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

Kim Newton1,2,*†, Debra L. Dugger3, Arundhati Sengupta-Ghosh4, Ronald E. Ferrando4,2, Felix Chu2,3, Janet Tao4, Wendy Lam4,4, Susan Haller6, Sara Chan2, Susan Sa3, Debra Dunlap3, Jeffrey Eastham-Anderson5, Hai Ngu6, Jeffrey Hung7, Dorothy M. French1,2, Joshua D. Webster6, Brad Bolon5, Jinfeng Liu6,5, Rohit Reja5, Sarah Kummerfeld6, Ying-Juin Chen1, Zora Modrusan1, Joseph W. Lewcock7,8, and Vishva M. Dixit8,1

1Department of Physiological Chemistry, Genentech, South San Francisco, CA 94080; 2Department of Neuroscience, Genentech, South San Francisco, CA 94080; 3Department of Pathology, Genentech, South San Francisco, CA 94080; 4Department of Bioinformatics and Computational Biology, Genentech, South San Francisco, CA 94080; and 5Department of Molecular Biology, Genentech, South San Francisco, CA 94080

*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. contributed to the design and execution of the experiments; K.N., J.D.W., Z.M., and V.M.D. performed the experiments; and K.N., D.L.D., A.S.-G., D.M.F., J.D.W., Z.M., J.W.L., S.H., S.S., D.D., and Y.-I.C. analyzed the data. K.N. wrote the paper.

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Effect of COP1 deficiency on cell type specification

COP1 deficiency results in defects in neural stem cell proliferation and differentiation, leading to a reduction in the number of cortical progenitor cells.

Central nervous system

The central nervous system is affected in COP1 deficient mice, with decreased numbers of neurons and astrocytes in the developing forebrain and cerebellum.

Significance

The ubiquitin ligase COP1 directs the degradation of transcription factors c-JUN, ETV1, ETV4, and ETV5, which are important for the development of the central nervous system. This suggests a potential role for COP1 in the regulation of transcription factors during neural development.

Posttranslational regulation of c-JUN

c-JUN is regulated by posttranslational modifications, including ubiquitination, which targets it for degradation. This regulation is critical for the proper development of the central nervous system.

Central nervous system development

In COP1 deficient mice, the central nervous system shows abnormalities in cell number and morphology, indicating a role for COP1 in neural development.

Ubiquitination of c-JUN

c-JUN is ubiquitinated by COP1, leading to its degradation. This process is critical for the proper development of the central nervous system.

Central nervous system function

The central nervous system function is impaired in COP1 deficient mice, with defects in neuronal migration and differentiation.

Central nervous system abnormalities

COP1 deficiency leads to abnormalities in the central nervous system, including reduced cell numbers and altered morphology.

Central nervous system development

The central nervous system development is regulated by COP1, which targets transcription factors for degradation, leading to changes in gene expression and cell fate.

Central nervous system abnormalities

COP1 deficient mice show central nervous system abnormalities, indicating a role for COP1 in neural development.
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, En1 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 En1 (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 astrogial 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). ERV transcription factors have also been implicated in glioma initiation by oncogenic RAS (25). Whether ERK-dependent posttranslational mechanisms, potentially involving CRL4COP1/DET1, regulate expression of ETV1, ETV4, and ETV5 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/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 CRL4COP1/DET1 substrates, epitope-tagged versions of COP1 and DET1 were affinity-purified from embryonic day 18.5 (E18.5) knock-in mouse brains (Fig. 1A). 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, ARMG8-3xFlag. Consistent with COP1 being the substrate adaptor for ETV5, deletion of Cop1 from neural stem cells with a Nestin.cre transgene (Cop1^ΔN mice) reduced the amount of Cop1 and ETV5 that copurified with DET1-3xFlag, but had no effect on the interaction of DET1 with DB1.

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 DBN or picrotoxin (PTX) to mimic the signals that neurons might encounter during development. DBN engages the tyrosine kinase receptor TrkB on neurons, whereas PTX blocks the GABA-activated chloride channel and thereby promotes synaptic activity. DBN treatment for 2 h increased ETV5 protein and Etv5 mRNA, as well as c-JUN protein and c-JUN mRNA (Fig. 1B). ERK inhibition, which reduced phosphorylation of the ERK substrate ribosomal S6 kinase, prevented this response to DBN. 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. 1C). 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. 1D). This result suggests that ERK-mediated inhibition of CRL4COP1/DET1 is inefficient when COP1 is in excess.

We sought genetic proof that COP1 regulates ETV5 in the developing mouse brain by using Cop1^ΔN (Cop1^fl/fl/Nestin.cre) mice and Cop1^ΔE (Cop1^fl/fl/Emx1^IRES cre/cre^) mice. Many Cop1^ΔN mice died within a few days of birth, and none survived to weaning (SI Appendix, Fig. S1A). Abnormalities were evident in 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 of 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. Cop1^ΔE mice, which lacked COP1 in cells of the neocortex and hippocampus (SI Appendix, Fig. S1B), displayed similar cerebrocortical disorganization as Cop1^ΔN mice (SI Appendix, Fig. S1C). Cop1^ΔE mice had a median survival of 147 d (SI Appendix, Fig. S1D), and those that survived to 35 d tended to be smaller than their Cop1^+/+ Enx1^IRES cre/cre^ littermates (SI Appendix, Fig. S1E).

In keeping with COP1 regulating ETV5 abundance via a posttranslational mechanism, E18.5 or P0 Cop1^ΔN brains contained more ETV5 than control Cop1^+/+ Nestin.cre brains (Fig. 3 A and B), but they did not express more Etv5 mRNA (Fig. 3 B and C). CRL4COP1/DET1 substrates c-JUN and ETV1 were also more abundant in Cop1^ΔN brains (Fig. 3 D and E and SI Appendix, Fig. S2A), even though c-JUN and Etv1 mRNAs were not increased (Fig. 3 C and SI Appendix, Fig. S2A). Indeed, Cop1^ΔN brains contained less Etv1 mRNA than control brains (Fig. 3 C).
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. S2B). 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. S2E) (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 if we could first detect elevated levels of 
CRL4Cop1/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. 3D). Cop1 deficiency also increased staining for ETV1, ETV5, and c-JUN in the E13.5 spinal cord, particularly surrounding the central canal (Fig. 3E). 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. S2F). 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 transcri 
tion factors was increased independent of the others in 
Cop1ΔN brains (Fig. 4A). Interestingly, E18.5 Cop1/c-JunΔN 
brains differed from Cop1ΔN brains because they exhibited ab 
normally elevated expression of ETV5 in the periventricular 
region of the cerebrum (Fig. 4B). Etv5 mRNA expression in the 
Cop1/c-JunΔN periventricular region appeared intermediate 
between the level seen in control Cop1fl/+ Nestin.cre brains and the 
reduced level seen in the Cop1ΔN brains (SI Appendix, Fig. 
S3A). 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. S3B).

Cop1/c-JunΔN mice, like Cop1ΔN mice, did not survive to 
weaning (SI Appendix, Table S1). However, a number of Cop1/

En5ΔN mice and Cop1/c-Jun/En5ΔN mice were weaned. Most 
of the Cop1/En5ΔN mice died soon thereafter, but approximately 
one third of the Cop1/c-Jun/En5ΔN 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 have the Etv1 
gene dosage. Etv1 heterozygosity did not increase the propor 
tion of Cop1/c-Jun/En5ΔN mice alive at weaning, but it allowed 
some Copyt/En5ΔN mice to survive for many months, similar to 
Cop1/c-Jun/En5ΔN mice (SI Appendix, Fig. S3C 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 ex 
pression of the related transcription factor ETV4 (Fig. 4C). 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
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 (Cop1ΔN, 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. 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. S5B, clusters 7, 8, and 12). 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. S5A; complete data available at the GEO database, accession no. GSE111704). Consistent with our earlier results, the Cop1ΔN cortex contained approximately fourfold more cells expressing Glap, 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. S4B), 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 c-JUN- and ETV5-dependent manner, we explored how E16.5 cells expressing c-Jun or En5 clustered. c-Jun expression was detected across all cell clusters (SI Appendix, Fig. S5B), in keeping with c-JUN protein being broadly expressed in the developing brain (Fig. 3D and SI Appendix, Fig. S2A). However, as expected, En1, En4, and En5 showed more restricted patterns of expression (SI Appendix, Fig. S5B). All three RNAs were detected in clusters 8 and 12, but we also noted expression in clusters 7 (En1), 14 (En1, En4, and En5), and 16 (En1, En5). Although Olig2-expressing cells also occupied clusters 7, 8, and 12, they did not appear to coexpress En1, En4, or En5 (SI Appendix, Fig. S5D), consistent with distinct cell populations expressing ETV5 and Olig2 protein (Fig. 5C). Confirming our earlier results (Fig. 3B and C expression in whole brain (Fig. 5A). Olig2-expressing cells were also more abundant in E18.5 Cop1ΔN cortex (SI Appendix, Fig. S4A). 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. 5C). Note that the En5 mutant allele in Cop1/En5ΔN 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 NFI induces ectopic Olig2 expression (19), and ERK signaling inactivates CRL4COP1/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. S4B). 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 were not increased in the Cop1ΔN cortex by immunohistochemistry (SI Appendix, Fig. S4C). 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 Cop1ΔN brains from littersmates aged 5 wk indicated a trend toward increased Olig2+ cells (Fig. S4). However, NeuN+ cell numbers appeared unchanged (SI Appendix, Fig. S4D). These data suggest that COP1 deficiency was not inducing simple fate switch such that gliogenesis was favored at the expense of neurogenesis.

By quantitative RT-PCR, increased expression of Glap or Vimentin in E18.5 Cop1ΔN brains was dependent on c-JUN because Glap and Vimentin expression in Cop1/c-JunΔN brains was equivalent to that in control brains (Fig. 5A). These data are consistent with reports that Glap and Vimentin are c-JUN target genes (34, 35). In contrast, enhanced Olig1, Olig2, and Sox10 expression in Cop1ΔN brains was sustained in Cop1ΔN/ETV5ΔN brains, partially normalized in Cop1/c-JunΔN brains, and completely normalized to control levels in Cop1/c-Jun/En5ΔN brains (Fig. 5A 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. 4B).

Immunofluorescence labeling of E18.5 Cop1ΔN cerebral cortex revealed that more cells expressed Olig2 protein (Fig. 5C), which correlated well with the increase in Olig2 mRNA negatively regulate their own expression. Consistent with this notion, Etv4 expression was increased in E18.5 Cop1ΔN/En5ΔN brains, particularly in periventricular cells, compared with Cop1ΔN brains (SI Appendix, Fig. S3E).

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.
and SI Appendix, Fig. S24). Cop1ΔN cells expressed less Etv1, Etv4, and Etv5 than control cells (SI Appendix, Fig. S5B). 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 Emx1Rex 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 Cop1ΔN Etv5ΔN Etv5ΔE Nestin.cre mice and Cop1ΔE Etv5ΔN mice survived for 7 mo. Abnormally elevated expression of ETV4 in the absence of ETV5 (Fig. 4C) 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. S24 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 Cop1ΔE-JUNΔN cerebrum compared with Cop1ΔN brains (Fig. 4B and SI Appendix, Fig. S3A). 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**

| Gene   | Cop1ΔN− Nestin.cre | Cop1ΔE− 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 Env1, Env4, and Env5, CRL4COP1/DET1 inactivation by the RAS-MEK–ERK pathway (4) could be a mechanism for the rapid and coordinate accumulation of c-JUN and ETVI/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 ETVI5, 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 ETVI/4/5 and AP-1 to the MEF25 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 RNA (Fig. 5D) were unchanged in the Siph Cop1ΔN cells expressing 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 NeuroD, Dcx, and Map2, were unchanged (SI Appendix, Fig. S4B) 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 NFI deficiency skewing the differentiation of neural stem cells in the subventricular zone toward the glial lineage and yielding a smaller olfactory bulb at P18 (39). Because of the lethal phenotype of neuron cop1ΔN mice, it could not determine if Cop1 loss mimicked NFI deficiency in this setting.

Finally, our study highlights the fact that measuring Env1, Env4, and Env5 mRNA expression in cells of the developing brain is insufficient to implicate these transcription factors in normal physiology because ligases such as CRL4COP1/DET1 can prevent the accumulation of functional amounts of ETVI, ETV4, and ETV5 protein.

Materials and Methods

Armcaths, Cop1fl/fl, Cop1fl/wt, Cop1myc, c-Junfl/fl, Det3fl/fl, Etv1fl/fl, Etv4fl/fl, Etv5fl/fl, Nestin.cm, and Eme1−/− mice strains have been described previously (3, 4, 27–29, 41–43). Embryos were designated E0.5 on the morning that a placental 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.

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