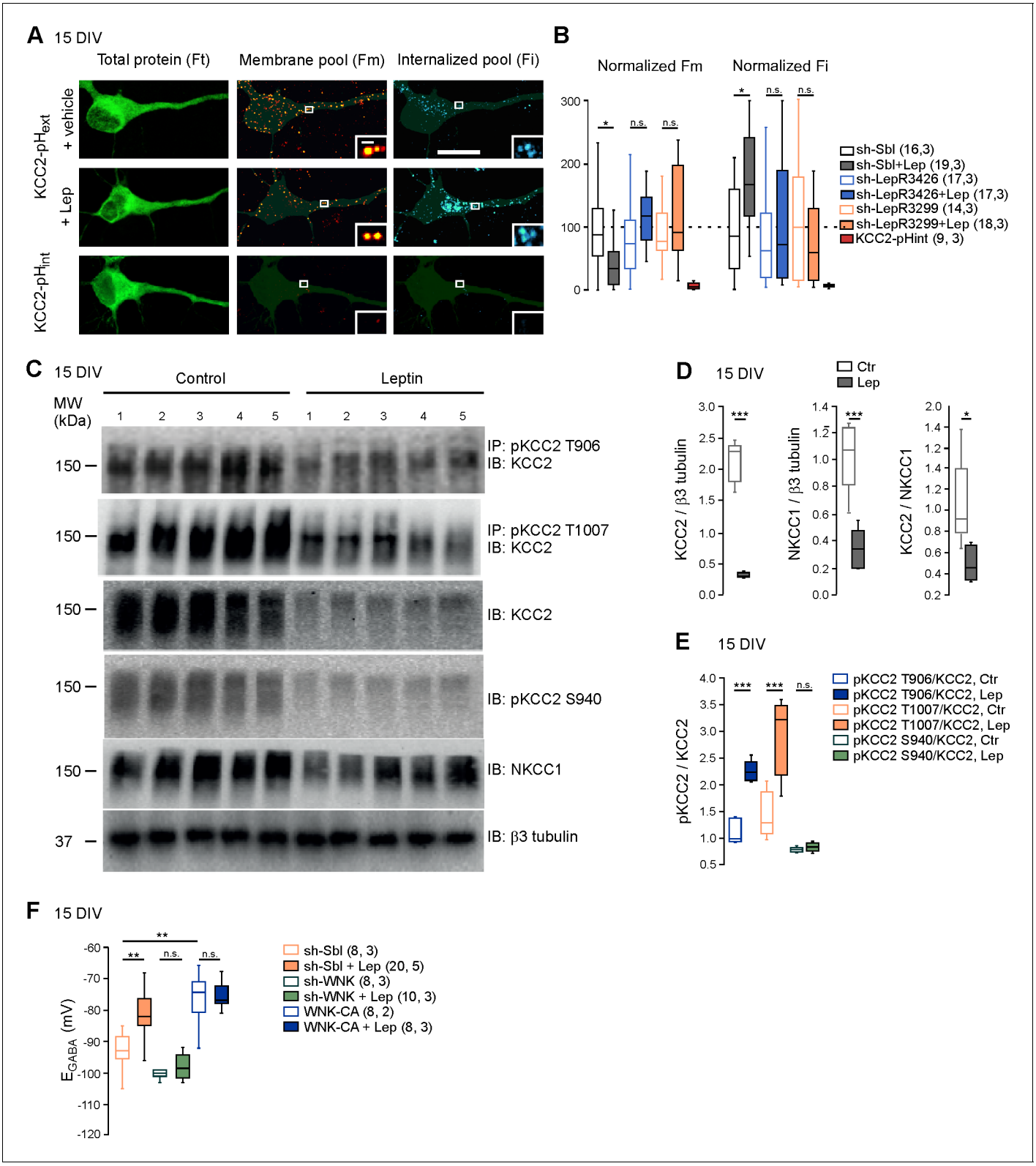

Figure 3. Leptin reduces the plasma membrane expression of KCC2 and modulates its phosphorylation state in cultured hippocampal neurons. (**A**) Representative images illustrating total, membrane and internalized pools of KCC2 with external tag (KCC2-pHext) in vehicle and leptin-treated (100 mM, 24 hr) cultured hippocampal neurons expressing a scramble Sh-RNA (Sh-Sbl). Neurons expressing KCC2 with internal tag (KCC2-pHint) were proceeded in parallel experiments to ensure that immunocytochemistry on living neurons does not permeabilized the membrane. Scale bars 20 µm and

Figure 3 continued on next page

Figure 3 continued

1 µm. (**B**) Box plots of normalized membrane (Fm) and internalized (Fi) fluorescence in vehicle and leptin-treated (+Lep) cultured neurons expressing the indicated constructs. *p<0.05, one way ANOVA followed by a Tukey's *post hoc* test. (**C**) Western blots and quantifications (**D and E**) of KCC2, NKCC1, KCC2/NKCC1 ratio and the threonine 906, threonine 1007 and serine 940-phosphorylated forms of KCC2 in control and leptin (100 nM, 24 hr)-treated hippocampal neuronal cultures (DIV15, five independent neuronal cultures). ***p<0.001, two-tailed unpaired Student's *t-test*. (**F**) Box plots of E_{GABA} in the indicated conditions. Gramicidin perforated patch clamp recordings were performed on hippocampal neuronal cultures at 15 DIV. **p<0.01, ***p<0.001, one way ANOVA followed by a Tukey's *post hoc* test. In B and F, the number of cells recorded and number of cultures used are indicated in parenthesis.

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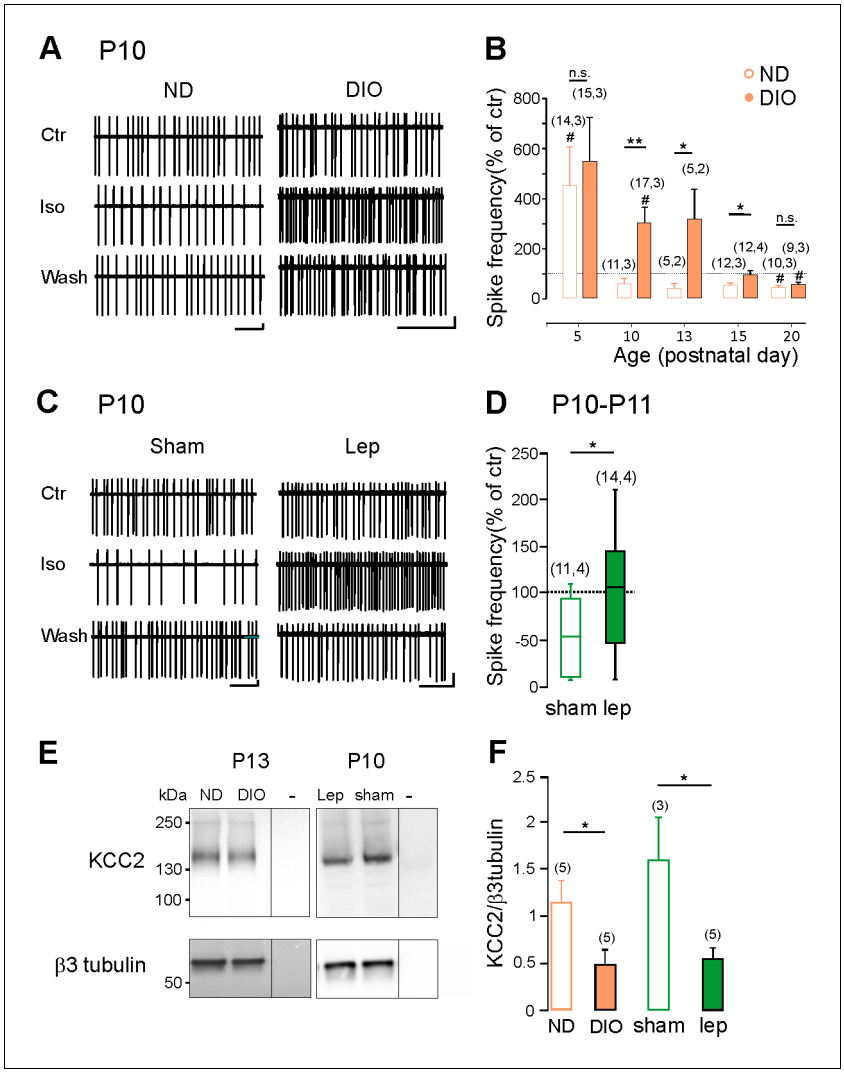

Figure 4. Hyperleptinemia and maternal obesity delay the GABA developmental sequence and downregulate KCC2 expression. (**A**) Cell attached recordings of CA3 pyramidal neurons on acute hippocampal slices obtained from pups of normal diet (ND) and diet-induced obese (DIO) dams at P10. (**B**) Developmental changes of isoguvacine action on spike frequency. Mean +SEM. (**C**) Cell attached recordings of CA3 pyramidal neurons on acute hippocampal slices obtained from vehicle-treated (sham) and leptin-treated mice at P10. (**D**) Box plots of isoguvacine action on spike activity. In *B* and *D*, number of cells recorded and number of mice used are indicated in parenthesis; $^{\#}p<0.05$ when compared to pre-isoguvacine values, two-tailed paired Student's *t*-test and *p<0.05 and **p<0.01 when compared to age matched ND-pups (**B and C**) or sham-pups (**E**), two-tailed unpaired Student's *t*-test. (**E**) Representative immuno-blots for hippocampal panKCC2 and β3-tubulin in offspring of DIO and ND dams at P13 and in control (sham) and leptin-treated (Lep) mice at P10. The third lanes (-) illustrate background (empty wells). (**F**) Normalized panKCC2 immunoreactivity in ND (n = 6 pups) and sham (n = 3 pups), in offspring of DIO (n = 5 pups) and in leptin-treated mice (n = 5 pups). Mean +SEM. *p<0.05, two-tailed unpaired Student's *t*-test.

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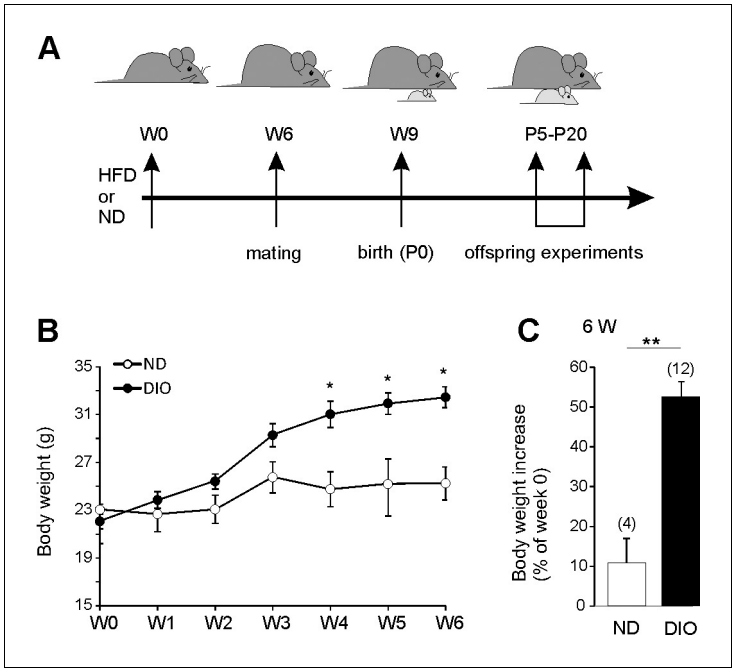

Figure 4—figure supplement 1. High fat diet induced obesity. (**A**) Experimental overview. (**B**) Body weight curves of adult (8 weeks old) female mice fed with normal diet (10% kcal from fat, ND white symbols, n = 4) or a high-fat diet (60% kcal from fat, HFD, n = 12). (**C**) Average body weight increase after 6 weeks of food supply. *p<0.05, **p<0.01, two-tailed unpaired Student's *t*-test.

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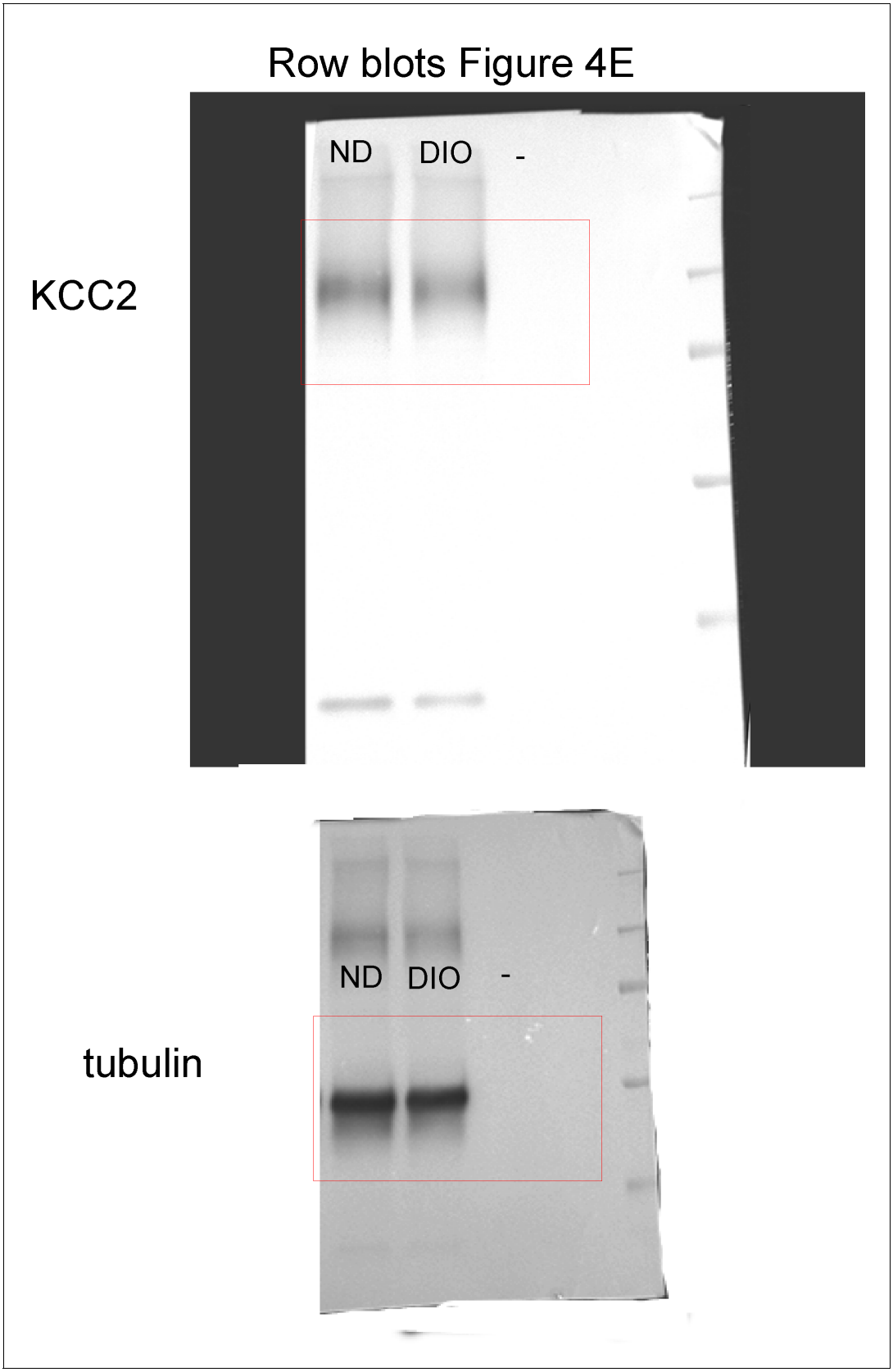

Figure 4—figure supplement 2. Raw blots for panel E (ND and DIO).
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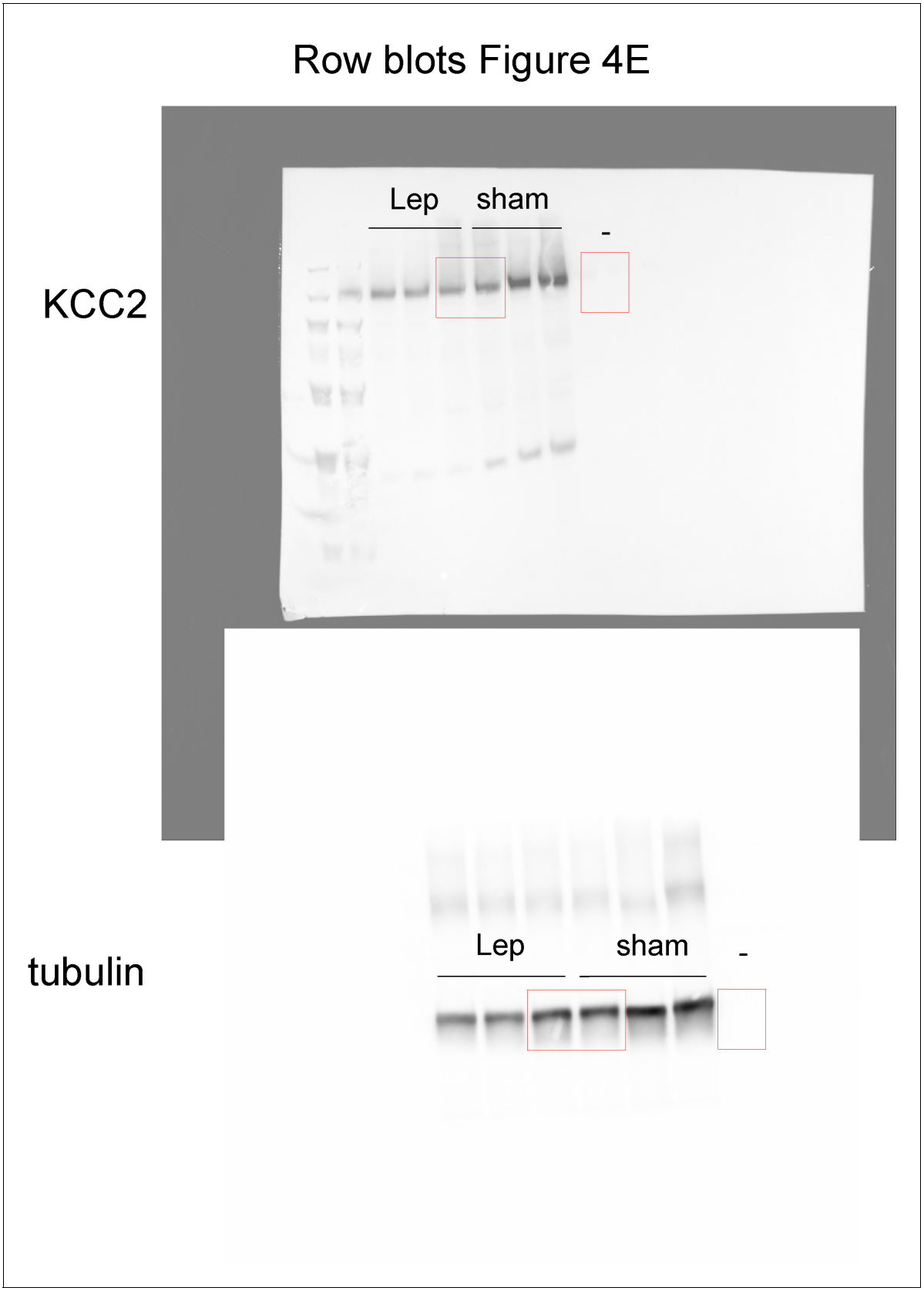

Figure 4—figure supplement 3. Raw blots for panel E (Lep and sham).
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