Ubiquitin ligase COP1 coordinates transcriptional programs that control cell type specification in the developing mouse brain

Kim Newton[a,1], Debra L. Dugger[a], Arundhati Sengupta-Ghosh[b], Ronald E. Ferrando[c,2], Felix Chu[c,3], Janet Tao[c], Wendy Lam[c,4], Susan Haller[c], Sara Chan[c], Susan Sa[c], Debra Dunlap[c], Jeffrey Eastham-Anderson[c], Hai Ngu[c], Jeffrey Hung[c], Dorothy M. French[c,2], Joshua D. Webster[c], Brad Bolon[d], Jinfeng Liu[e,5], Rohit Reja[e], Sarah Kummerfeld[e,6], Ying-Jiun Chen[f], Zora Modrusan[f], Joseph W. Lewcock[b,7], and Vishva M. Dixit[a,1]

[a]Department of Physiological Chemistry, Genentech, South San Francisco, CA 94080; [b]Department of Neuroscience, Genentech, South San Francisco, CA 94080; [c]Department of Pathology, Genentech, South San Francisco, CA 94080; [d]GEMpath, Longmont, CO 80504; [e]Department of Bioinformatics and Computational Biology, Genentech, South San Francisco, CA 94080; and [f]Department of Molecular Biology, Genentech, South San Francisco, CA 94080

Contributed by Vishva M. Dixit, September 18, 2018 (sent for review March 23, 2018; reviewed by Wenyi Wei and Yuan Zhu)

The E3 ubiquitin ligase CRL4$^{COP1/DET1}$ is active in the absence of ERK signaling, modifying the transcription factors ETV1, ETV4, ETV5, and c-JUN with polyubiquitin that targets them for proteasomal degradation. Here we show that this posttranslational regulatory mechanism is active in neurons, with ETV5 and c-JUN accumulating within minutes of ERK activation. Mice with *constitutive photomorphogenesis 1* (*Cop1*) deleted in neural stem cells showed abnormally elevated expression of ETV1, ETV4, ETV5, and c-JUN in the developing brain and spinal cord. Expression of c-JUN target genes *Vimentin* and *Gfap* was increased, whereas ETV5 and c-JUN both contributed to an expanded number of cells expressing genes associated with gliogenesis, including *Olig1*, *Olig2*, and *Sox10*. The mice had subtle morphological abnormalities in the cerebral cortex, hippocampus, and cerebellum by embryonic day 18 and died soon after birth. Elevated c-JUN, ETV5, and ETV1 contributed to the perinatal lethality, as several *Cop1*-deficient mice also lacking *c-Jun* and *Etv5*, or lacking *Etv5* and heterozygous for *Etv1*, were viable.

COP1 | ETV1 | ETV4 | ETV5 | c-JUN

Constitutive photomorphogenesis 1 (COP1; also called RFWD2) is the evolutionarily conserved substrate adaptor of the cullin-RING ubiquitin ligase CRL4$^{COP1/DET1}$ (1). COP1 binds to De-etiolated 1 (DET1), which in turn binds to Damage-specific DNA binding protein 1 (DDB1) that is in complex with scaffold protein CUL4A and Ring-box 1 (2–4). Substrates of mammalian CRL4$^{COP1/DET1}$ include the transcription factors c-JUN, ETV1, ETV4, ETV5, ETS1, ETS2, and C/EBPα, the metabolic enzyme acetyl-CoA carboxylase, and CREB regulated transcription coactivator 2 (2, 3, 5–8). Ubiquitination of these substrates targets them for proteasomal degradation.

Studies with COP1-deficient mice suggest that regulation of c-JUN, ETV1, and ETV4 abundance contributes to tumor suppression by COP1 (3, 9). For example, COP1 deficiency in mouse prostate epithelial cells results in elevated ETV1, ETV4, and c-JUN and early prostate intraepithelial neoplasia (3). COP1 also has an important role in regulating ETV1, ETV4, and ETV5 levels in pancreatic β-cells so that insulin secretion is not perturbed (10). CRL4$^{COP1/DET1}$ activity is tightly regulated, with phosphorylation of DET1 by ERK contributing to inactivation of the ligase. Consequently, cells exposed to growth factors exhibit rapid accumulation of CRL4$^{COP1/DET1}$ substrates (4).

Although COP1 deficiency in many tissues is deleterious, inappropriate CRL4$^{COP1/DET1}$ activity can also promote disease. For example, C/EBPα degradation orchestrated by COP1 in combination with the pseudokinase TRIB1 (or TRIB2) is linked to impaired myeloid differentiation and the development of acute myeloid leukemia (7, 11). Inappropriate CRL4$^{COP1/DET1}$ activity may also have consequences for the development of the central nervous system because increased *COP1* copy number is linked to autism in humans (12).

Potential substrates of CRL4$^{COP1/DET1}$ in the brain include the transcription factors c-JUN, ETV1, ETV4, and ETV5. Activator protein-1 (AP-1) transcription factor c-JUN is broadly expressed in the developing brain, regulates neuronal apoptosis in response to phosphorylation by upstream kinases (13), and is a substrate of both CRL4$^{COP1/DET1}$ and the ubiquitin ligase SCF^{FBW7}. Posttranslational regulation of c-JUN is critical to neurogenesis because deletion of *Fbw7* from neural stem cells elevates c-JUN and reduces cell viability (14). Regulation of c-JUN abundance by FBW7 in the granule cell layer also plays an important role in development of the cerebellum (15).

Significance

The ubiquitin ligase CRL4$^{COP1/DET1}$ modifies specific transcription factor substrates with polyubiquitin so that they are degraded. However, the Ras–MEK–ERK signaling pathway can inactivate CRL4$^{COP1/DET1}$ and thereby promote the rapid accumulation of these transcription factors. Here we show that constitutive photomorphogenesis 1 (COP1) has a critical role in mouse brain development because its deletion from neural stem cells stabilizes the transcription factors c-JUN, ETV1, ETV4, and ETV5, leading to perturbation of normal gene expression patterns; anatomic anomalies in cerebral cortex, hippocampus, and cerebellum; and perinatal lethality.

Author contributions: K.N., D.L.D., A.S.-G., D.M.F., J.D.W., Z.M., J.W.L., and V.M.D. designed research; D.L.D., A.S.-G., R.E.F., F.C., J.T., W.L., S.H., S.C., S.S., D.D., and Y.-J.C. performed research; J.E.-A., H.N., J.H., D.M.F., J.D.W., B.B., J.L., R.R., and S.K. analyzed data; and K.N. wrote the paper.

Reviewers: W.W., Harvard University; and Y.Z., Children's National Medical Center.

Conflict of interest statement: All authors were employees of Genentech.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession nos. GSE111564 and GSE111704).

[1]To whom correspondence may be addressed. Email: knewton@gene.com or dixit@gene.com.

[2]Present address: Abbvie Biotherapeutics, South San Francisco, CA 94080.

[3]Present address: Atreca, Redwood City, CA 94063.

[4]Present address: Department of Pathology, Sutter Health, Palo Alto, CA 94301.

[5]Present address: Department of Bioinformatics, Gilead Sciences, Foster City, CA 94404.

[6]Present address: Kinghorn Centre for Clinical Genomics, Garvin Institute of Medical Research, Darlinghurst, NSW 2010, Australia.

[7]Present address: Denali Therapeutics, South San Francisco, CA 94080.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1805033115/-/DCSupplemental.

Published online October 15, 2018.

ETS family members ETV1, ETV4, and ETV5 have several roles in the developing nervous system (16–20), but less is known about their posttranslational regulation in the brain. In developing mouse cortex, *Etv1* mRNA marks a subpopulation of early Cajal–Retzius neurons that are specified by FGF8 signaling, whereas, at later stages, it serves as a marker of layer five cerebrocortical neurons (21–23). *Etv4* and *Etv5* are also expressed early in cortical development, but they show a distinct pattern of expression to *Etv1* (23, 24). Expression of *Etv5* in the ventricular zone of the mouse cerebral cortex is induced by the MAPKs MEK1 (also called MAP2K1) and MEK2 (MAP2K2), and this is proposed to confer an astrocytic fate on neural stem and progenitor cells (18). MEK1 and MEK2 are part of the RAF–MEK–ERK kinase cascade that is engaged by RAS GTPases. Consistent with this pathway regulating the switch from neurogenesis to gliogenesis, deletion of the RAS negative regulator neurofibromatosis 1 (NF1) from neural stem cells promotes ERK-dependent gliogenesis at the expense of neurogenesis in the olfactory bulb during perinatal stages (19). ETV transcription factors have also been implicated in glioma initiation by oncogenic RAS (25). Whether ERK-dependent posttranslational mechanisms, potentially involving $CRL4^{COP1/DET1}$, regulate expression of ETV1, ETV4, and/or ETV5 protein in the brain has not been examined to our knowledge. We investigated how COP1 impacts brain development by deleting *Cop1* in neural stem and progenitor cells with Nestin.cre transgenic mice, or in cells of the neocortex and hippocampus with $Emx1^{IRES\ cre/+}$ knock-in mice.

Results

COP1 Mediates Posttranslational Regulation of c-JUN, ETV1, and ETV5 During Brain Development. To determine whether COP1 and DET1 expressed in the developing mouse brain interact with known $CRL4^{COP1/DET1}$ substrates, epitope-tagged versions of COP1 and DET1 were affinity-purified from embryonic day 18.5 (E18.5) knock-in mouse brains (Fig. 1*A*). ETV5 and DET1 copurified with Flag-HA-COP1. These interactions were specific because ETV5 and COP1 also copurified with DET1-3xFlag, but not with an unrelated protein, ARMC8-3xFlag. Consistent with COP1 being the substrate adaptor for ETV5, deletion of *Cop1* from neural stem cells with a Nestin.cre transgene (*Cop1ΔN* mice) reduced the amount of COP1 and ETV5 that copurified with DET1-3xFlag, but had no effect on the interaction of DET1 with DDB1.

One consequence of posttranslational regulation of protein stability is that protein abundance may not correlate with mRNA abundance. Evidence for posttranslational regulation of ETV5 was obtained with E15.5 cortical neural cultures, which we treated with BDNF or picrotoxin (PTX) to mimic the signals that neurons might encounter during development. BDNF engages the tyrosine kinase receptor TrkB on neurons, whereas PTX blocks the GABA-activated chloride channel and thereby promotes synaptic activity. BDNF treatment for 2 h increased ETV5 protein and *Etv5* mRNA, as well as c-JUN protein and *c-Jun* mRNA (Fig. 1*B*). ERK inhibition, which reduced phosphorylation of the ERK substrate ribosomal S6 kinase, prevented this response to BDNF. However, PTX increased ETV5 and c-JUN protein abundance in an ERK-dependent manner within 5 min without increasing *Etv5* and *c-Jun* mRNA expression (Fig. 1*C*). This rapid accumulation of ETV5 and c-JUN is consistent with them being subject to posttranslational regulation. Interestingly, ectopic COP1 prevented the PTX-induced increase in ETV5, whereas mutant COP1Δ24 that is unable to bind to DET1 (2) had no effect (Fig. 1*D*). This result suggests that ERK-mediated inhibition of $CRL4^{COP1/DET1}$ is inefficient when COP1 is in excess.

We sought genetic proof that COP1 regulates ETV5 in the developing mouse brain by using *Cop1ΔN* ($Cop1^{fl/-}$ Nestin.cre) mice and *Cop1ΔE* ($Cop1^{fl/fl}$ $Emx1^{IRES\ cre/+}$) mice. Many *Cop1ΔN* mice died within a few days of birth, and none survived to weaning (*SI Appendix*, Fig. S1*A*). Abnormalities were evident in

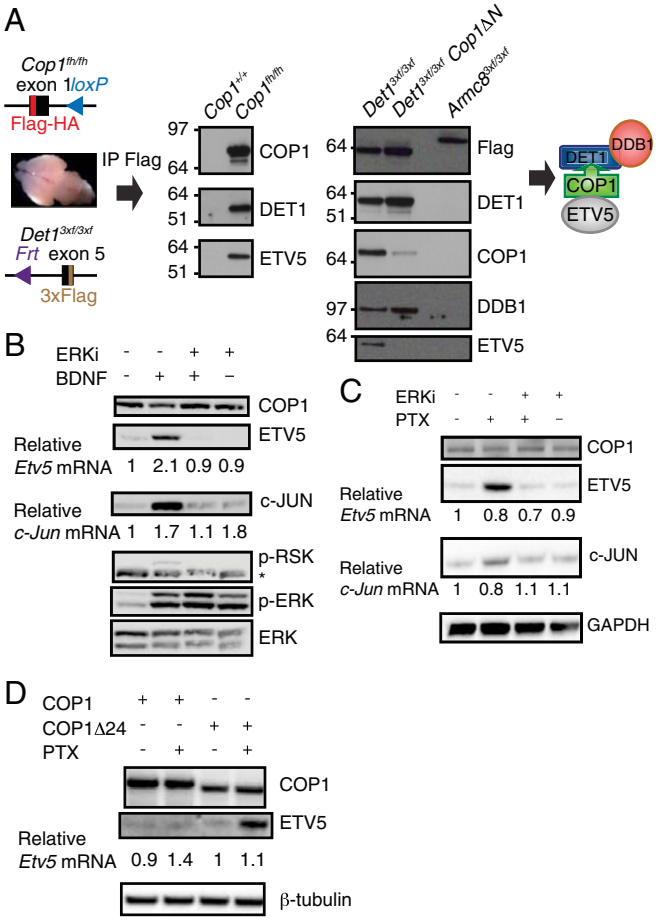

Fig. 1. Posttranslational regulation of ETV5 and c-JUN in neurons. (*A*) Western blots of E18.5 mouse brain lysates after immunoprecipitation (IP) with anti-FLAG M2 beads. (*B*) Western blots of cortical neurons cultured for 2 d and then stimulated with 100 ng/mL BDNF with or without 1 μM ERK inhibitor (ERKi) for 2 h. mRNA expression was determined by quantitative RT-PCR. (*C*) Western blots of cortical neurons cultured for 14 d and then stimulated with 100 μM PTX with or without 1 μM ERKi for 5 min. (*D*) Western blots of cortical neurons infected with adeno-associated viruses expressing WT or mutant COP1. Results in *A*–*D* are representative of three independent experiments.

the postnatal day 0 (P0) cerebral cortex, hippocampus, and cerebellum (Fig. 2). Neurons in cortical layers 2 and 3 of the frontal and parietal regions appeared more densely packed than in littermate controls, the thickness of the molecular layer in the hippocampus was reduced, and the granular cell layer of the cerebellum exhibited indistinct lobulation and hypocellularity. *Cop1ΔE* mice, which lacked COP1 in cells of the neocortex and hippocampus (*SI Appendix*, Fig. S1*B*), displayed similar cerebrocortical disorganization as *Cop1ΔN* mice (*SI Appendix*, Fig. S1*C*). *Cop1ΔE* mice had a median survival of 147 d (*SI Appendix*, Fig. S1*D*), and those that survived to 35 d tended to be smaller than their $Cop1^{+/+}$ $Emx1^{IRES\ cre/+}$ littermates (*SI Appendix*, Fig. S1*E*).

In keeping with COP1 regulating ETV5 abundance via a posttranslational mechanism, E18.5 or P0 *Cop1ΔN* brains contained more ETV5 than control $Cop1^{fl/+}$ Nestin.cre brains (Fig. 3 *A* and *B*), but they did not express more *Etv5* mRNA (Fig. 3 *B* and *C*). $CRL4^{COP1/DET1}$ substrates c-JUN and ETV1 were also more abundant in *Cop1ΔN* brains (Fig. 3*A* and *SI Appendix*, Fig. S2*A*), even though *c-Jun* and *Etv1* mRNAs were not increased (Fig. 3*C* and *SI Appendix*, Fig. S2*A*). Indeed, *Cop1ΔN* brains contained less *Etv1* or *Etv5* mRNA than control brains (Fig. 3*C*),

DEVELOPMENTAL BIOLOGY

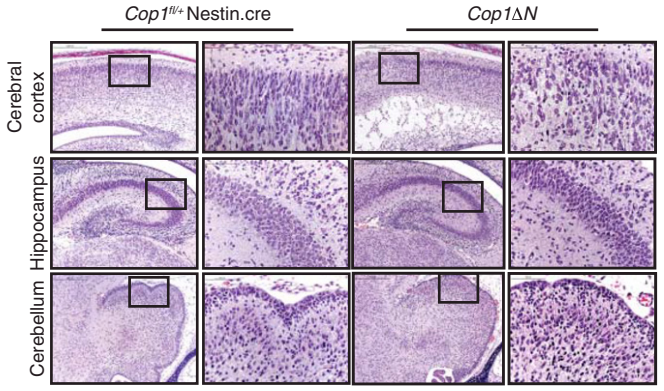

Fig. 2. COP1 is required for normal brain development. Brain sections from P0 littermates stained with H&E. Boxed regions in columns 1 and 3 are shown at higher magnification in columns 2 and 4, respectively. (Scale bars: 50 μm; boxed regions, 200 μm.) Results are representative of four mice per genotype.

suggestive of a negative feedback signaling loop in response to elevated ETV1 and ETV5. ETV1, ETV5, and c-JUN were also more abundant in *Cop1ΔE* brains (*SI Appendix*, Fig. S2*B*). We confirmed the specificity of our ETV1 and ETV5 monoclonal antibodies with ETV1- and ETV5-deficient mouse brains (*SI Appendix*, Fig. S2 *C* and *D*).

Given that *Cop1*, *Etv1*, *Etv5*, and *c-Jun* are also expressed in the brain and spinal cord at earlier stages of embryogenesis (*SI Appendix*, Fig. S2*E*) (16, 23, 26) and that cre activity in Nestin.cre mice is detected by E11, we examined *Cop1ΔN* brains between E12.5 and E16.5 to see when we could first detect elevated levels of $CRL4^{COP1/DET1}$ substrates. Labeling of *Cop1ΔN* brains at E12.5 was variable, but, at E14.5, the *Cop1ΔN* cerebral cortex exhibited more intense c-JUN staining and contained significantly more $ETV5^{+}$ cells than control cortex (Fig. 3*D*). COP1 deficiency also increased staining for ETV1, ETV5, and c-JUN in the E13.5 spinal cord, particularly surrounding the central canal (Fig. 3*E*). *Cop1ΔN* brains were indistinguishable morphologically from control brains at these earlier stages of embryogenesis.

Similar to what we observed in whole brain, cortical neural cultures from E16.5 *Cop1ΔN* embryos contained more ETV5 protein than control cultures, even though *Etv5* mRNA was not increased (*SI Appendix*, Fig. S2*F*). In addition, BDNF treatment did not further increase ETV5 in the *Cop1ΔN* cells, suggesting that the BDNF-induced increase in ETV5 in control cells could partly reflect ERK-mediated inactivation of COP1.

Combined ETV5 and c-JUN Deficiency Reduces Lethality in *Cop1ΔN* Mice. To determine the contribution of abnormally high c-JUN, ETV1, and ETV5 protein expression to the lethal phenotype of *Cop1ΔN* mice, we introduced floxed *c-Jun*, *Etv1*, and *Etv5* alleles (27–29). In addition, we confirmed that each of the three transcription factors was increased independent of the others in *Cop1ΔN* brains (Fig. 4*A*). Interestingly, E18.5 *Cop1/c-JunΔN* brains differed from *Cop1ΔN* brains because they exhibited abnormally elevated expression of ETV5 in the periventricular region of the cerebrum (Fig. 4*B*). *Etv5* mRNA expression in the *Cop1/c-JunΔN* periventricular region appeared intermediate between the level seen in control $Cop1^{fl/+}$ Nestin.cre brains and the reduced level seen in the *Cop1ΔN* brains (*SI Appendix*, Fig. S3*A*). These data suggest that c-JUN negatively regulates *Etv5* expression in the periventricular region, although whether this is direct or indirect regulation is unclear. c-JUN deficiency alone also increased ETV5 expression in the periventricular region (*SI Appendix*, Fig. S3*B*).

Cop1/c-JunΔN mice, like *Cop1ΔN* mice, did not survive to weaning (*SI Appendix*, Table S1). However, a number of *Cop1/Etv5ΔN* mice and *Cop1/c-Jun/Etv5ΔN* mice were weaned. Most of the *Cop1/Etv5ΔN* mice died soon thereafter, but approximately one third of the *Cop1/c-Jun/Etv5ΔN* mice appeared healthy at 3 mo of age (*SI Appendix*, Fig. S3 *C* and *D*). Deletion of *Etv1* with Nestin.cre was lethal around weaning, similar to what has been reported for $Etv1^{-/-}$ mice (16), so we could only halve the *Etv1* gene dosage. *Etv1* heterozygosity did not increase the proportion of *Cop1/c-Jun/Etv5ΔN* mice alive at weaning, but it allowed some *Cop1/Etv5ΔN* mice to survive for many months, similar to *Cop1/c-Jun/Etv5ΔN* mice (*SI Appendix*, Fig. S3*C* and Table S1). We conclude from these data that aberrant c-JUN, ETV1, and ETV5 protein expression, although not entirely responsible, contributes to the lethality of *Cop1ΔN* mice.

Complete rescue might not have been achieved because loss of ETV5 alone or in combination with ETV1 loss increased expression of the related transcription factor ETV4 (Fig. 4*C*). This finding is reminiscent of combined ETV4 and ETV5 deficiency causing ETV1 expression in pancreatic β-cells lacking COP1 (10). Such observations suggest that ETV1, ETV4, and ETV5

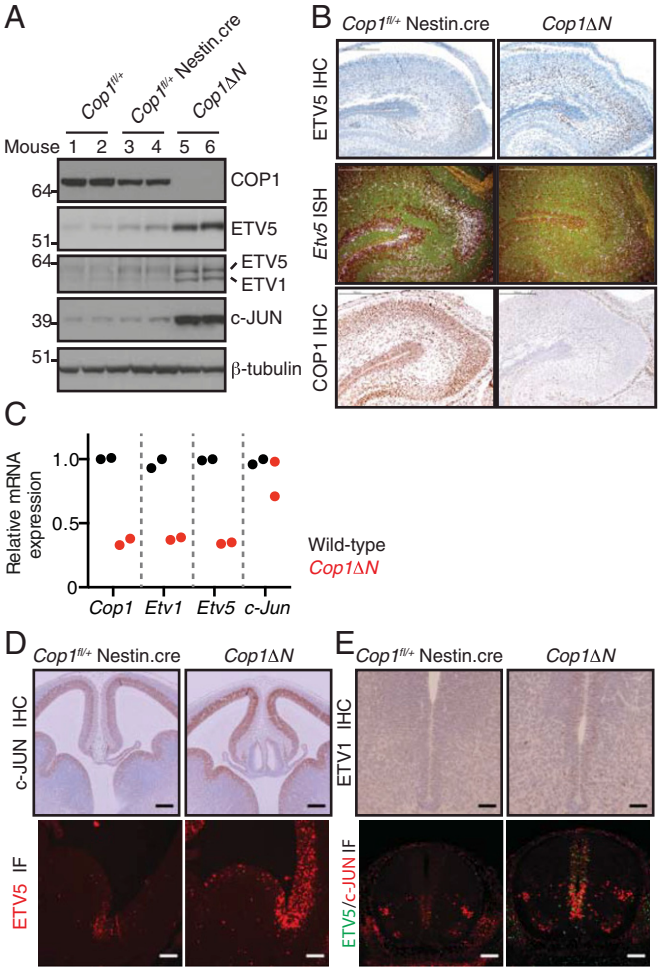

Fig. 3. COP1 limits expression of ETV1, ETV5, and c-JUN during brain development. (*A*) Western blots of P0 mouse brains. (*B*) E18.5 cerebral cortices labeled for ETV5 (brown), *Etv5* (white), or COP1 (brown). (Scale bars: 200 μm.) IHC, immunohistochemistry; ISH, in situ hybridization. Results are representative of two mice per genotype. (*C*) Relative *Etv1*, *Etv5*, and *c-Jun* mRNA expression in E18.5 brains. Circles represent individual embryos. (*D*) E14.5 cerebral cortices labeled for ETV5 (red) or c-JUN (brown). (Scale bars: ETV5, 100 μm; c-JUN, 200 μm.). IF, immunofluorescence. (*E*) E13.5 spinal cords labeled for ETV1 (brown) or ETV5 (green) with c-JUN (red). (Scale bars: ETV1, 50 μm; ETV5/c-JUN, 100 μm.) Results in *D* and *E* are representative of three to four mice per genotype.

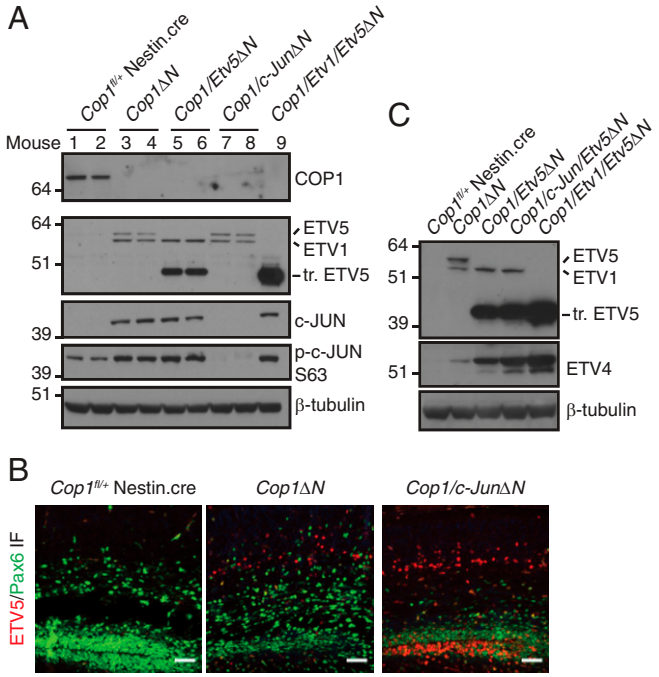

Fig. 4. Negative regulation of *Etv5* by c-JUN and of *Etv4* by ETV1 and ETV5. (*A*) Western blots of E18.5 mouse brains. tr. ETV5, truncated ETV5. (*B*) E18.5 cerebral cortices labeled for Pax6 (green) and ETV5 (red). (Scale bars: 50 μm.) Results are representative of three mice per genotype. (*C*) Western blots of E18.5 brains.

negatively regulate their own expression. Consistent with this notion, *Etv4* expression was increased in E18.5 *Cop1/Etv1/Etv5ΔN* brains, particularly in periventricular cells, compared with *COP1ΔN* brains (*SI Appendix*, Fig. S3*E*).

COP1 Deficiency Enhances Expression of Genes Associated with Gliogenesis in a c-JUN/ETV5–Dependent Manner. To determine the gene-expression changes caused by aberrant expression of c-JUN, ETV1, and ETV5, we analyzed E18.5 control (*Cop1*$^{fl/+}$ Nestin.cre), *Cop1ΔN*, *Cop1/c-JunΔN*, *Cop1/c-Jun/Etv1ΔN*, and *Cop1/c-Jun/Etv1/Etv5ΔN* brains by RNA sequencing [complete data available at the Gene Expression Omnibus (GEO) database, accession no. GSE111564]. Compared with controls, 97 genes were dysregulated in *Cop1ΔN* brains (based on an adjusted *P* value <0.05 and fold change >1.5). Of these 97 genes, 57 seemed to be regulated by c-JUN, ETV1, and/or ETV5 because their expression reverted to control levels in *Cop1/c-Jun/Etv1/Etv5ΔN* brains. Within this subset of 57 genes, *Gfap*, *Vimentin*, *Olig1*, *Olig2*, *Sox10*, and *Cspg4* were up-regulated 1.6–4.0-fold in the *Cop1ΔN* brains and stood out because of their involvement in gliogenesis (30–33).

By quantitative RT-PCR, increased expression of *Gfap* or *Vimentin* in E18.5 *Cop1ΔN* brains was dependent on c-JUN because *Gfap* and *Vimentin* expression in *Cop1/c-JunΔN* brains was equivalent to that in control brains (Fig. 5*A*). These data are consistent with reports that *Gfap* and *Vimentin* are c-JUN target genes (34, 35). In contrast, enhanced *Olig1*, *Olig2*, and *Sox10* expression in *Cop1ΔN* brains was sustained in *Cop1/Etv5ΔN* brains, partially normalized in *Cop1/c-JunΔN* brains, and completely normalized to control levels in *Cop1/c-Jun/Etv5ΔN* brains (Fig. 5 *A* and *B*). We speculate that elimination of both c-JUN and ETV5 is needed to normalize expression of *Olig1*, *Olig2*, and *Sox10* because c-JUN loss increased ETV5 in the periventricular region of *Cop1ΔN* brains (Fig. 4*B*).

Immunofluorescence labeling of E18.5 *Cop1ΔN* cerebral cortex revealed that more cells expressed OLIG2 protein (Fig. 5*C*), which correlated well with the increase in *Olig2* mRNA expression in whole brain (Fig. 5*A*). OLIG2-expressing cells were also more abundant in E18.5 *Cop1ΔE* cortex (*SI Appendix*, Fig. S4*A*). OLIG2 and ETV5 were largely expressed in different cell populations, although a small number of periventricular cells showed overlapping expression in *Cop1/c-JunΔN* brains (Fig. 5*C*). Note that the *Etv5* mutant allele in *Cop1/Etv5ΔN* brains encodes truncated, transcriptionally inactive ETV5 (29), and we detect this protein with our ETV5 antibody.

Sustained RAS–MEK–ERK signaling in neural stem cells lacking NF1 induces ectopic OLIG2 expression (19), and ERK signaling inactivates CRL4$^{COP1/DET1}$ (4), so we wondered whether COP1 deficiency enhanced gliogenesis at the expense of neurogenesis in the cerebral cortex. However, E18.5 control and *Cop1ΔN* cerebral cortices contained comparable numbers of cells expressing the neuronal marker NeuN (*SI Appendix*, Fig. S4*B*). In addition, even though we detected a 1.7-fold increase in *Cspg4* expression in E18.5 *Cop1ΔN* whole brain by RNA sequencing, cells expressing CSPG4/NG2 (chondroitin sulfate proteoglycan 4; also called neuron-glial antigen 2) were not increased in the *Cop1ΔN* cortex by immunohistochemistry (*SI Appendix*, Fig. S4*C*). It is possible that the subtle increase in *Cspg4* expression in E18.5 *Cop1ΔN* whole brain reflects differences outside the cerebral cortex. Colabeling of NeuN and OLIG2 in control and *Cop1ΔE* brains from littermates aged 5 wk indicated a trend toward increased OLIG2$^+$ cells, whereas NeuN$^+$ cell numbers appeared unchanged (*SI Appendix*, Fig. S4*D*). These data suggest that COP1 deficiency was not inducing a simple fate switch such that gliogenesis was favored at the expense of neurogenesis.

To further validate these findings, we performed single-cell RNA sequencing on E16.5 cerebral cortices isolated from *Cop1ΔN* and control brains (*SI Appendix*, Fig. S5*A*; complete data available at the GEO database, accession no. GSE111704). Consistent with our earlier results, the *Cop1ΔN* cortex contained a greater proportion of cells expressing *Olig2*, *Olig1*, or *Sox10* (Table 1). These cells were assigned to similar clusters as control cells expressing *Olig2*, *Olig1*, or *Sox10*, and the level of gene expression per cell was comparable between the two genotypes (*SI Appendix*, Fig. S5*B*, clusters 7, 8, and 12). These data suggest that the extra *Olig2*-expressing cells in the *Cop1ΔN* cerebral cortex represent the expansion of a normally occurring cellular subset rather than a population of cells aberrantly expressing *Olig2*. For example, the *Cop1ΔN* cortex did not contain a preponderance of cells aberrantly coexpressing *Olig2* and the neuronal marker gene *Microtubule associated protein 2* (*Map2*; *SI Appendix*, Fig. S5*C*). The *Cop1ΔN* cortex also contained approximately fourfold more cells expressing *Gfap*, whereas the increase in the number of cells expressing *Vimentin* was minimal (Table 1). Consistent with COP1 deficiency not reducing NeuN$^+$ neurons in the E18.5 cerebral cortex (*SI Appendix*, Fig. S4*B*), numbers of immature neurons expressing *Doublecortin* (*Dcx*) or postmitotic neurons expressing *Map2* were comparable between E16.5 control and *Cop1ΔN* cortices (Table 1). Therefore, markers of neurogenesis in the embryonic cerebral cortex do not appear to be suppressed by COP1 deficiency despite aberrant expression of markers of gliogenesis.

Given that OLIG2-expressing cells were increased in E18.5 *Cop1ΔN* cerebral cortex in a cJUN- and ETV5-dependent manner, we explored how E16.5 cells expressing *c-Jun* or *Etv5* clustered. *c-Jun* expression was detected across all cell clusters (*SI Appendix*, Fig. S5*B*), in keeping with c-JUN protein being broadly expressed in the developing brain (Fig. 3*D* and *SI Appendix*, Fig. S2*A*). However, as expected, *Etv1*, *Etv4*, and *Etv5* showed more restricted patterns of expression (*SI Appendix*, Fig. S5*B*). All three RNAs were detected in clusters 8 and 12, but we also noted expression in clusters 7 (*Etv1*), 14 (*Etv1*, *Etv4*), and 16 (*Etv1*, *Etv5*). Although *Olig2*-expressing cells also occupied clusters 7, 8, and 12, they did not appear to coexpress *Etv1*, *Etv4*, or *Etv5* (*SI Appendix*, Fig. S5*D*), consistent with distinct cell populations expressing ETV5 and OLIG2 protein (Fig. 5*C*). Confirming our earlier results (Fig. 3 *B* and *C*

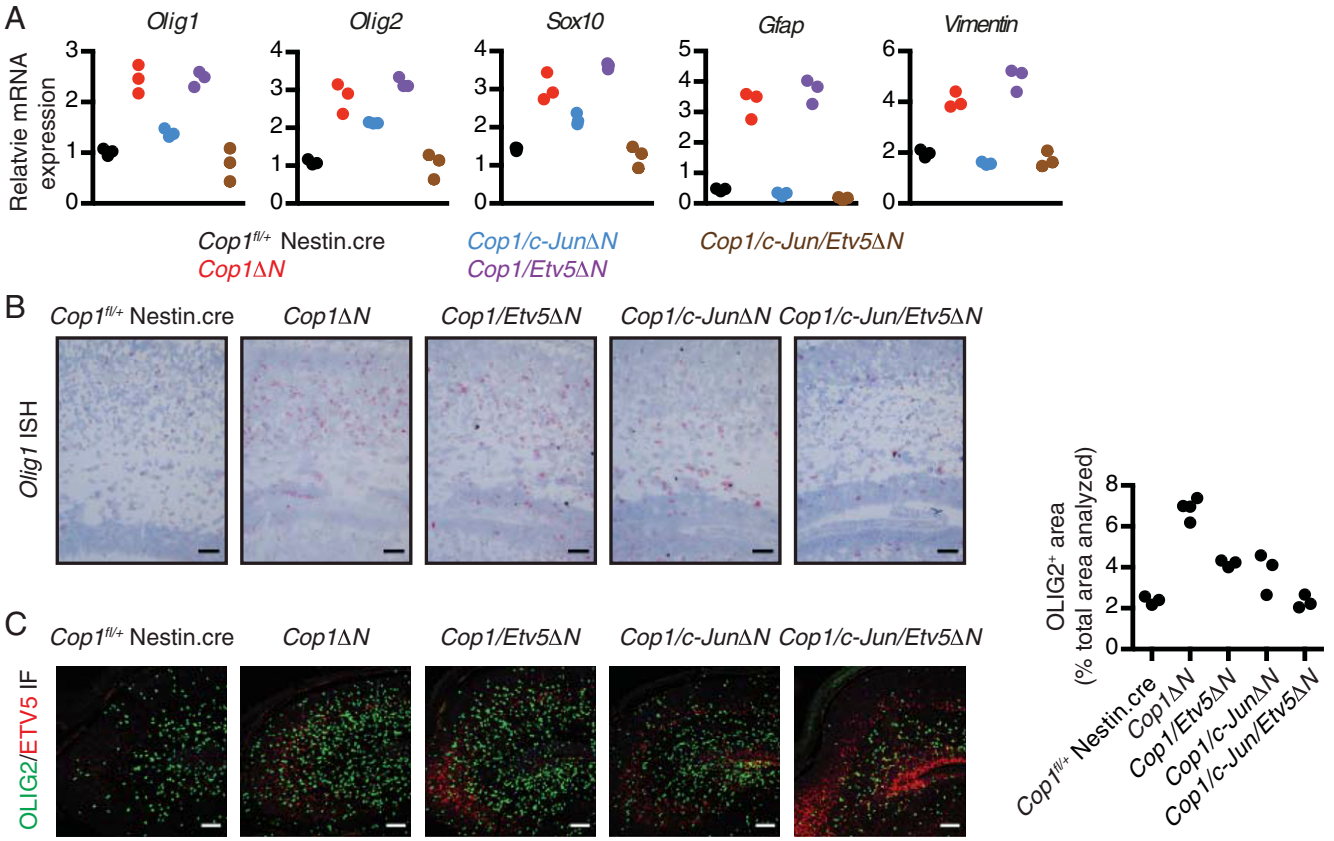

Fig. 5. c-JUN/ETV5-dependent changes in gene expression in COP1-deficient brains. (*A*) Relative *Olig1*, *Olig2*, *Sox10, Gfap*, and *Vimentin* expression in E18.5 brains by quantitative RT-PCR. Circles represent individual embryos. (*B*) E18.5 cerebral cortices labeled for *Olig1* mRNA (purple). (Scale bars: 50 μm.) Results are representative of three mice per genotype. (*C*) E18.5 cerebral cortices labeled for OLIG2 (green) and ETV5 (red). (Scale bars: 100 μm.) Graph shows quantification of labeling. Circles represent individual embryos.

and *SI Appendix*, Fig. S2*A*), *Cop1ΔN* cells expressed less *Etv1*, *Etv4*, and *Etv5* than control cells (*SI Appendix*, Fig. S5*B*). Collectively, our results suggest that stabilization of c-JUN and/or ETV1/4/5 in a subset of cells lacking COP1 promotes the expansion of a population of cells expressing glial marker genes. Determination of the precise relationship between these cell populations will require further study.

Discussion

Complete loss of COP1 is deleterious to the developing mouse embryo around E9.5 (9), so we explored the role of COP1 in brain development by restricting *Cop1* deletion to neural stem and progenitor cells with a Nestin.cre transgene. These *Cop1ΔN* mice died soon after birth with morphological abnormalities in the cerebral cortex, hippocampus, and cerebellum (Fig. 2). Isolated heart and kidney cells of Nestin.cre mice also exhibit cre activity, but brain abnormalities in the *Cop1ΔN* mice probably caused the perinatal lethality because 22% (22 of 101) of *Cop1ΔE* mice also died before weaning, compared with 5% (6 of 125) of $Emx1^{IRES\ cre/+}$ littermate controls.

The precise cause of death of *Cop1ΔN* or *Cop1ΔE* mice was unclear, but aberrant expression of ETV5, c-JUN, and ETV1 contributed to the *Cop1ΔN* phenotype because several $Cop1^{fl/-}$ $Etv5^{fl/fl}$ $Etv1^{fl/+}$ Nestin.cre mice and *Cop1/c-Jun/Etv5ΔN* mice survived for 7 mo. Abnormally elevated expression of ETV4 in the absence of ETV5 (Fig. 4*C*) might explain why not all mice were rescued. Consistent with ETV1 and ETV5 suppressing *Etv1*, *Etv4*, and *Etv5* gene expression, increased amounts of ETV1 and ETV5 in the *Cop1ΔN* brains coincided with reduced expression of all three genes (Fig. 3 *B* and *C* and *SI Appendix*, Figs. S2*A* and S3 *A* and *E*). It is unclear if this represents direct transcriptional repression by ETV1 and ETV5. c-JUN may also suppress *Etv5* gene expression because ETV5 protein and *Etv5* mRNA were more abundant in the periventricular region of the *Cop1/c-JunΔN* cerebrum compared with *Cop1ΔN* brains (Fig. 4*B* and *SI Appendix*, Fig. S3*A*). A c-JUN binding site is detected between exons 6 and 7 of *Etv5*, albeit without an underlying AP-1 motif (36). Regardless of the exact mechanism, suppression of *Etv1*, *Etv4*, or *Etv5* gene expression by ETV1/4/5 or c-JUN appears to be a negative feedback loop for limiting the ETV1/4/5 transcriptional response.

Neighboring ETS and AP-1 transcription factor binding sites are recognized RAS-response elements (37). Both ETV1 and ETV4 of the ETS family are capable of binding to these genomic

Table 1. Single-cell RNA sequencing of E16.5 cortical cells

	Positive (%)	
Gene	$Cop1^{fl/+}$ Nestin.cre	$Cop1^{fl/-}$ Nestin.cre (*Cop1ΔN*)
Olig1	124 (1.4)	419 (4.2)
Olig2	186 (2.1)	513 (5.1)
Sox10	40 (0.4)	88 (0.9)
Dcx	7,227 (80.8)	7,616 (76.0)
Map2	5,763 (64.4)	6,076 (60.6)
Vim	2,867 (32.1)	3,749 (37.4)
Gfap	68 (0.8)	330 (3.3)

Cells expressing the genes listed in column 1 are enumerated and shown as percentages of all cortical cells analyzed. Data represent cortical cells from two mice of each genotype. A gene is considered expressed if the expression value in that cell is greater than zero.

DNA sequences (38). Therefore, in cells expressing *c-Jun* and one or more of *Etv1*, *Etv4*, and *Etv5*, $CRL4^{COP1/DET1}$ inactivation by the RAS–MEK–ERK pathway (4) could be a mechanism for the rapid and coordinate accumulation of c-JUN and ETV1/4/5 for binding to RAS-response elements. Indeed, ERK-dependent expression of the transcription factor c-MAF in the lens of the developing mouse eye has been linked to c-JUN and ETV5, which bind to ETS-AP1 sites in the *c-Maf* locus and synergistically activate transcription in reporter studies (39). This synergy might reflect simultaneous binding of ETV1/4/5 and AP-1 to the MED25 subunit of the mediator transcriptional coactivator complex that engages RNA polymerase II (40).

The genes that were up-regulated in E18.5 *Cop1ΔN* whole brain in a c-JUN/ETV5–dependent manner included *Olig1*, *Olig2*, and *Sox10* (Fig. 5*A*), which are markers of oligodendrocyte precursor cells. Single-cell RNA sequencing of E16.5 cerebral cortex (*SI Appendix*, Fig. S5) confirmed OLIG2 immunohistochemistry experiments (Fig. 5*C*) and showed that more *Cop1ΔN* cells expressed these genes (Table 1). Given that *Cop1ΔN* cells expressing *Olig1*, *Sox10*, or *Olig2* fell into similar clusters as their control counterparts (*SI Appendix*, Fig. S5), these data suggest that COP1 deficiency promotes expansion of a normal cellular subset rather than promoting aberrant gene expression in cells that do not normally express *Olig1*, *Sox10*, or *Olig2*. Cells expressing markers of neurogenesis, including NeuN, *Dcx*, and *Map2*, were unchanged (*SI Appendix*, Fig. S4*B*) or minimally reduced in the *Cop1ΔN* cortex (Table 1), indicating that gliogenesis was not enhanced at the expense of neurogenesis. Perhaps COP1 loss enhanced proliferation and/or reduced apoptosis in a progenitor population that was already committed to a glial fate. In contrast to neurogenesis in the embryonic cerebral cortex, neurogenesis in the olfactory bulb perinatally is perturbed by enhanced RAS–MEK–ERK signaling, with NF1 deficiency skewing the differentiation of neural stem cells in the subventricular zone toward the glial lineage and yielding a smaller olfactory bulb at P18 (19). Because of the lethal phenotype of newborn *Cop1ΔN* mice, we could not determine if COP1 loss mimicked NF1 deficiency in this setting.

Finally, our study highlights the fact that measuring *Etv1*, *Etv4*, and *Etv5* mRNA expression in cells of the developing brain is insufficient to implicate these transcription factors in normal physiology because ligases such as $CRL4^{COP1/DET1}$ can prevent the accumulation of functional amounts of ETV1, ETV4, and ETV5 protein.

Materials and Methods

Armc8$^{3xfl/3xf}$, *Cop1*$^{fl/fl}$, *Cop1*$^{lacZ/+}$, *Cop1*$^{fh/fh}$, *c-Jun*$^{fl/fl}$, *Det1*$^{3xfl/3xf}$, *Etv1*$^{fl/fl}$, *Etv5*$^{fl/fl}$, Nestin.cre, and *Emx1*$^{IRES\ cre/+}$ mouse strains have been described previously (3, 4, 27–29, 41–43). Embryos were designated E0.5 on the morning that a vaginal plug was observed. Pups were defined as P0 on the day of birth. Images in Fig. 1 *B–D* used cortical cells from E15–E16 CD-1 mouse embryos (Charles River Laboratories). The Genentech Animal Care and Use Committee approved all animal protocols. Detailed methods and reagents are provided in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Thinh Pham, Linda Rangell, and Carmina Espiritu for coordinating sample processing; Kelli Veliz, Jerome Anunciacion, and Raymond Asuncion for animal husbandry; Karen O'Rourke and Merone Roose-Girma for technical assistance; and Silvia Arber, Xin Sun, and John Hassell for providing mutant mice.

1. Marine JC (2012) Spotlight on the role of COP1 in tumorigenesis. *Nat Rev Cancer* 12: 455–464.
2. Wertz IE, et al. (2004) Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science* 303:1371–1374.
3. Vitari AC, et al. (2011) COP1 is a tumour suppressor that causes degradation of ETS transcription factors. *Nature* 474:403–406.
4. Zhang Z, et al. (2017) Transcription factor Etv5 is essential for the maintenance of alveolar type II cells. *Proc Natl Acad Sci USA* 114:3903–3908.
5. Qi L, et al. (2006) TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism. *Science* 312:1763–1766.
6. Dentin R, et al. (2007) Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. *Nature* 449:366–369.
7. Keeshan K, et al. (2010) Transformation by Tribbles homolog 2 (Trib2) requires both the Trib2 kinase domain and COP1 binding. *Blood* 116:4948–4957.
8. Lu G, et al. (2014) Phosphorylation of ETS1 by Src family kinases prevents its recognition by the COP1 tumor suppressor. *Cancer Cell* 26:222–234.
9. Migliorini D, et al. (2011) Cop1 constitutively regulates c-Jun protein stability and functions as a tumor suppressor in mice. *J Clin Invest* 121:1329–1343.
10. Suriben R, et al. (2015) β-Cell insulin secretion requires the ubiquitin ligase COP1. *Cell* 163:1457–1467.
11. Yoshida A, Kato JY, Nakamae I, Yoneda-Kato N (2013) COP1 targets C/EBPα for degradation and induces acute myeloid leukemia via Trib1. *Blood* 122:1750–1760.
12. Glessner JT, et al. (2009) Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. *Nature* 459:569–573.
13. Behrens A, Sibilia M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet* 21:326–329.
14. Hoeck JD, et al. (2010) Fbw7 controls neural stem cell differentiation and progenitor apoptosis via Notch and c-Jun. *Nat Neurosci* 13:1365–1372.
15. Jandke A, et al. (2011) The F-box protein Fbw7 is required for cerebellar development. *Dev Biol* 358:201–212.
16. Arber S, Ladle DR, Lin JH, Frank E, Jessell TM (2000) ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell* 101:485–498.
17. Livet J, et al. (2002) ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. *Neuron* 35:877–892.
18. Li X, et al. (2012) MEK is a key regulator of gliogenesis in the developing brain. *Neuron* 75:1035–1050.
19. Wang Y, et al. (2012) ERK inhibition rescues defects in fate specification of Nf1-deficient neural progenitors and brain abnormalities. *Cell* 150:816–830.
20. Fontanet PA, Ríos AS, Alsina FC, Paratcha G, Ledda F (2018) Pea3 transcription factors, Etv4 and Etv5, are required for proper hippocampal dendrite development and plasticity. *Cereb Cortex* 28:236–249.
21. Hasegawa H, et al. (2004) Laminar patterning in the developing neocortex by temporally coordinated fibroblast growth factor signaling. *J Neurosci* 24:8711–8719.
22. Yoneshima H, et al. (2006) Er81 is expressed in a subpopulation of layer 5 neurons in rodent and primate neocortices. *Neuroscience* 137:401–412.
23. Zimmer C, et al. (2010) Role of Fgf8 signalling in the specification of rostral Cajal-Retzius cells. *Development* 137:293–302.
24. Li S, et al. (2014) RAS/ERK signaling controls proneural genetic programs in cortical development and gliomagenesis. *J Neurosci* 34:2169–2190.
25. Breunig JJ, et al. (2015) Ets factors regulate neural stem cell depletion and gliogenesis in Ras pathway glioma. *Cell Rep* 12:258–271.
26. Wilkinson DG, Bhatt S, Ryseck RP, Bravo R (1989) Tissue-specific expression of c-jun and junB during organogenesis in the mouse. *Development* 106:465–471.
27. Raivich G, et al. (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron* 43:57–67.
28. Patel TD, et al. (2003) Peripheral NT3 signaling is required for ETS protein expression and central patterning of proprioceptive sensory afferents. *Neuron* 38:403–416.
29. Zhang Z, Verheyden JM, Hassell JA, Sun X (2009) FGF-regulated Etv genes are essential for repressing Shh expression in mouse limb buds. *Dev Cell* 16:607–613.
30. Zhou Q, Wang S, Anderson DJ (2000) Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. *Neuron* 25: 331–343.
31. Zhou Q, Anderson DJ (2002) The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* 109:61–73.
32. Zhu X, Bergles DE, Nishiyama A (2008) NG2 cells generate both oligodendrocytes and gray matter astrocytes. *Development* 135:145–157.
33. Küspert M, Hammer A, Bösl MR, Wegner M (2011) Olig2 regulates Sox10 expression in oligodendrocyte precursors through an evolutionary conserved distal enhancer. *Nucleic Acids Res* 39:1280–1293.
34. Rinehart-Kim J, Johnston M, Birrer M, Bos T (2000) Alterations in the gene expression profile of MCF-7 breast tumor cells in response to c-Jun. *Int J Cancer* 88:180–190.
35. Gao K, et al. (2013) Traumatic scratch injury in astrocytes triggers calcium influx to activate the JNK/c-Jun/AP-1 pathway and switch on GFAP expression. *Glia* 61: 2063–2077.
36. Yue F, et al.; Mouse ENCODE Consortium (2014) A comparative encyclopedia of DNA elements in the mouse genome. *Nature* 515:355–364.
37. Wasylyk B, et al. (1990) The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature* 346:191–193.
38. Hollenhorst PC, et al. (2011) Oncogenic ETS proteins mimic activated RAS/MAPK signaling in prostate cells. *Genes Dev* 25:2147–2157.
39. Xie Q, et al. (2016) Regulation of c-Maf and αA-crystallin in ocular lens by fibroblast growth factor signaling. *J Biol Chem* 291:3947–3958.
40. Currie SL, et al. (2017) ETV4 and AP1 transcription factors form multivalent interactions with three sites on the MED25 activator-interacting domain. *J Mol Biol* 429: 2975–2995.
41. Dey A, et al. (2012) Loss of the tumor suppressor BAP1 causes myeloid transformation. *Science* 337:1541–1546.
42. Tronche F, et al. (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23:99–103.
43. Gorski JA, et al. (2002) Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J Neurosci* 22:6309–6314.