Supplementary Information

Title: "Loss of Usp9x disrupts cell adhesion, and components of the Wnt and Notch signaling pathways in neural progenitors."

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Supplementary Figures

FigS1. Reduced N-cadherin and AF-6 immunoreactivity in E12.5 *Usp9x$^{-/Y}$* neocortices. **(A)** Coronal sections of a further two independent E12.5 *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* neocortices stained for N-cadherin and AF-6 (in addition to Fig.1A). Compared to *Usp9x$^{+/Y}$* (a, c, e) N-cadherins immunoreactivity was clearly reduced at the apical surface of *Usp9x$^{-/Y}$* VZ (white arrows) (b, d, f). **(B)** Coronal sections of two independent E12.5 *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* neocortices stained for AF-6. Compared to *Usp9x$^{+/Y}$* (a, c, e) AF-6 immunoreactivity was clearly reduced at the apical surface of *Usp9x$^{-/Y}$* VZ (white arrows) (b, d, f). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.

FigS2. Heat map showing 1047 genes differently expressed between E12.5 *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* neocortices. Expression intensity of E12.5 Usp9x-/Y compared to littermate controls (n=4 for each genotype).

FigS3. Heat map showing 1397 genes differently expressed between E14.5 *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* neocortices. Expression intensity of E14.5 Usp9x-/Y compared to littermate controls (n=4 for each genotype).

FigS3. Neurogenesis is intact in *Usp9x$^{-/Y}$* neocortices. (A) Tbr1^{+} neuronal expression in *Usp9x$^{-/Y}$* neocortices. E12.5 (a, b), E14.5 (c, d) and E16.5 (e, f) neocortices stained with Tbr1 antibodies (representative of n=3 for each embryonic stage). **(B)** The graph represents the number of Tbr1^{+} cells expressed per unit area of neocortex. No difference was observed between *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* neocortices at any stage. **(C)** E12.5 (a, b), E14.5 (c, d), E16.5 (e, f) and E18.5 (g, h) neocortices stained with Dcx antibodies (n=3 for each embryonic stage). Dotted lines demark the borders of the ventricular zone and cortical plate. **(D)** The graph represents Dcx expression as the ratio of Dcx^{+} cortical plate thickness (orange double headed arrow) over total cortical thickness (red double headed arrow) (n=3). No difference was observed for Dcx^{+} neuroblasts expression between *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* neocortices in all the tested embryonic stages. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.

FigS4. Apoptosis in *Usp9x$^{-/Y}$* neocortices. (A) Cleaved Caspase 3 stained *Usp9x$^{-/Y}$* brains. E12.5 (a, b), E14.5 (c, d), E16.5 (e, f) and E18.5 (g, h) neocortices stained with cleaved caspase 3 antibodies (n=3 for each embryonic stage). Caspase staining failed to detect apoptotic cells in *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* brains at the tested embryonic stages. **(B)** TUNEL assay of *Usp9x$^{-/Y}$* brains. TUNEL assay on sections from E12.5 (a, b), E14.5 (c, d), E16.5 (f, g) and E18.5 (i, j) neocortices stained (n=3 for each embryonic stage). (e, h) Brain sections exposed to DNase1 were used as the positive controls. No TUNEL positive cells were observed at any tested embryonic stages. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.

FigS5. (A) Co-immunoprecipitation of N-cadherin from E12.5 *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* cortical lysates, with anti-β-catenin antibodies. Relative to inputs, similar amounts of N-cadherin were pulled down from *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* lysates suggesting the catenin-cadherin heterodimer is intact in E12.5 *Usp9x$^{-/Y}$* NPs. **(B-E)** Immunoblot analysis of total APC protein levels in *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* cortices at E12.5 and E14.5 (n=3). (C, E) No difference was observed for total APC protein levels between *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* brains at E12.5 and E14.5. **(F-I)** Immunoblot analysis of GSK-3β protein levels in *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* cortices at E12.5 and E14.5 (n=3). (G, I**)** No difference was observed for total GSK-3β protein levels between *Usp9x$^{+/Y}$* and *Usp9x$^{-/Y}$* cortices at E12.5 and E14.5**. (J)** USP9X protein is depleted and Ser33/37/ Thr41 pβ-catenin protein is increased in HEK293 cells 72 hours after siRNA transfection. β-tubulin used as loading control.

FigS6. qRT-PCR analyses of the dysregulated Wnt pathway genes identified by Ingenuity pathway analysis. (a-f) qRT-PCR analyses results of dysregulated Wnt signaling genes in E12.5 *Usp9x$^{-/Y}$* cortices. Expression of all the tested genes was significantly altered in *Usp9x$^{-/Y}$* cortices. (g-n) qRT-PCR analyses results of some of the dysregulated Wnt signaling genes in E14.5 *Usp9x$^{-/Y}$* cortices. Only Sox4 and Sox1 expression are significantly different in *Usp9x$^{-/Y}$* cortices. *, $p < 0.05$, **,$p<0.01$, ***, $p<0.001$

FigS7. Astroglial differentiation is normal in the *Usp9x*$^{-/Y}$ neocortex. (A) Coronal sections E12.5 *Usp9x*$^{+/Y}$ and *Usp9x*$^{-/Y}$ neocortices co-stained with BLBP and the astrocytic marker GFAP. No difference was observed for the GFAP expression (a, b) between *Usp9x*$^{+/Y}$ and *Usp9x*$^{-/Y}$ brains, despite increased BLBP expression (c, d) in *Usp9x*$^{-/Y}$ brains. **(B)** Similar pattern of GFAP (g, h) and BLBP (i, j) staining in E18.5 *Usp9x*$^{+/Y}$ and *Usp9x*$^{-/Y}$ brains. **(C, D)** *HES5* and *HES1* gene expression were significantly increased in USP9X-depeleted ReNcell VM NPs. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. *, $p < 0.05$.

FigS8. Mindbomb1 expression in *Usp9x*$^{-/Y}$ neocortices. (A, C) Immunoblot analysis revealed Mindbomb1 protein levels are unaltered in E12.5 and E14.5 *Usp9x*$^{-/Y}$ neocortices (n=3). **(B, D)** Quantification of Mindbomb1 protein levels relative to β-tubulin.

FigS9. **Usp9x co-localises with Numb in embryonic mouse brains.** Coronal sections of E14.5 neocortices immunostained with Usp9x (red arrows) and Numb (green arrows) antibodies. Yellow arrows show the extensive co-localization of Usp9x and Numb proteins most notably at the VZ.

Table S1. Microarray was conducted using cortical tissues of E12.5 *Usp9x*$^{+/Y}$ and *Usp9x*$^{-/Y}$ neocortices (n=4). Differentially expressed genes were annotated using the functional annotation tool of Ingenuity pathway analysis software.

Table S2. Microarray was conducted using cortical tissues of E14.5 *Usp9x*$^{+/Y}$ and *Usp9x*$^{-/Y}$ neocortices (n=4). Differentially expressed genes were annotated using the functional annotation tool of Ingenuity pathway analysis software.

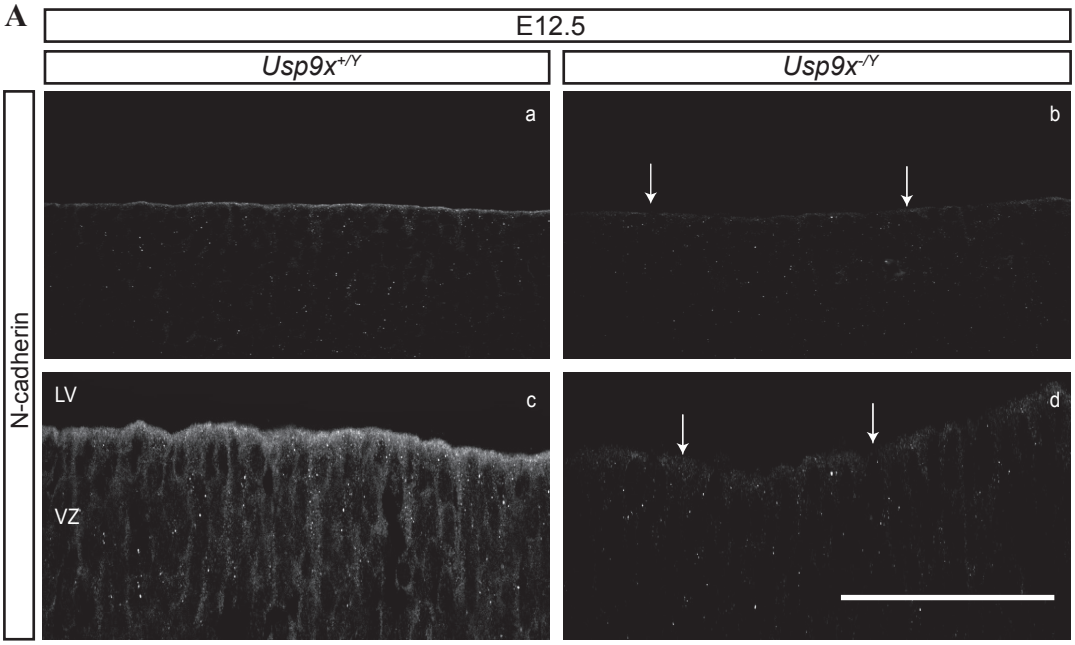
Table S3. 147 genes commonly expressed between E14.5 and E12.5 U*sp9x*$^{-/Y}$ brains.

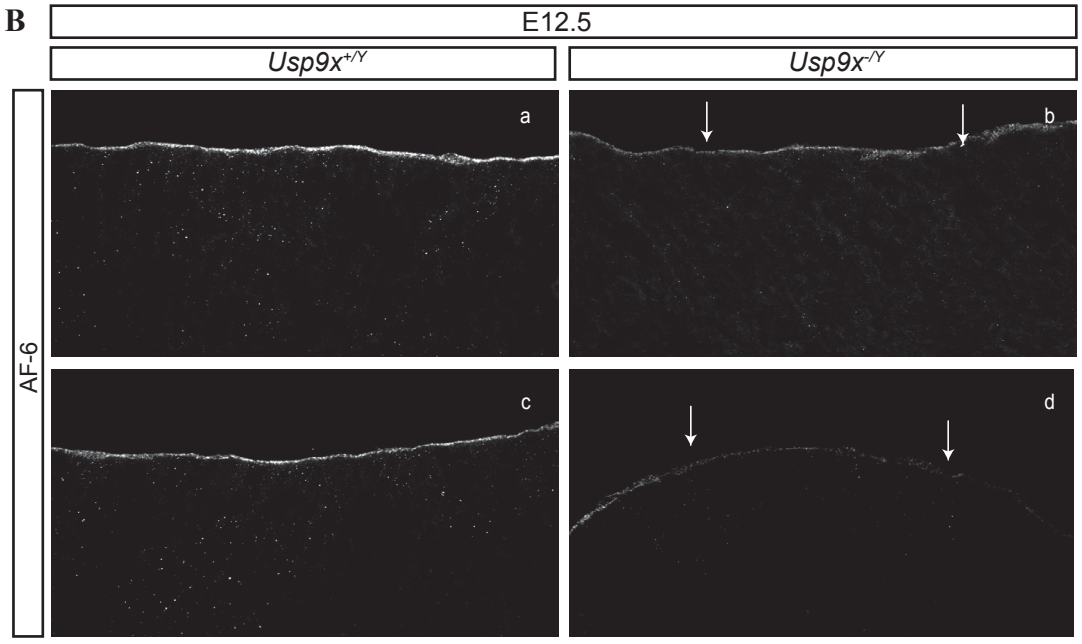
Table S4. Ingenuity pathway analysis identified Wnt signalling as one of the most significantly affected pathway in *Usp9x*$^{-/Y}$ neocortices (n=4).

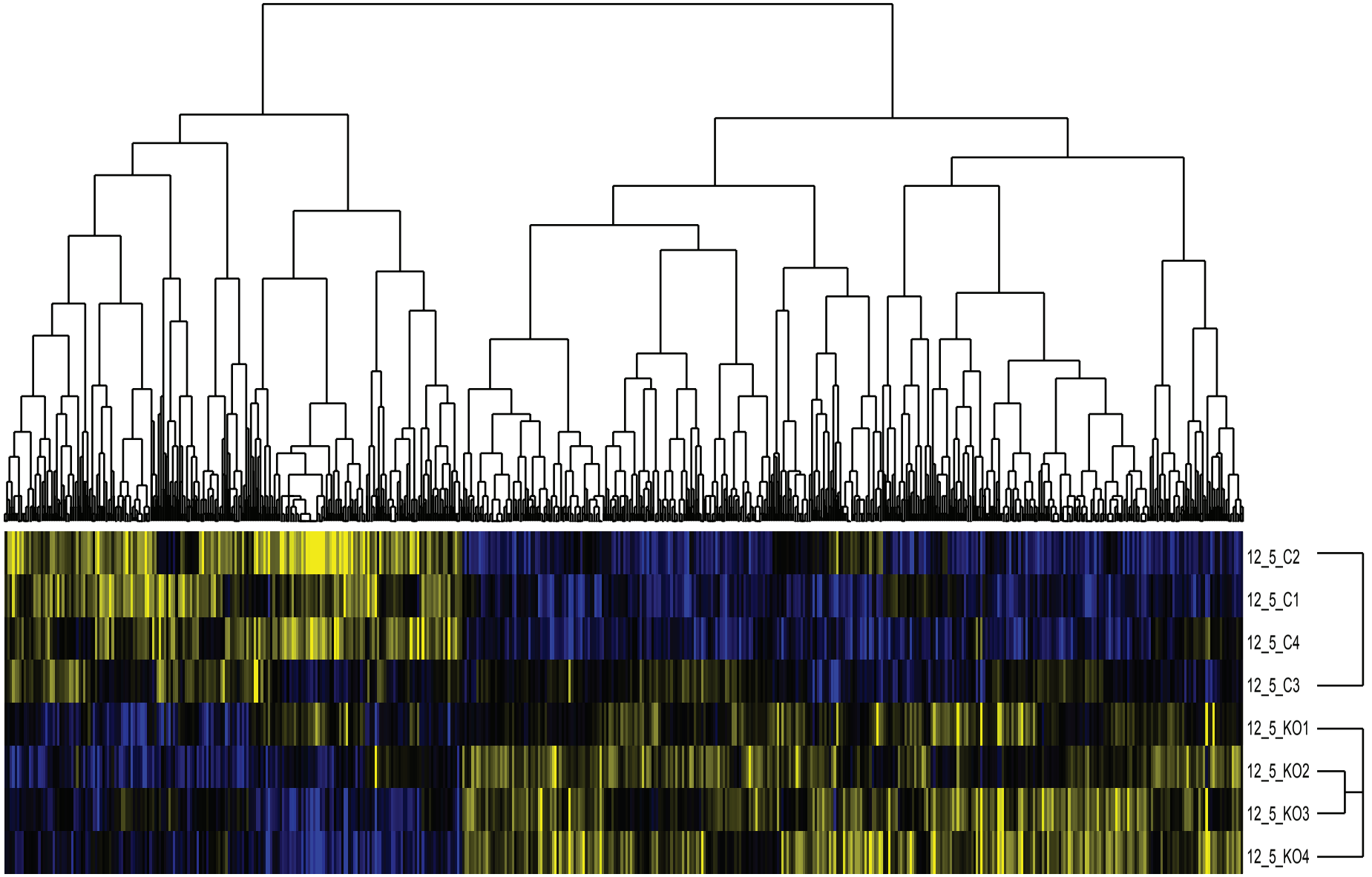
Table S5. Details of the antibodies and antibody dilutions used for IHC analysis.

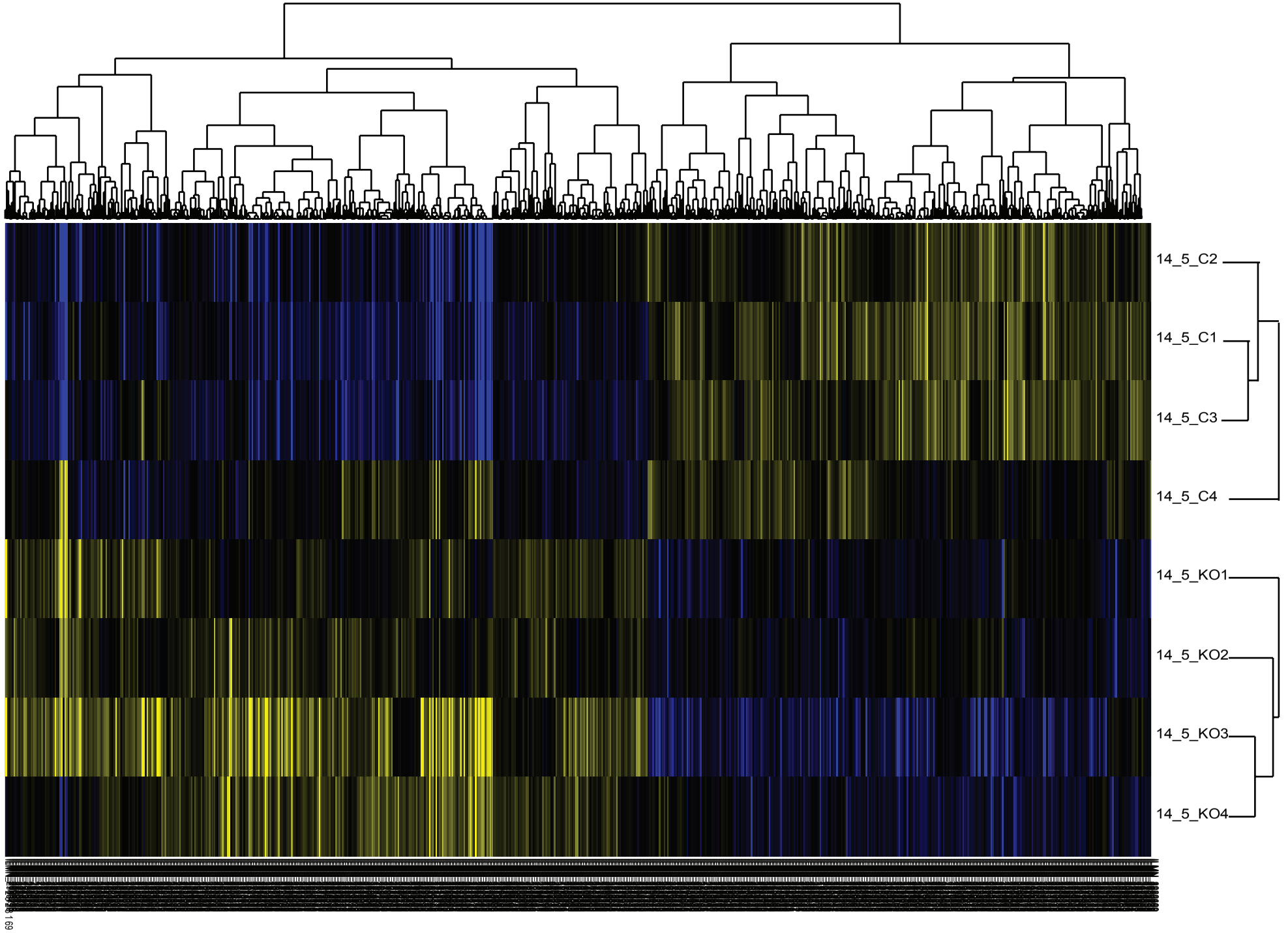
Table S6. Details of the antibodies and antibody dilutions used for IB analysis.

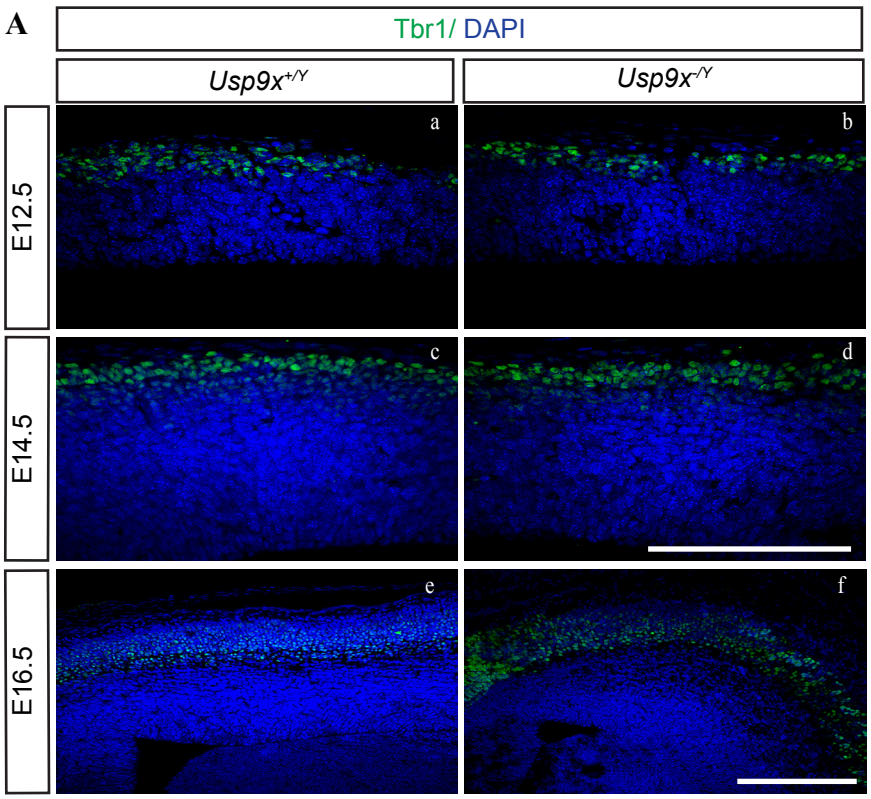
Table S7. Primer sequences.

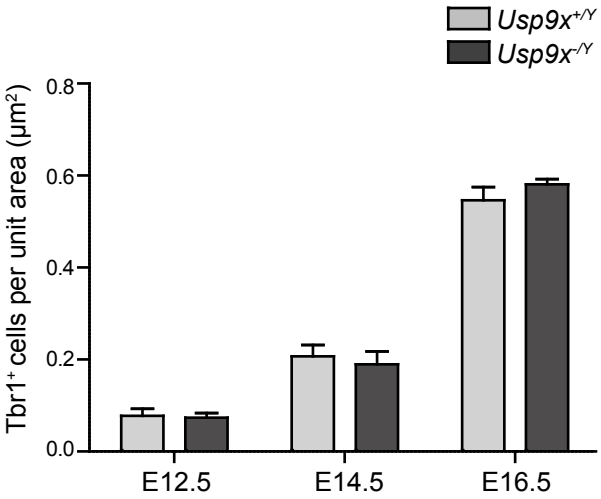

A
E12.5
Usp9x+/Y
Usp9x-/Y
N-cadherin
a
b
c
d
LV
VZ

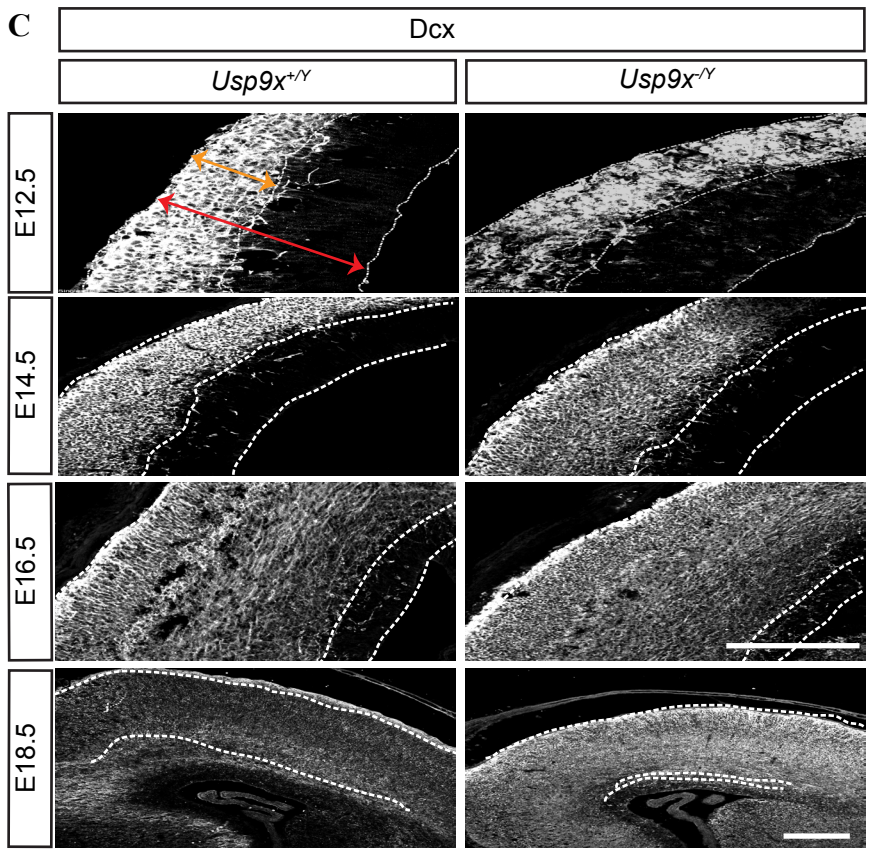

B
E12.5
Usp9x+/Y
Usp9x-/Y
AF-6
a
b
c
d

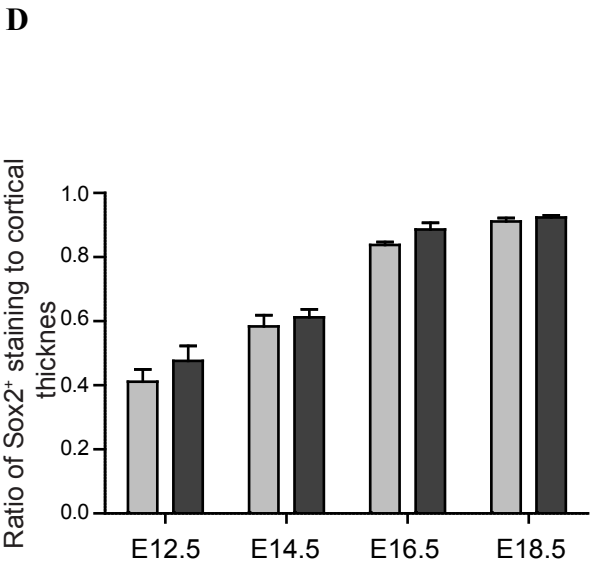

12_5_C2
12_5_C1
12_5_C4
12_5_C3
12_5_KO1
12_5_KO2
12_5_KO3
12_5_KO4

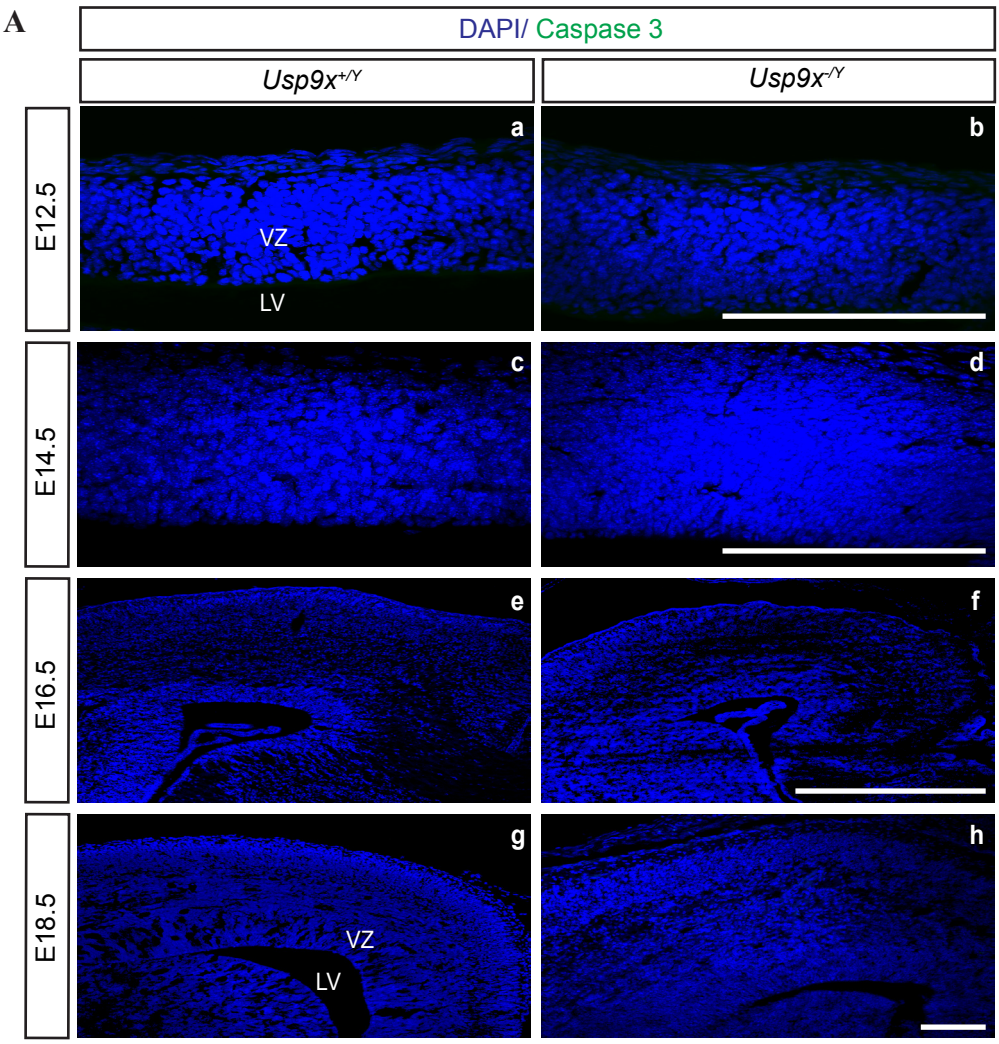

14_5_C2
14_5_C1
14_5_C3
14_5_C4
14_5_KO1
14_5_KO2
14_5_KO3
14_5_KO4

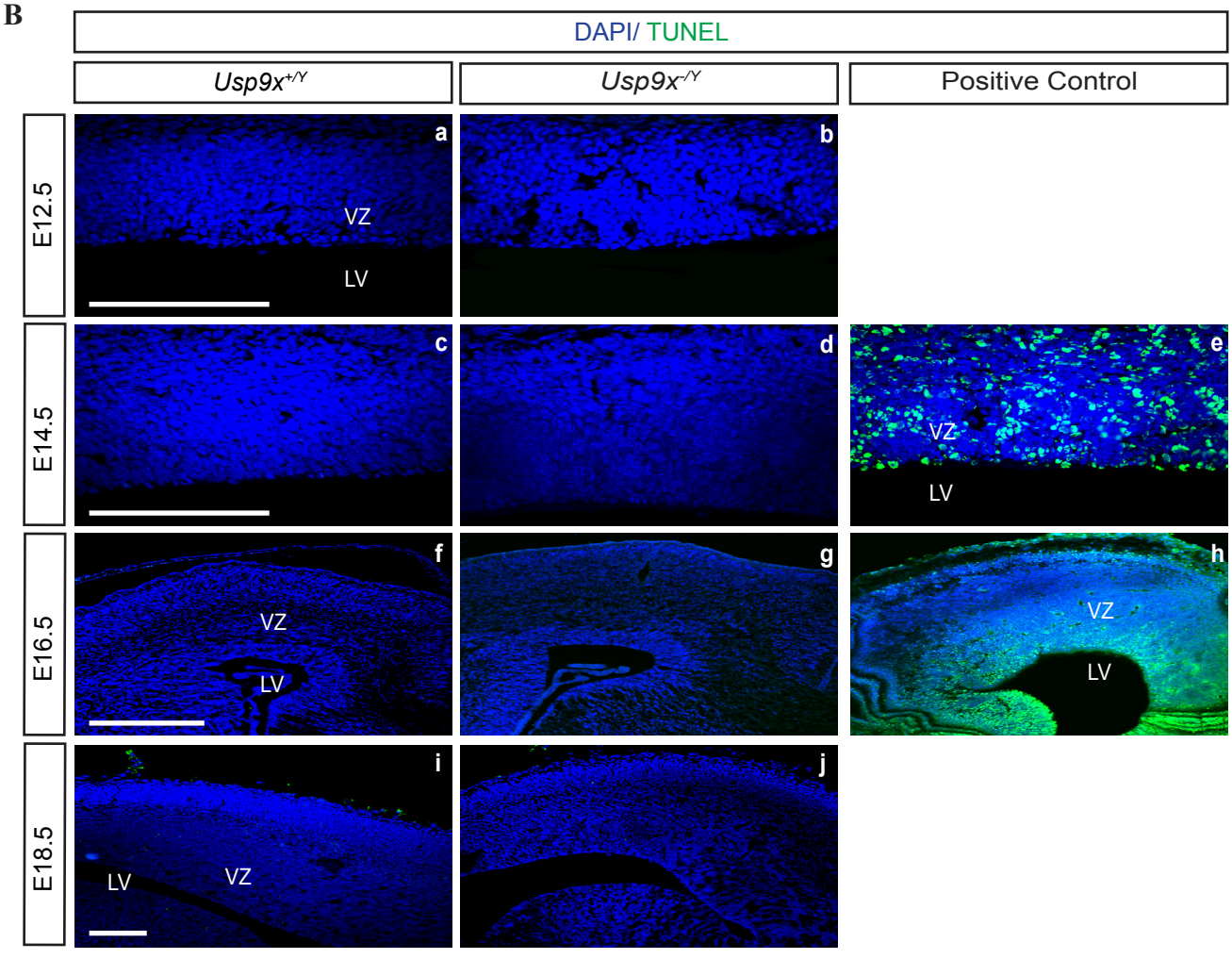

A
Tbr1/ DAPI
Usp9x+/Y
Usp9x-/Y
E12.5
E14.5
E16.5
a
b
c
d
e
f

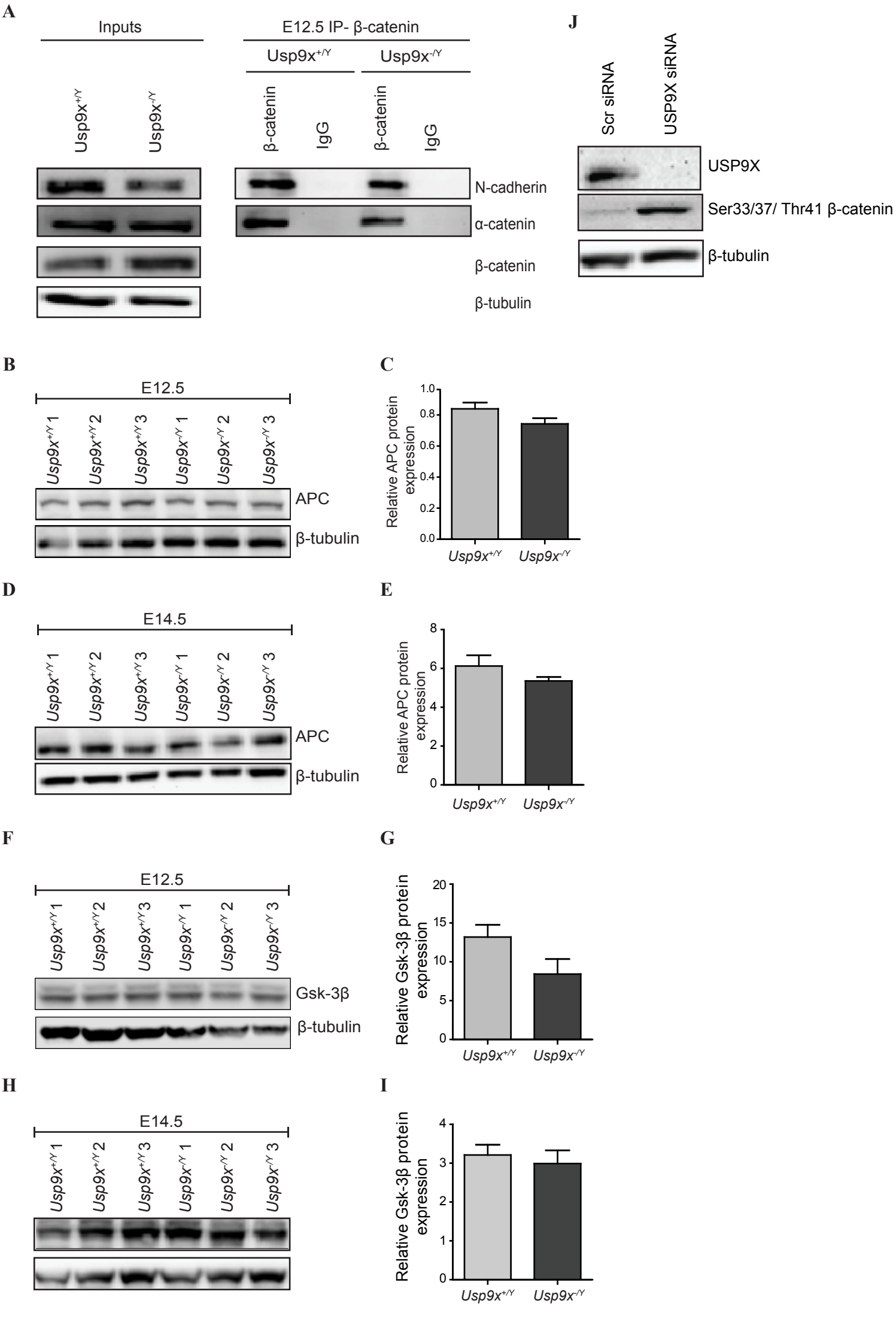

B
Usp9x+/Y
Usp9x-/Y
Tbr1+ cells per unit area (μm2)
0.0
0.2
0.4
0.6
0.8
E12.5
E14.5
E16.5

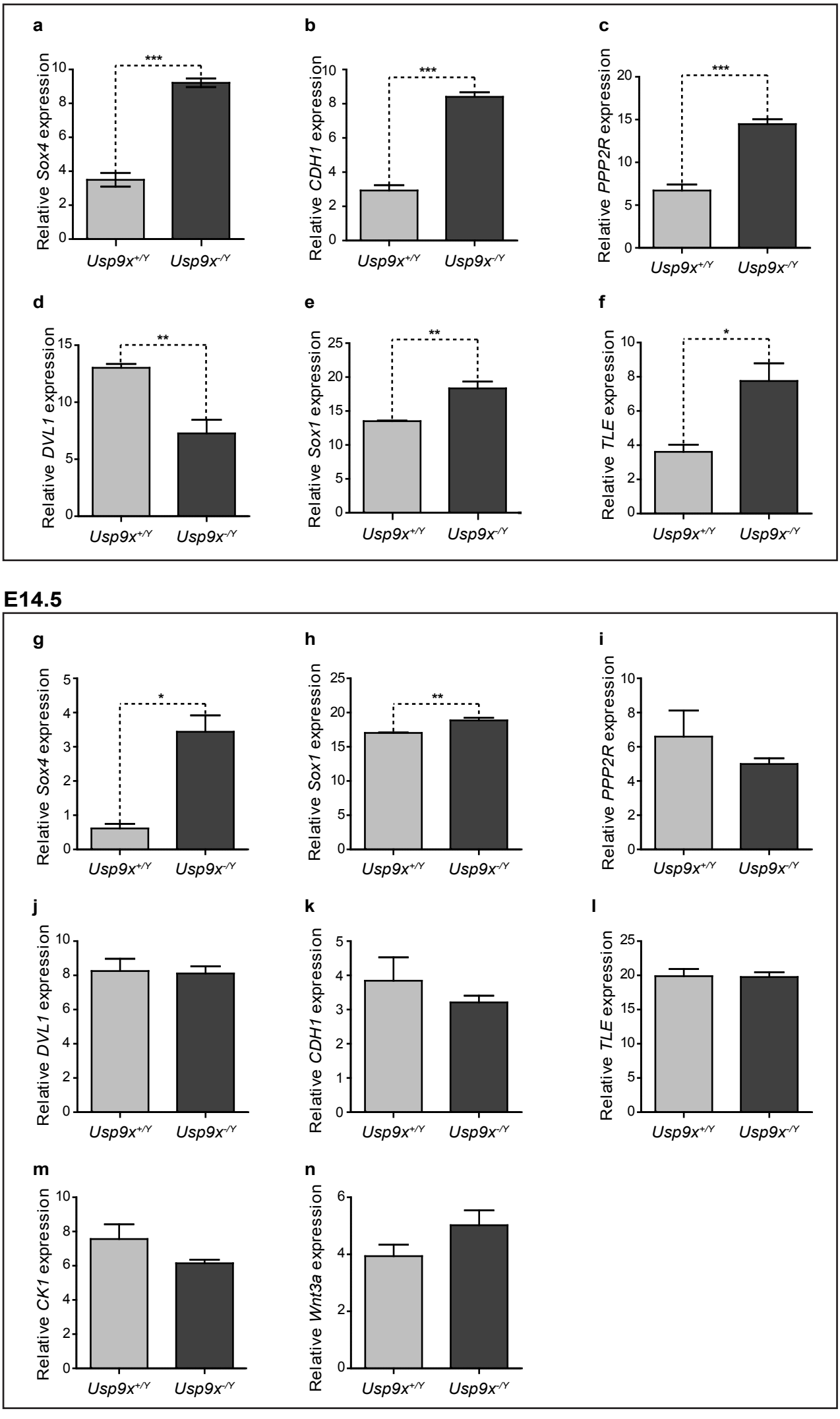

C
Dcx
Usp9x+/Y
Usp9x-/Y
E12.5
E14.5
E16.5
E18.5

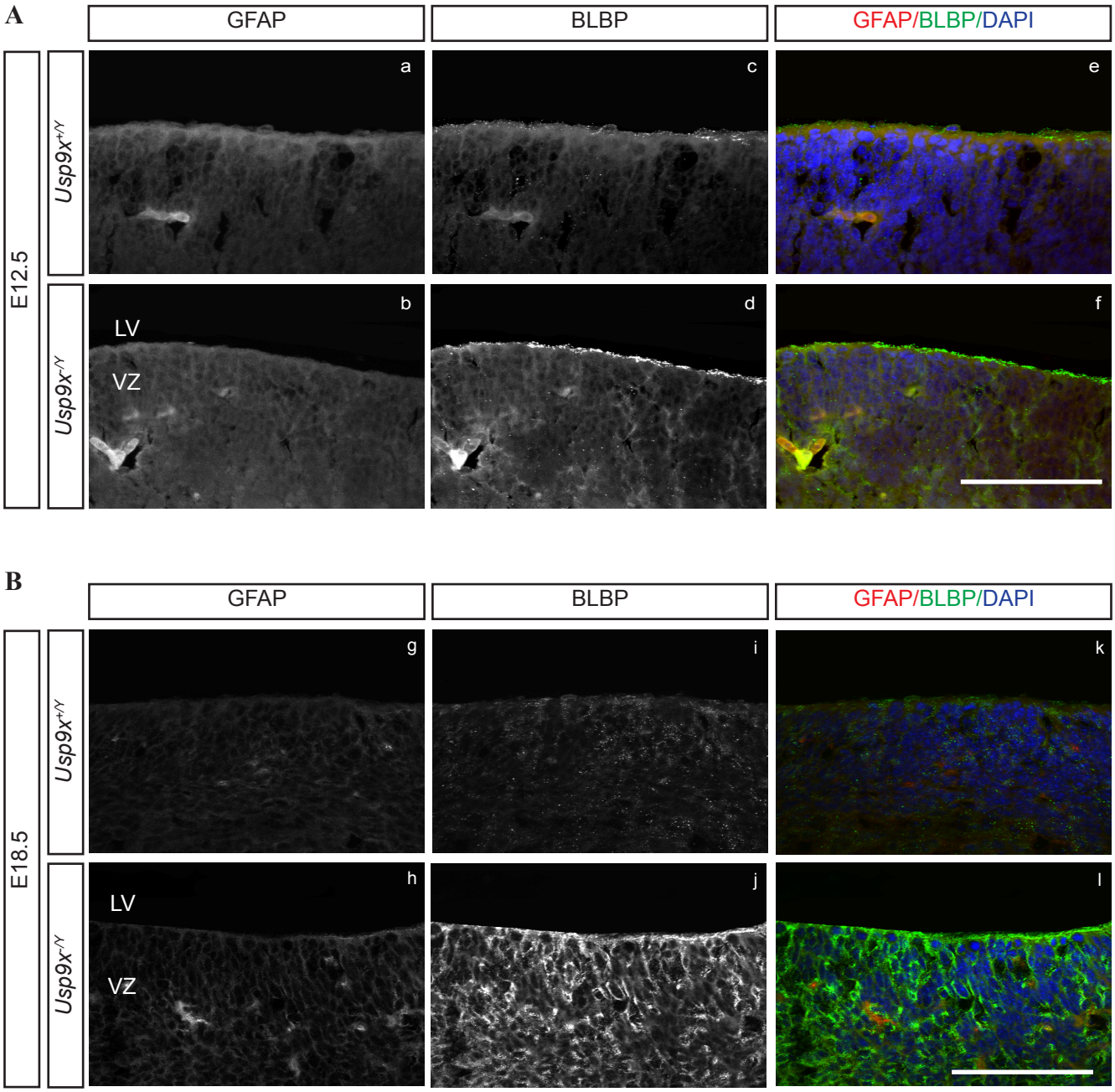

D
Ratio of Sox2+ staining to cortical thickness
0.0
0.2
0.4
0.6
0.8
1.0
E12.5
E14.5
E16.5
E18.5

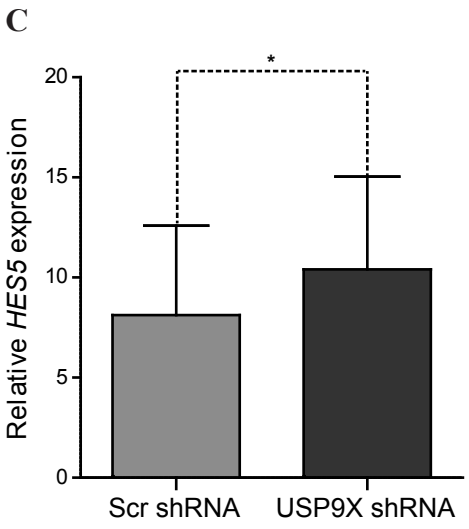

A
DAPI/ Caspase 3
Usp9x+/Y
Usp9x-/Y
E12.5
E14.5
E16.5
E18.5
a
b
c
d
e
f
g
h
VZ
LV

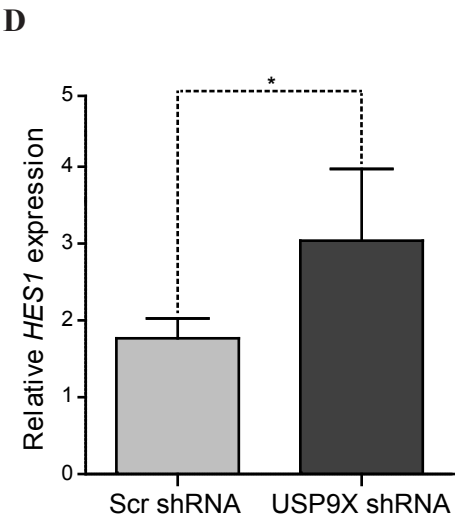

B
DAPI/ TUNEL
Usp9x+/Y
Usp9x-/Y
Positive Control
E12.5
E14.5
E16.5
E18.5
a
b
c
d
e
f
g
h
i
j
VZ
LV

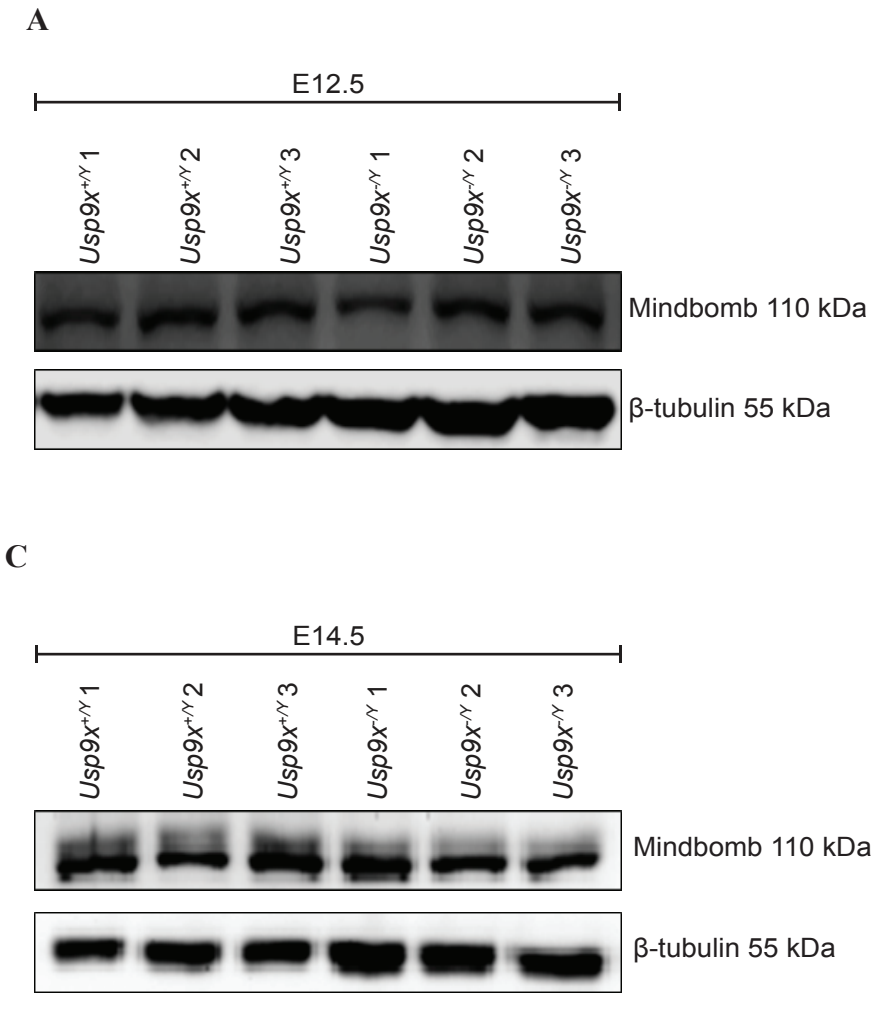

A
Inputs
E12.5 IP- β-catenin
Usp9x+/Y
Usp9x-/Y
Usp9x+/Y
Usp9x-/Y
β-catenin
IgG
β-catenin
IgG
N-cadherin
α-catenin
β-catenin
β-tubulin
J
Scr siRNA
USP9X siRNA
USP9X
Ser33/37/ Thr41 β-catenin
β-tubulin
B
E12.5
Usp9x+/Y 1
Usp9x+/Y 2
Usp9x+/Y 3
Usp9x-/Y 1
Usp9x-/Y 2
Usp9x-/Y 3
APC
β-tubulin
C
Relative APC protein expression
1.0
0.8
0.6
0.4
0.2
0.0
Usp9x+/Y
Usp9x-/Y
D
E14.5
Usp9x+/Y 1
Usp9x+/Y 2
Usp9x+/Y 3
Usp9x-/Y 1
Usp9x-/Y 2
Usp9x-/Y 3
APC
β-tubulin
E
Relative APC protein expression
8
6
4
2
0
Usp9x+/Y
Usp9x-/Y
F
E12.5
Usp9x+/Y 1
Usp9x+/Y 2
Usp9x+/Y 3
Usp9x-/Y 1
Usp9x-/Y 2
Usp9x-/Y 3
Gsk-3β
β-tubulin
G
Relative Gsk-3β protein expression
20
15
10
5
0
Usp9x+/Y
Usp9x-/Y
H
E14.5
Usp9x+/Y 1
Usp9x+/Y 2
Usp9x+/Y 3
Usp9x-/Y 1
Usp9x-/Y 2
Usp9x-/Y 3
I
Relative Gsk-3β protein expression
4
3
2
1
0
Usp9x+/Y
Usp9x-/Y

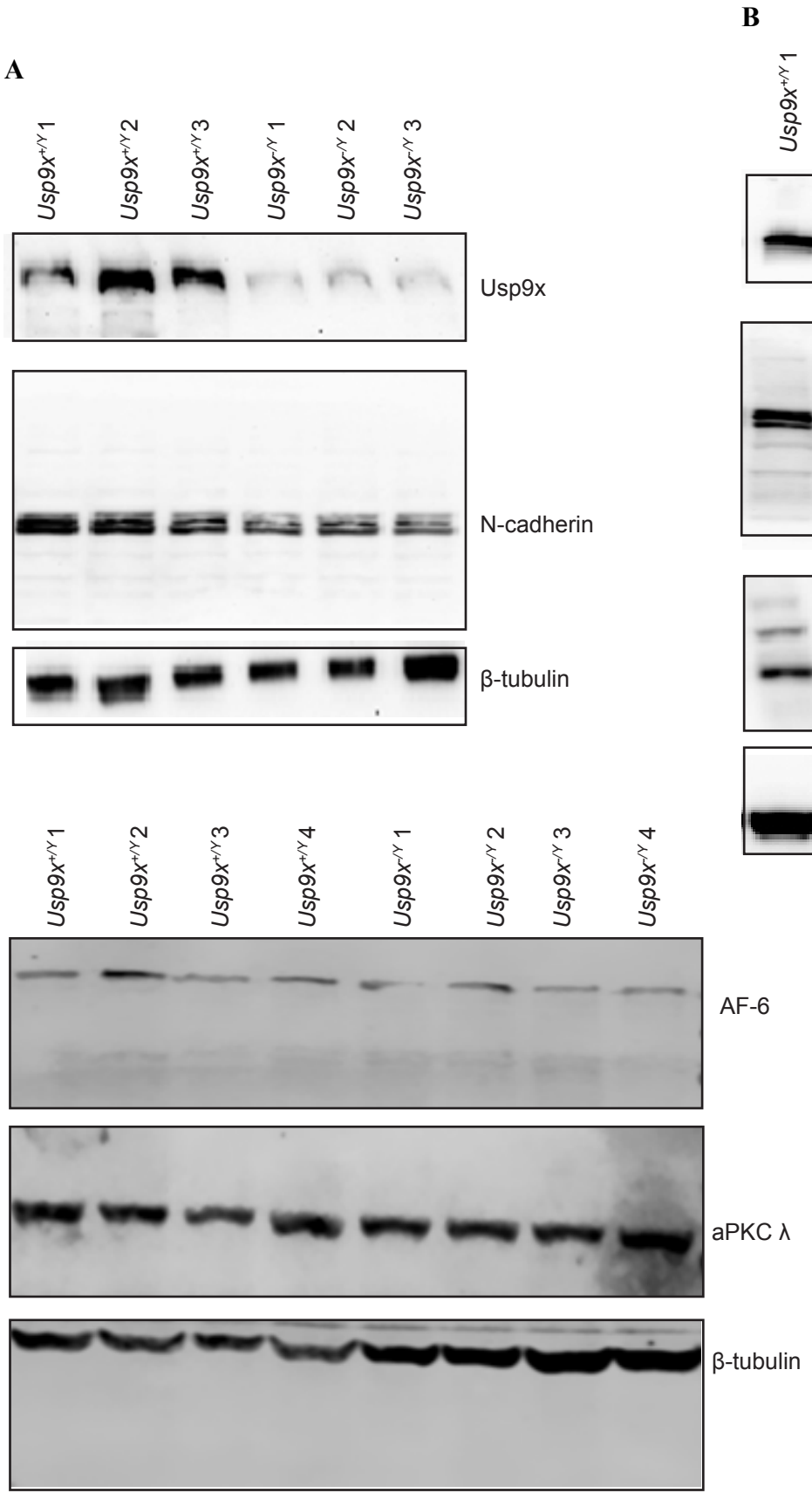

E12.5
a
Relative Sox4 expression
***
Usp9x+/Y
Usp9x-/Y
b
Relative CDH1 expression
***
Usp9x+/Y
Usp9x-/Y
c
Relative PPP2R expression
***
Usp9x+/Y
Usp9x-/Y
d
Relative DVL1 expression
**
Usp9x+/Y
Usp9x-/Y
e
Relative Sox1 expression
**
Usp9x+/Y
Usp9x-/Y
f
Relative TLE expression
*
Usp9x+/Y
Usp9x-/Y
E14.5
g
Relative Sox4 expression
*
Usp9x+/Y
Usp9x-/Y
h
Relative Sox1 expression
**
Usp9x+/Y
Usp9x-/Y
i
Relative PPP2R expression
Usp9x+/Y
Usp9x-/Y
j
Relative DVL1 expression
Usp9x+/Y
Usp9x-/Y
k
Relative CDH1 expression
Usp9x+/Y
Usp9x-/Y
l
Relative TLE expression
Usp9x+/Y
Usp9x-/Y
m
Relative CK1 expression
Usp9x+/Y
Usp9x-/Y
n
Relative Wnt3a expression
Usp9x+/Y
Usp9x-/Y

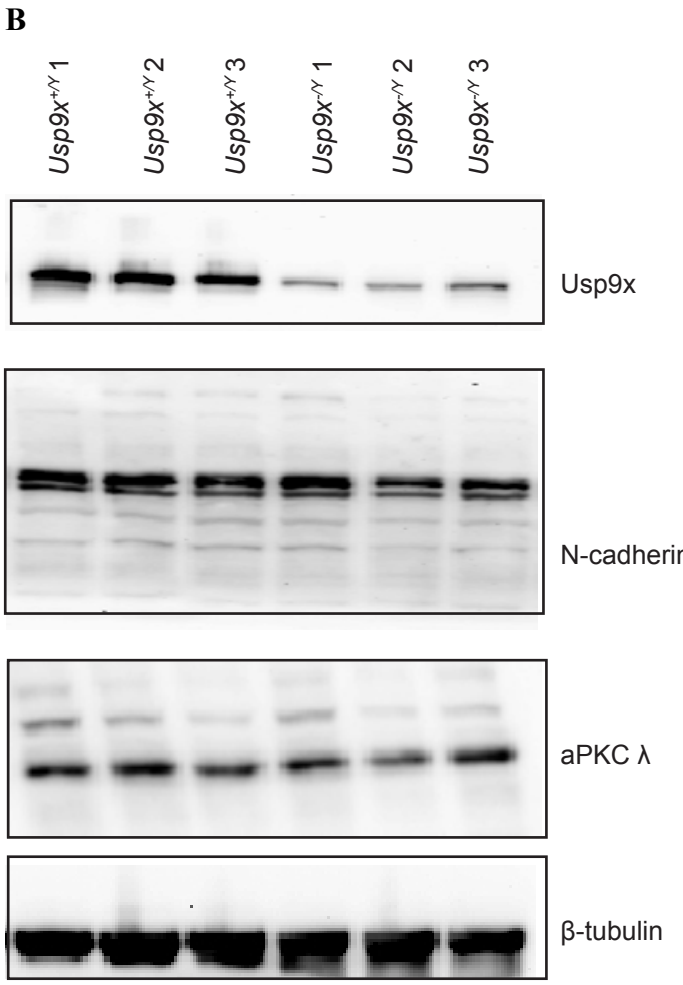

A
GFAP
BLBP
GFAP/BLBP/DAPI
E12.5
Usp9x+/Y
Usp9x-/Y
a
b
c
d
e
f
LV
VZ
B
GFAP
BLBP
GFAP/BLBP/DAPI
E18.5
Usp9x+/Y
Usp9x-/Y
g
h
i
j
k
l
LV
VZ


C
Relative HES5 expression
20
15
10
5
0
*
Scr shRNA
USP9X shRNA

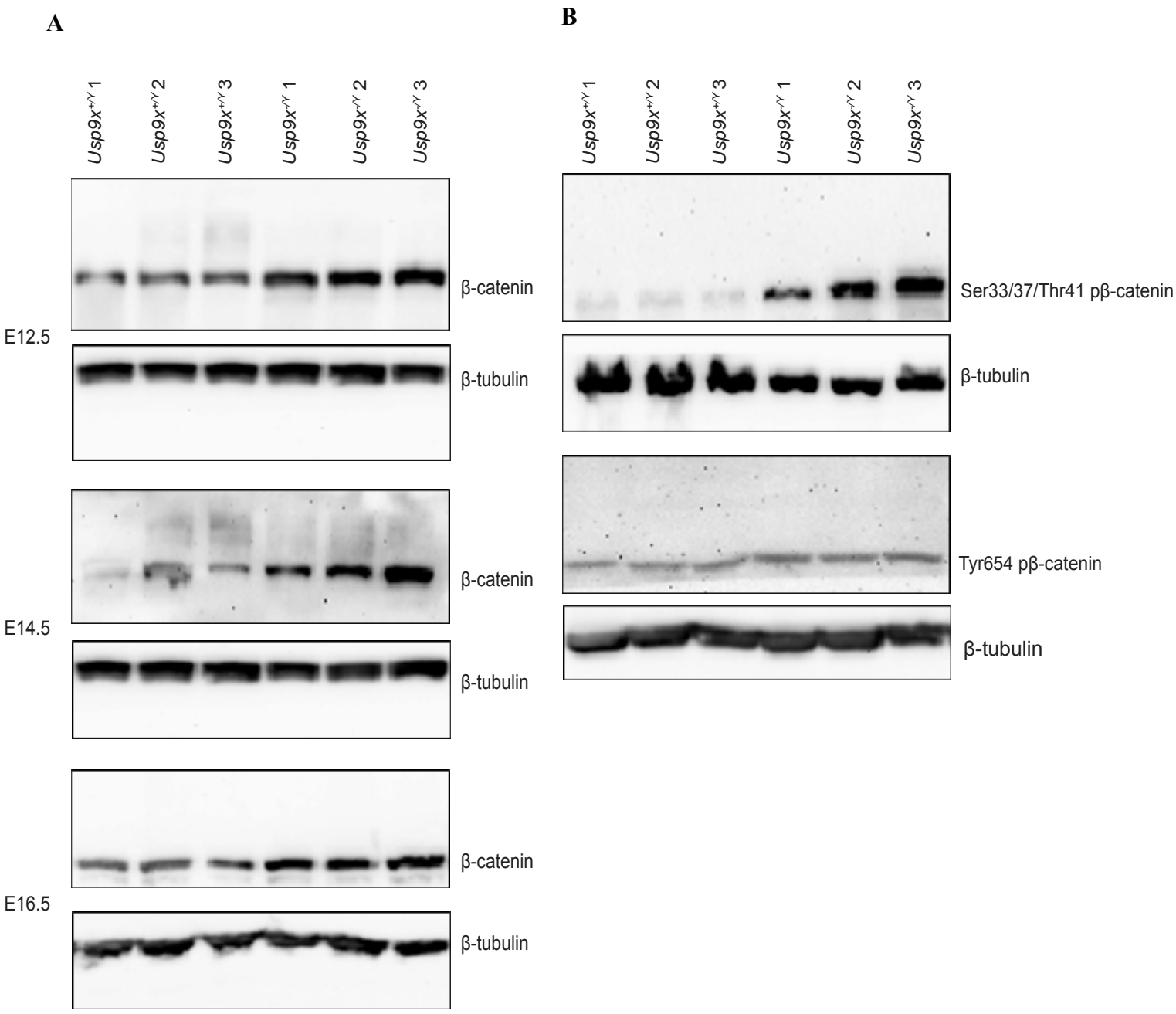

D
Relative HES1 expression
5
4
3
2
1
0
*
Scr shRNA
USP9X shRNA


A
E12.5
Usp9x+/Y 1
Usp9x+/Y 2
Usp9x+/Y 3
Usp9x-/Y 1
Usp9x-/Y 2
Usp9x-/Y 3
Mindbomb 110 kDa
β-tubulin 55 kDa
B
Relative Mindbomb protein Expression
0.0
0.1
0.2
0.3
Usp9x+/Y
Usp9x-/Y
C
E14.5
Usp9x+/Y 1
Usp9x+/Y 2
Usp9x+/Y 3
Usp9x-/Y 1
Usp9x-/Y 2
Usp9x-/Y 3
Mindbomb 110 kDa
β-tubulin 55 kDa
D
Relative Mindbomb protein Expression
0.0
0.5
1.0
1.5
Usp9x+/Y
Usp9x-/Y



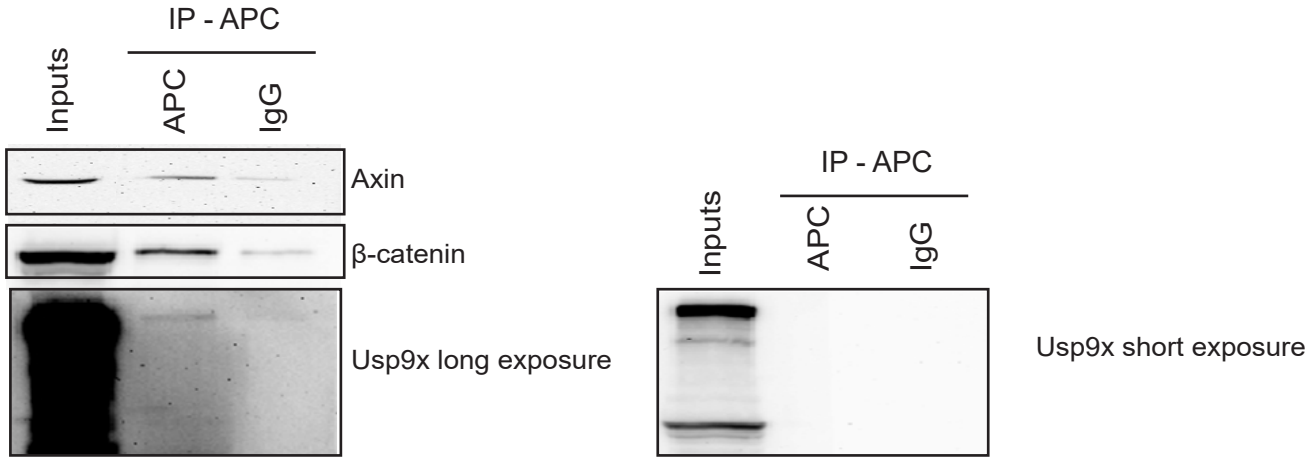


Wood Supplementary Figure 10 to match Figure 1B and 1E full length westernblots



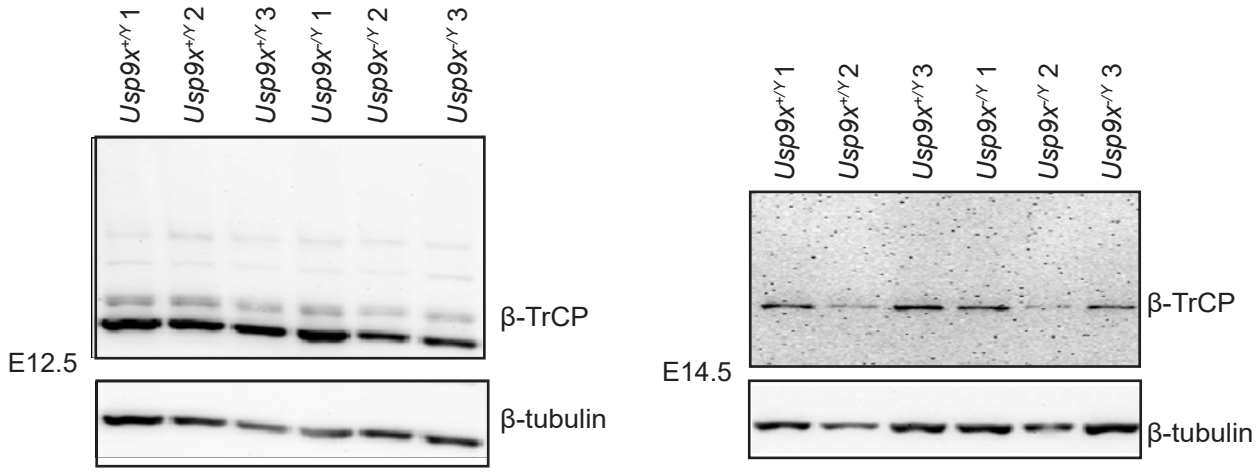
Wood Supplementary Figure 11 to match Figure 3B and 3F full length westernblots

A



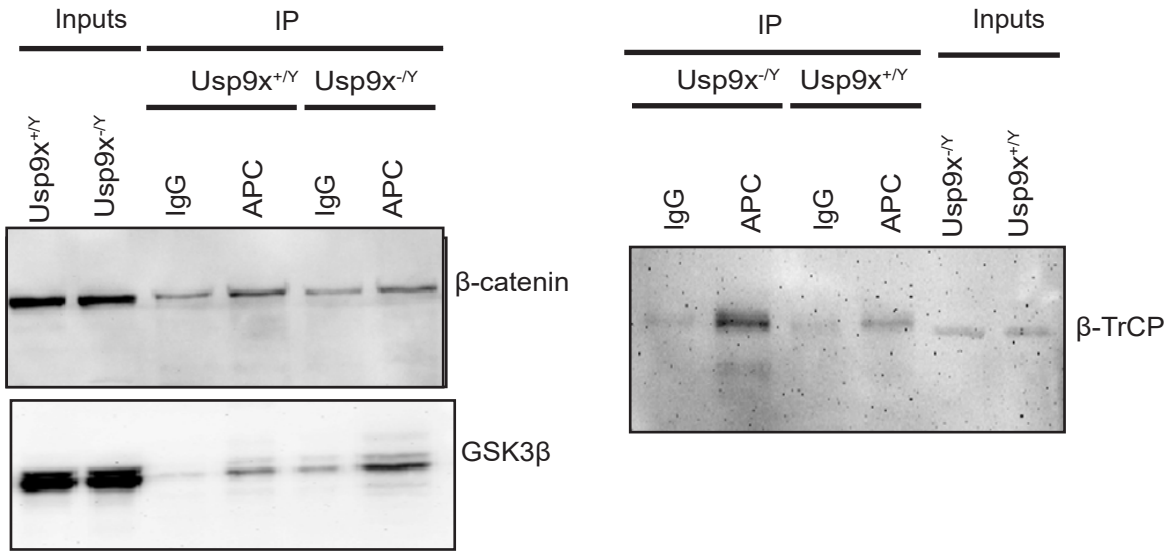
Full length westernblots of Figure 4B

B



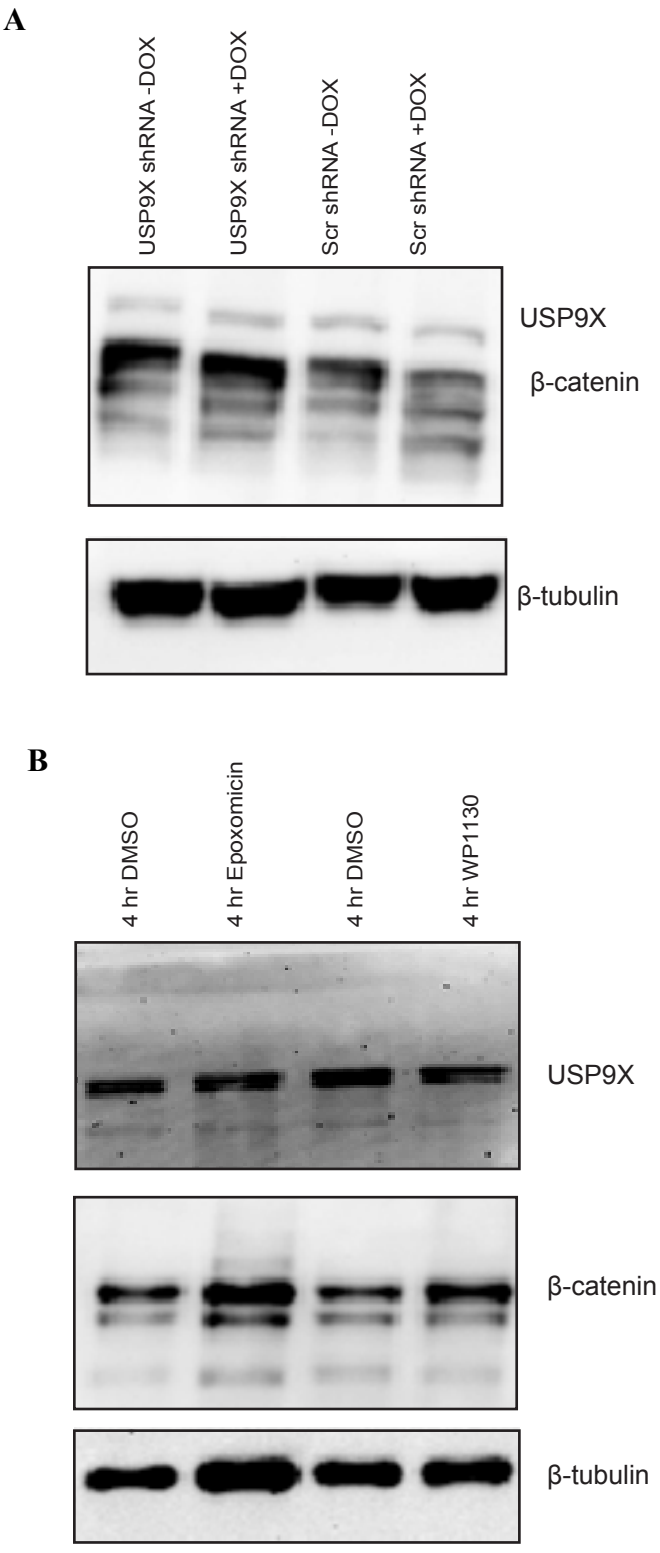
Full length westernblots of Figure 4D

C

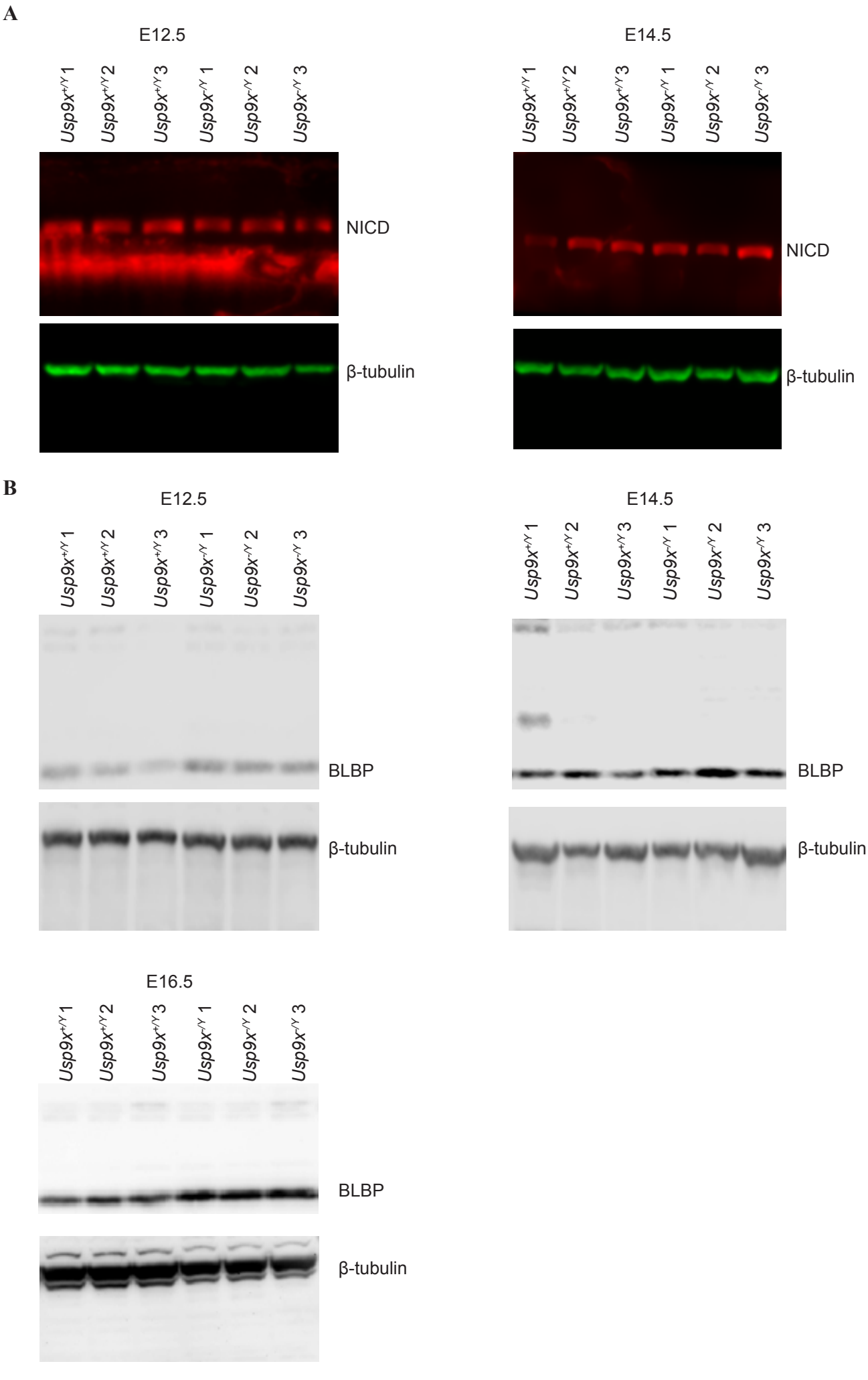


Full length westernblots of Figure 4F

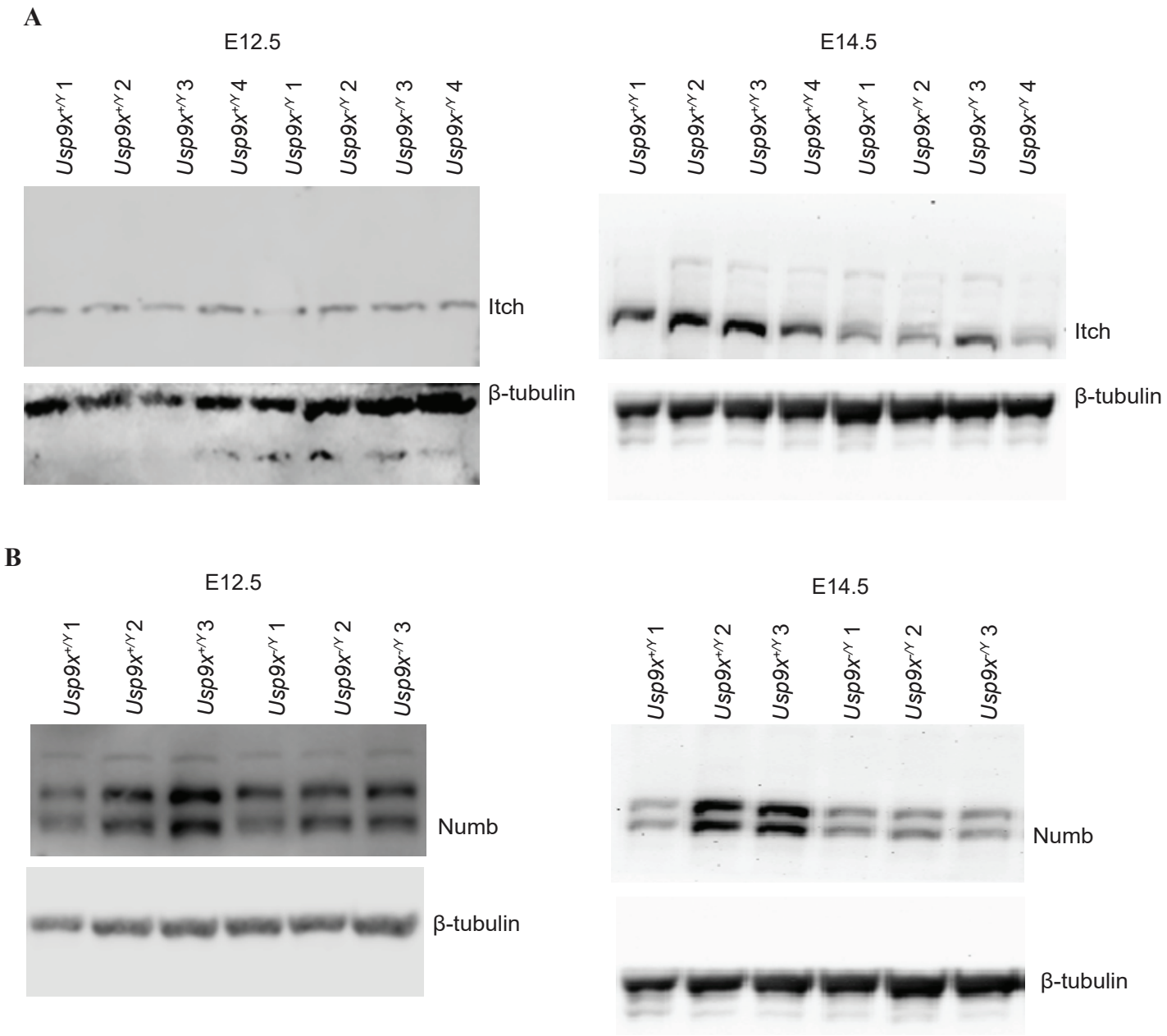
Wood Supplementary Figure 12 to match Figure 4B, F and D full length westernblots



Wood Supplementary Figure 13 to match Figure 5D and Figure 5G full length westernblots



Wood Supplementary Figure 14 to match Figure 6A and Figure 6E full length westernblots



Wood Supplementary Figure 15 to match Figure 7A, D and H full length westernblots