Capicua regulates neural stem cell proliferation and lineage specification through control of Ets factors

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Capicua (Cic) is a transcriptional repressor mutated in the brain cancer oligodendroglioma. Despite its cancer link, little is known of Cic’s function in the brain. We show that nuclear Cic expression is strongest in astrocytes and neurons but weaker in stem cells and oligodendroglial lineage cells. Using a new conditional Cic knockout mouse, we demonstrate that forebrain-specific Cic deletion increases proliferation and self-renewal of neural stem cells. Furthermore, Cic loss biases neural stem cells toward glial lineage selection, expanding the pool of oligodendrocyte precursor cells (OPCs). These proliferation and lineage effects are dependent on de-repression of Ets transcription factors. In patient-derived oligodendroglioma cells, CIC re-expression or ETV5 blockade decreases lineage bias, proliferation, self-renewal, and tumorigenicity. Our results identify Cic as an important regulator of cell fate in neurodevelopment and oligodendroglioma, and suggest that its loss contributes to oligodendroglioma by promoting proliferation and an OPC-like identity via Ets overactivity.
The identification of cancer genes often presents opportunities to uncover previously unappreciated mechanisms regulating development. The transcriptional repressor Capicua (CIC) has been identified as a likely tumor suppressor, as CIC mutations and/or reduced expression are found in several cancers. In the brain, CIC mutations are nearly exclusively found in oligodendrogliomas (ODGs)—tumors composed of cells resembling oligodendrocyte precursor cells. Indeed, concurrent IDH1/2 mutation, single-copy losses of 1p and 19q, and mutation of the remaining copy of CIC on chr 19q13 are together highly characteristic of ODG. These associations suggest a unique relationship between CIC and glioblastoma.

Prior work has shown that Cic is a transcriptional repressor downstream of receptor tyrosine kinase (RTK) signaling. Binding of Cic to the sequence (G/C)AATG(G/A)A in enhancers and promoters leads to transcriptional repression of its target genes. This default repression is relieved upon RTK mediated ERK-mediated Cic phosphorylation, permitting transcription of targets—among which are PEA3/ETS transcription factors. By date, knowledge of Cic’s function in mammalian development or in the brain is limited. Yang et al. using Cic conditional knockout mice, reported that Cic loss increases a population of proliferating Olig2 + cells in the brain, and potentiates tumorigenesis in a PDGFR-driven glioma model. The mechanisms underlying those findings, however, remained undefined. Meanwhile, in the non-neoplastic context, Lu et al. showed that impairing the interaction of Cic with Ataxin1 results in neurobehavioral and neurocognitive phenotypes, and alteration in cortical neuronal populations—indicating a role for Cic in neuronal biology as well. Deciphering the molecular mechanisms of Cic function may shed light on these varied phenotypes.

Here, we examine Cic expression in the mammalian forebrain, and take a loss-of-function approach to determine its role in specifying neuronal-glial identity. Our results reveal an important role for Cic in regulating the proliferation and lineage specification of neural stem cells—i.e., loss favoring neural stem cell (NSC) proliferation and glial production at the expense of neuronal production. Furthermore, we show that these effects are mediated largely through Cic’s regulation of Ets factors. The proliferative dysregulation is recapitulated in ODG cells, where Cic re-expression or Ets blockade reduces tumorigenicity. Our findings reveal an important role for Cic in development and ODG, and identify Ets5 as a potential therapeutic target for ODG.

Results

Cic loss increases glial cell expression of neural genes. Domains in Cic include an HMG box and a C-terminal domain that together mediate DNA binding, and a C-terminal Gro-L domain that mediates protein–protein interactions. We generated Cic conditional knockout mice in which exons 2–11 of Cic were flanked by loxP sites, with the floxed region containing all exons encoding the HMG box. Upon Cre expression, exons 2–11 are excised and the remaining exons 12–20 are frameshifted, ablating all of these critical domains. We used these animals for in vivo studies and for cell line generation to dissect Cic’s functions.

We crossed Cic-floxed mice to Foxg1-Cre mice, to generate forebrain-specific Cic deletion starting at E10.5. Cicfllox/foxg1-Cre/+ animals were born in approximate Mendelian ratios and were grossly normal at birth, but became visible runts by P7, and were lethal by P22. The reason for lethality is unclear, but we suspect that poor feeding secondary to impaired neurologic function may be related to their decline. Although all major forebrain structures (e.g., cortex, white matter, deep nuclei, hippocampus) were present, and the cortex was laminated; Cic-null cerebra were smaller than those of littermate controls. Cic-null cortices were more than those of littermate controls (Fig. 2c). The smaller cerebral size was due to global decreases in gray matter and white matter (Supplementary Fig. 2a–c). Despite decreased thickness/size of corpus callosum and cortex, however, the tissue showed increased cellularity (Supplementary Fig. 2d–e). As neuronal density was not significantly lower in Sox2 + cells than in NeuN + cells, Tbr1 + cells, or Gfap + cells (Fig. 1j). As both NSCs and OPCs are proliferation-competent cell types, this pattern of expression raised the possibility that Cic may repress proliferation-related genes and/or early oligodendroglial-promoting programs.

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Evaluation of P21 cortices confirmed shifted cell proportions in Cic-null brains. NeuN + cells were decreased in Cic-null cortices compared with Cic-wildtype or heterozygote control cortices (Fig. 2d, e). In contrast, Gfap + cells were increased in knockouts relative to controls (Fig. 2d, e). Olig2 + cells were also
significantly increased (Fig. 2f, g)—a finding corroborated by increased Sox10+ (Fig. 2d, e). In oligodendrogial lineage progression, earlier stages were particularly increased, whereas later stages were decreased in Cic-null animals. Olig2+Sox2+ cells and Olig2+Pdgfra+OPCs were increased (Fig. 2f, g and Supplementary Fig. 3), whereas CNPase+immature oligodendrocytes were decreased (Fig. 2f, g) in Cic-null animals. Mbp, the product of mature oligodendrocytes, was also decreased in Cic-null brains (Fig. 2f, g).

We found no change in apoptosis in the knockout brains (Supplementary Fig. 4f), suggesting that the phenotype of altered cell proportions was not due to increased cell death. Other possibilities were that Cic loss could be affecting NSC lineage selection, or that Cic loss could have specific effects on OPCs.
In the following work, we focus our investigations on Cic’s role at the NSC stage, and examine its role in proliferation and lineage selection.

**Cic deficiency increases NSC proliferation and self-renewal.** To determine whether Cic loss affects NSC proliferation, we electroporated pCIG2-Cre (or pCIG2 empty control) into E13 Cic<sup>Fli/Fli</sup> embryos and performed EdU labeling in the last 30 min prior to sacrifice. Forty-eight hours post electroporation, the fraction of GFP+ cells that was EdU+ was markedly increased in cre- vs. control-electroporated brains (Fig. 3a, b). These findings supported a cell-autonomous increase in NSC proliferation with Cic loss. There was also an increase in EdU+ cells among non-GFP cells in the electroporated areas, suggesting additional non-cell-autonomous effects that we did not pursue (Supplementary Fig. 6e). To confirm the cell-autonomous gains in NSC proliferation, we turned to cell culture. Cic-null and control NSCs were derived from E15 Cic-floxed cells transfected ex vivo with Cre or control plasmids (Fig. 3c). 3 days after plating equal cell numbers in NSC proliferation media, however, we found a threefold increase in numbers of Cic-null cells relative to controls (Fig. 3d). These data were corroborated by Alamar blue assay (Supplementary Fig. 4a), Ki67 immunostaining (Fig. 3e) and cell cycle analysis (Fig. 3f), which showed increased viability and cycling in Cic-null cells. There were no differences in the numbers of dead cells between the two (Supplementary Fig. 4c). Together, the data show that Cic is a strong negative regulator of proliferation in forebrain NSCs.

**Self-renewal of Cic-null cells was also assessed by clonogenic assay (Fig. 3g).** In this assay, after plating equal numbers of control-electroporated brains (Fig. 3a, b). These findings were supported by clonal sphere formation efficiency, which was increased in Cic-deficient cells. In this assay, after plating equal numbers of control-electroporated brains (Fig. 3a, b). These findings were supported by clonal sphere formation efficiency, which was increased in Cic-deficient cells.
of cell division, and the fraction of cells remaining in or exiting the cell cycle. Both Cic-null and control NSCs generated spheres, but Cic-null NSCs generated more spheres (Fig. 3g, h) and larger spheres (Fig. 3g, i) compared with controls. Thus, Cic loss confers not only higher proliferation but higher self-renewal in NSCs, at least when cells are in conditions promoting NSC proliferation.

**Cic regulates NSC cell division asymmetry.** During neurodevelopment, the NSC pool is first expanded by symmetric proliferative divisions, followed by rounds of asymmetric divisions of stem and progenitor cells generating neurons and glia, and finally by terminal symmetric differentiative divisions. The balance between symmetric and asymmetric divisions is important;
deficiency in asymmetric divisions and an increase in symmetric divisions can lead to neoplasia. To investigate whether alteration of cell division mode was a feature of the Cic-deficient NSCs, we performed paired cell assays. Cic-null and -control NSCs were seeded at low density in adherent cultures for 24 h, then fixed and stained for Ki67 (Fig. 3). Pairs where both daughter cells were Ki67+ were scored as symmetric proliferative. Pairs where daughter cells differed in Ki67 (i.e., Ki67+/Ki67−) were scored as asymmetric. Pairs where both daughter cells were Ki67− were scored as symmetric terminal. Cic-null cells underwent more frequent symmetric proliferative divisions and fewer asymmetric divisions compared with controls (Fig. 3k). Although not significant, there was a trend to decreased symmetric terminal divisions between Cic-null and control cells (Fig. 3k). The net effect of these alterations in cell division symmetry/asymmetry is the presence of more cycling and self-renewing cells.

Consistent with the above increased NSC proliferation and self-renewal observed in vitro, examination of our electroporated brains showed that the fraction of GFP+ cells that were Sox2+ was higher in cre- versus control-electroporated brains (Fig. 4a–c). There was no change in activated Caspase-3 (Supplementary Fig. 4d, e), indicating that the increased Sox2+ fraction in vivo was not owing to increased apoptosis of other cells. Cumulatively, the findings of increased NSC proliferation, decreased asymmetric divisions, and increased Sox2+ cells suggested that Cic deficiency increases a population of self-renewing (albeit dysregulated) stem-like cells.

Gliogenic determinants are elevated in Cic-deficient NSCs. Although the proliferative effects of Cic deficiency in NSCs were clear, what was intriguing was the added possibility that Cic loss may be affecting lineage selection. As the CicFlox/FoxG1Cre/+ phenotype was characterized by fewer neurons and increased glia, we asked whether Cic loss altered the expression of glial lineage selection factors and, thus, gliogenic potential.

To this end, we examined Sox9 and Olig2 expression in the NSCs and their progeny after E13 Cre- or control-electroporation. Sox9 is an HMG box transcription factor present in a range of central nervous system cells, including stem cells, glial precursors, and later glia. It has key roles in stem cell maintenance and in driving differentiation programs away from neurogenesis, toward gliogenesis, at the stage of gliogenic initiation. Similarly, Olig2 is expressed in many cells including stem cells, oligodendroglial lineage cells, and some neurons; but it has a major role in establishing oligodendroglial competence. Two days post electroporation, the fraction of GFP+ cells expressing Sox9 was significantly increased over controls (Fig. 4d, e). Five days post electroporation, a smaller fraction of Cic-deleted NSCs became Tbr1+ early-born (Fig. 4f, g). Conversely, greater fractions became Aldh1+ astrocytes and Pdgfra+ OPCs (Fig. 4h–i).

These findings were echoed in the expression of stem and lineage markers in cultured NSCs. When maintained in serum-free NSC proliferation media, both Cic-null and control NSCs strongly expressed Nestin, as expected, and were devoid of staining for Gfap, αIII Tubulin (as detected by Tuj1), and committed oligodendroglial markers (Fig. 5a–c). There, was, however, a marked increase in the percentage of cells expressing Sox9 and Olig2 in Cic-null cultures (Fig. 5a–c). It is reasonable to posit that the increased Sox9 and Olig2 present in Cic-null NSCs then sets an intrinsic foundation for pro-glial

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**Fig. 4** Deletion of Cic in the neurogenic period biases NSCs to glial lineage selection. a Localized deletion of Cic in neural stem cells via in utero electroporation into VZ cell of Cic-floxed embryos; targeted cells carry a fluorescent marker and express either cre recombinase (Cre), dominant negative ETV5 (DNETV5), or not (Empty control), depending on the electroporated plasmid. b–l Immunofluorescence staining and quantitation of cells in the electroporated patch. Sox2+ stem cells b, c and Sox9+ glioblasts d, e in n = 4 per group 2 days after E13 electroporation, analyzed at E15. Late-born Tbr1+ neurons f, g, Aldh1+ astrocytes h, i, Olig2+ oligodendroglial lineage cells j, k, and Pdgfra+ oligodendrocyte precursors cells l detected 5 days after E13 electroporation, analysis at E18. Data from n = 5 for control and n = 6 mice for Cre for Tbr2 staining; n = 6 for control and n = 5 for cre for Aldh1 staining; and n = 4 per group for Olig2 and Pdgfra staining. Statistical analyses between control and experimental groups performed by ANOVA with Tukey’s post hoc test. Scale bars: 50 μm. Source data are provided as a Source Data file. Data shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ns-not significant. Labels: Ctx-cortex, LV-lateral ventricle, VZ-ventricular zone.
or pro-oligodendroglial programs starting early in the neural cellular hierarchy.

Cic-deficient NSCs are biased to the oligodendroglial lineage.

To directly test cell type specification capacity, we challenged Cic-null and control NSCs with exposure to different lineage-promoting culture conditions. Neuronal differentiation was induced by culturing cells with B27 and cAMP. Astrocytic differentiation was induced by culturing cells in media with B27 and tri-iodo-thyronine. After 10-day exposures to these conditions, cultures were analyzed for cellular identity and morphology.

Following exposure to neuronal-promoting conditions, Tuj1+ cells comprised the majority of cells in Cic-null cultures, but were only a minority in Cic-null cultures (Fig. 5d, g). Furthermore, the Tuj1+ Cic-null cells that were present had fewer and less complex cell processes than in their control counterparts (Fig. 5d). Likewise, in response to astrocyte-promoting conditions, Gfap+ cells comprised the majority of cells in control cultures, but only a minority in Cic-null cultures (Fig. 5e, h); and the Gfap+ Cic-null cells that were present displayed more rudimentary processes than controls (Fig. 5e, h). In the oligodendrocyte-promoting condition, there was a higher fraction of Olig2+ cells in Cic-null cultures compared with control, as well as increased Olig2 by western blotting (Fig. 5f–j). When analyzed for markers of OPCs versus more mature oligodendrocytes, Cic-null cultures also had a greater percentage of Olig2+ Pdgfra+ cells than Cic-wildtype cultures (Fig. 5f–j). Conversely, the percentage of Olig2+ MBP+ cells was decreased in Cic-null cultures (Fig. 5f–j). Furthermore, in

Fig. 5 Cultured Cic-null cells are oligodendrocyte lineage-biased. a-c Cic-null and control mouse NSCs, cultured in serum-free stem cell proliferation media and analyzed for expression of Nestin, Sox9, Olig2, Gfap, and βIII-Tubulin (Tuj1). Representative images of immunofluorescence staining c quantitation of cell percentages expressing Nestin, Sox9, and Olig2 b, and western blots for Nestin, Sox9, Olig2, Gfap, and Tuj1, with β-Actin loading control. c-d Responses of Cic-null and control NSCs to 10-day exposure to lineage-directed differentiation conditions for neurons, astrocytes, and oligodendrocytes. Representative staining and quantitation of Tuj1+ cells d, g, and morphology. e-h Representative staining and quantitation of Olig2+ cells e, h in cultures exposed to astrocyte-promoting conditions. Representative staining and quantitation of Gfap+ cells f, i in cultures exposed to oligodendrocyte-promoting conditions, with western blotting j for Olig2, Pdgfra, and Mbp. Data from n = 3 biologic replicates per condition for all experiments. Source data are provided as a Source Data file. Statistical analysis performed by unpaired t test. Scale bar: 10 μm. Data shown as mean ± SD. ns—not significant, **p < 0.01, ****p < 0.0001
the few Olig2 + MBP + cells that were present in Cic-null cultures, process formation was rudimentary compared with Cic-wildtype cells (Fig. 5f). Thus, Cic is required for appropriate NSC responsiveness to extrinsic specification/differentiation cues. Our findings indicate that Cic-deficient NSCs have more limited ability to specify neuronal or astrocytic fates, but have increased permissiveness to the oligodendroglial lineage. Some findings suggest an additional maturation defect after lineage commitment, but we did not address this here.

We then asked what Cic-null NSCs became in the astrocytic and neuronal conditions, if not astrocytes and neurons. In the astrocyte-promoting condition, the Gfap- population was comprised predominantly of Sox2 + cells and, to a lesser extent Olig2 + cells, both of which were significantly increased in Cic-null cultures (Fig. 6a, b). Tuj1 + cells were rare in this condition, and did not differ between Cic-null cells and controls. In the neuronal-promoting condition, the Tuj1- population was similarly comprised predominantly of Sox2 + cells and Olig2 + cells.

**Fig. 6** Alternate lineage selection of Cic-null NSCs in response to extrinsic differentiation cues. **a, b** NSCs cultured for 10 days in astrocyte-promoting media and stained for Sox2, Olig2, GFAP, and Tuj1. Representative images of cultures stained for oligodendrocytes (Olig2 +), neurons (Tuj1 +) and stem cells (Sox2 +) in **a,** and corresponding quantitations of Olig2 + cells, Tuj1 + cells, and Sox2 + cells as a percentage of total cell enumerated by DAPI nuclear counterstain **b, c, d** NSCs cultured for 10 days under neuronal-promoting conditions, with representative images **c** of cultures stained for oligodendrocytes (Olig2 +), astrocytes (Gfap +) and stem cells (Sox2 +), and corresponding quantitations **d** of Olig2 + cells, Gfap + cells, and Sox2 + cells as a percentage of total cells enumerated by DAPI. **e, f** NSCs cultured for 10 days under oligodendroglial-promoting conditions, with representative images **e** of cultures stained for neurons (Tuj1 +), astrocytes (Gfap +) and stem cells (Sox2 +), and corresponding quantitations **f** of Tuj1 + cells, Gfap + cells, and Sox2 + cells as a percentage of total cells enumerated by DAPI. **g** Percentage of Sox2 + cells positive for EdU incorporation after 10 days exposure to neuronal-promoting conditions. **h** Percentage of Olig2 + positive for EdU incorporation after 10 days exposure to neuronal-promoting conditions. Absolute counts of Gfap + cells/well after differentiating 4 \times 10^3 NSCs in astrocytic conditions for 10 days. j Absolute counts of Tuj1 + cells/well after differentiation 4 \times 10^3 NSCs in neuronal conditions for 10 days. Data from n = 3 biologic replicates per condition for all experiments. Source data are provided as a Source Data file. Statistical analysis performed by unpaired t test. Bars indicate mean ± SD. Scale bar: 10 μm. ns—not significant, *p < 0.05, **p < 0.001.
(Fig. 6c, d), with a small but non-significant increase also in the percentage of Gfap + cells in the Cic-null cultures (Fig. 6d). With respect to oligodendroglial-promoting conditions, Sox2 + cells were increased, whereas both TuJ1 + and Gfap + cells were reduced compared with controls (Fig. 6c, f). These data further support that Cic-deficient NSCs are less responsive to neuronal and astrocytic cues, and instead remain as stem cells or are oligodendroglial-biased.

Because we previously found that Cic loss increases NSC proliferation, it was important to exclude that the decreased neuronal and astrocytic fractions observed in our experiments were owing to a dilutional effect from greater NSC/OPC proliferation. In both conditions, after the 10-day differentiation protocol, only rare proliferating cells remained (Supplementary Fig. 4j). Of the Sox2 + and Olig2 + cells present in the neuronal or astrocytic conditions, <1% were EdU +, and there were no significant differences between the proliferation of Sox2 + or Olig2 + cells in the Cic-null vs. control cultures (Fig. 6g, h). Moreover, when the data were analyzed for absolute numbers of cells rather than percentages of cells, we found fewer total Gfap + cells in the astrocytic conditions and a fewer total TuJ1 + cells in the neuronal conditions in Cic-null cultures compared with controls (Fig. 6i, j). No differences in apoptosis were found between Cic-null and control cultures (Supplementary Fig. 4j). Together, these data indicate that the cell type skewing is not a result of increased NSC proliferation, but rather is owing to intrinsic fate bias.

Cumulatively, we conclude that Cic is a critical regulator of proliferation, self-renewal, and cell fate of NSCs. Loss of Cic expands the OPC pool by not only increasing NSC proliferation but also by biasing their specification towards oligodendrocytes.

**Cic represses Ets factor expression in the forebrain.** As Cic is a transcriptional repressor, one mechanism for our findings is that Cic loss de-represses specific genes driving NSC proliferation and lineage selection. In this respect, Etv genes, encoding Ets-domain transcription factors, are candidate targets-of-interest. *Etv1, 4, 5* have been identified as Cic targets in various mammalian cells, and are overexpressed in ODGs (Supplementary Fig. 5f–h and 32). Furthermore, previous studies have documented Cic occupancy at the *Etv5* promoter, at least in cerebellar tissue. Consistent with a functional relationship between Cic and Ets expression in the forebrain, we found that NSCs and OPCs (the cells with the lowest levels of nuclear Cic), expressed the highest levels of Etv4 and 5 (Fig. 7f–k; Supplemental Fig. 5e). We also confirmed that in forebrain tissue, there is evidence of *Etv5* promoter occupancy by Cic (Fig. 7c).

If Ets factors are the mediators of the observed effects of Cic loss, we would expect their levels to be elevated in our experimental systems of Cic loss. Indeed, after 2 days of growth in oligodendroglial conditions, *Etv5* mRNA was increased ~37-fold, whereas *Etv4* transcripts were increased ~19-fold in Cic-null cells (Fig. 7a)—a finding also confirmed at the protein level (Fig. 7b). Etv1 levels, however, were much lower, and did not significantly differ between Cic-null and -wildtype cells. Although both *Etv4* and *Etv5* were both de-repressed in the absence of Cic, because of the comparatively higher levels of *Etv5* relative to *Etv4*, as well as previous studies implicating *Etv5* in mediating glial fate decisions, we focused additional studies on this Ets factor. Electroporation of Cic shRNA into the telencephalic VZ resulted in upregulation of *Etv5* transcript in the electroporated patch within 48 h (Fig. 7d). Our Cic-floxed, Cre-electroporated brains also showed an increase in *Etv5* protein in the electroporated patch (Fig. 7e). These data support that the PEAs3 Ets transcription factors, particularly *Etv4* and *Etv5*, are transcriptional repressive targets of Cic in the forebrain.

**Etv5 de-repression mediates effects of Cic loss in NSCs.** To determine whether the proliferative increase and OPC bias that we observed with Cic ablation was mediated by Etv5, we first asked whether *Etv5* overexpression alone was sufficient to mimic the phenotype of Cic deficiency. Electroporation of wildtype *Etv5* phenocopied the increased proliferation of Cic-null cells (Fig. 8g, h; Supplementary Figs. 5i, 6a). Similarly, *Etv5* overexpression acutely increased proliferation of cultured NSCs (Fig. 8k, Supplementary Fig. 6c). We also performed epistasis experiments introducing a dominant negative form (*DNETV5*) in which *Etv5* was fused to the *Engrailed* transcriptional repressor domain. Introduction of *DNETV5* or knockdown of *Etv5* with siRNA or shRNA reduced the proliferation of cultured Cic-deficient NSCs back to control levels (Fig. 8l; Supplementary Fig. 6d). In vivo, the increased proliferation observed upon deletion of Cic by cre electroporation was abrogated by co-electroporation with *DNETV5*, resulting in proliferation that was comparable to baseline (Fig. 8i, j compared with 8g, h; Supplementary Fig. 6b).

Based on the extent of the effects of *Etv5* overexpression and *DNETV5* rescue in these assays, we conclude that the proliferative effects of Cic loss are largely driven by de-repression of *Etv5*.

With respect to the altered proportions of cell types that we had observed in Cic-deficient NSCs exposed to the different lineage-specific culture conditions, overexpression of wildtype *Etv5* also phenocopied Cic loss in many respects. There were decreased fractions of TuJ1 + cells and Gfap + cells in the neuronal and astrocytic conditions, and increased fractions of Olig2 + and Olig2 + Pdgfra + cells in the oligodendrocytic condition when *Etv5* was overexpressed—although the severity of the changes was somewhat less marked with *Etv5* overexpression compared with Cic loss. *DNETV5* was also able to substantially (although incompletely) rescue the phenotype of Cic-deficient cells in these assays. In the neuronal and astrocytic conditions, *DNETV5* significantly increased the populations of TuJ1 + and Gfap + cells, respectively (Fig. 8a, b, d, e). The shifts in Olig2 + and Olig2 + Pdgfra + OPCs also returned to control levels (Fig. 8c, f). The MBP + fraction similarly showed a substantial although partial rescue (Fig. 8c, f). That the *DNETV5* rescue in several of the differentiation assays was significant but only partial suggests that other factors may contribute to the lineage specification effects. Nevertheless, the results point to *Etv5* as playing a large part in the lineage-biased phenotype.

Thus, *Etv5* overexpression is both sufficient for inducing proliferation and cell fate bias effects, and is necessary for the proliferative dysregulation and lineage bias resulting from Cic loss.

**Etv5 blockade decreases tumorigenicity of human ODG.** The above studies provide a plausible framework for understanding how disruption of Cic, a cancer-associated gene, can perturb a cell’s normal developmental trajectory. We then asked whether in established ODG, persistent loss of Cic or the resulting *ETV5* upregulation is required for maintaining malignancy. We used two patient-derived ODG cell lines, BT54 and BT88, to investigate this. Both lines harbor 1p19q co-deletion. BT54 harbors a splice acceptor site mutation in the remaining *CIC* exon 6, whereas BT88 harbors a missense mutation in the remaining *CIC* exon 20. In both BT54 and BT88, either stable re-expression of wildtype Cic or stable expression of *DNETV5* significantly reduced proliferation as measured by EdU incorporation (Fig. 9a, c, f). Sphere-forming ability was also decreased by Cic expression or by introduction of *DNETV5* (Fig. 9b, d, e). These anti-proliferative and anti-self-renewal effects were also seen using ETV5 shRNA (Supplementary Fig. 7a, b). Consistent with an ongoing requirement for Cic loss or ETV5 expression for
proliferation and self-renewal in ODG, in vivo tumorigenicity of BT88 cells was markedly reduced, and survival of animals was increased, by stable expression of either wildtype CIC or DNETV5 (Fig. 10). Thus, in cells that are already transformed to ODG, CIC loss and the subsequent elevation of ETV5 remains important to sustain the proliferative phenotype. CIC loss and ongoing ETV5 elevation are also required for preventing oligodendroglial differentiation in established ODG.

**Fig. 7** Etv5 is a direct target of CIC transcriptional repression. a, b Etv1, Etv4, and Etv5 mRNA expression levels a and protein expression levels b in cultured CIC-null and -control cells after 48-hour exposure to oligodendrocytic differentiating conditions; transcript levels normalized to average of three (Actin, GAPDH, Tubulin beta chain) housekeeping genes; data from n = 3 biologic replicates. Statistical analyses performed by unpaired t test. Data shown as mean ± SD. *p < 0.05. c ChIP-PCR for CIC at the Etv5 promoter. d Electroporation of CIC shRNA or non-targeting (NT) shRNA at E13 followed by in situ hybridization for Etv5 and GFP at E15; Etv5 transcripts are upregulated in areas of CIC knockdown indicated by the GFP expression; data from n ≥ 3 mice per group. e Immunofluorescence staining for Etv5 and GFP protein expression 2 days after Cre electroporation into E13 VZ of CIC-floxed embryos. GFP + cells show increased Etv5 protein. Scale bar: 50 μm. f Immunofluorescence staining for Etv5 expression in Sox2 + NSCs at mid-neurogenesis. g–i Immunofluorescence staining for Etv5 expression in Glia + astrocytes, Pdgfra + OPCs, and CNPase + oligodendrocytes in P56 adult cortex. Scale bar: 50 μm. j Quantitation of Etv5 nuclear staining for n ≥ 20 cells for each cell type marker shown; each point represents one cell quantitated. Data from n = 5 mice per group. Data shown as mean ± SD. Source data are provided as a Source Data file. Statistical analyses performed with ANOVA with Tukey's post hoc test. ns—not significant, ****p < 0.0001. SVZ—subventricular zone, LV—lateral ventricle, Ctx—cortex. k Schematic depiction of relationship of CIC and Etv5 expression as neural stem cells differentiate to mature myelinating oligodendrocytes.
In response to oligodendrocyte differentiation conditions, ODG cells with CIC re-expression or DNETV5 introduction showed increased expression of CNPase compared with controls, indicating improvement in capacity for the tumor cells to differentiate and mature along the oligodendroglial progression (Figure 9g, h; Supplementary Fig. 7c, d). Interestingly, however, effects on neuronal and astrocytic differentiation were mixed. CIC re-expression increased the ability of cells to respond to neuronal differentiation cues by increasing expressing bIII-Tubulin (Tuj1+) and extending neurites; however this was not phenocopied by DNETV5 (Fig. 9g, h; Supplementary Fig. 7c, d). With respect to expression of GFAP in response to astrocytic differentiation cues, no significant differences were detected with either CIC re-expression or DNETV5 introduction compared with BT88 control (Fig. 9g, h; Supplementary Fig. 7c, d). The results suggest that although CIC loss and ET5 overexpression maintain ODG cell proliferation and self-renewal, and prevent progression of oligodendroglial differentiation in established ODG; other aspects such as the capacity for neuronal or astrocytic differentiation are determined by additional genetic (or epigenetic) alterations, at least in our culture system. The effects of CIC and ET5 on ODG differentiation were not evaluated in vivo, however, as the lesions resulting from implantation of CIC- or DNETV5-expressing BT88 cells were too small to provide meaningful cell numbers for assessment.
Overall, our results indicate that Cic regulates NSC proliferation and cell fate in neurodevelopment and ODG, and that the pro-proliferative and pro-OPC phenotypes observed with Cic loss are largely mediated through Ets transcriptional de-repression.

**Discussion**

Genomic analyses of brain cancers have implicated CIC as a tumor suppressor gene in diffuse gliomas, particularly ODGs\(^3,4,36\). Here, we report for the first time cell type-specific differences in CIC expression in the brain. Moreover, our work provides new insight into the roles of this putative tumor suppressor in regulating the developmental fate of neural stem/progenitor cells.

We found that Cic loss biases neural stem cells away from selection of the neuronal lineage towards selection of glial lineages. Adding to prior knowledge that RAS/MAPK pathway signaling and ETV5 are both involved in gliogenic competence\(^17,34\), our results firmly place Cic at the intersection of...
two, providing a missing link between extrinsic differentiation signals and the execution of transcriptional programs critical for normal neuro- and gliogenesis. With respect to CIC and the anti-neuronal bias that we observed, recent work by Lu et al.14 found that disrupting Ataxin1-Cic complexes resulted in abnormal maturation and maintenance of upper-layer cortical neurons—with effects on behavior, learning, and memory. Though our studies focused on early lineage selection events, and did not systematically address questions of cell maturation, some of our in vitro morphological findings are consistent with neuronal and oligodendrocyte maturation defects being part of the Cic phenotype. Future work would be needed to define the complex cellular dependencies on Cic, not only in the forebrain but also in the cerebellum where Cic pathology has also been implicated37,38.

Our findings build on observations by Yang et al.13 that Cic deficiency increases a population of proliferating OPCs in the brain. We now provide mechanistic foundations for those observations—identifying symmetric cell division alterations, lineage selection effects, and a downstream effector. These provide tangible links between Cic loss and ODG biology—as both dysregulated proliferation and the persistence of an immature OPC phenotype are cardinal features of this cancer. Many parallels exist between normal OPCs and the cells comprising ODG. In common, both OPCs and ODGs express PDGF, PDGFR, and NG2, which control OPC differentiation39,40. Together, the results may explain some of these ODG features, and are consistent with other reports, suggesting that dysregulation of OPCs or OPC-like cells are amongst the early changes in gliomagenesis24,41. There are some notable differences between the work by Yang et al.13, however, and our studies. One difference is that the prior study used HOG cells that, despite their historic name, do not carry the ODG-defining genetic features of lp19q loss or Cic mutation. Similarly, the Pdgfra-amplified/overexpressed mouse glioma model that was used is more akin to an RTK-driven GBM than ODG. In deploying the BT88 and BT54 models (which genetically and phenotypically recapitulate ODG), our work may be closer to clinical relevance for ODG. Another difference is that our studies also further clarify the early neuronal-glial fate decision effects of NSCs, with the bias away from neuronal fates and toward glial fates in the setting of Cic loss. Most importantly, however, we now identify Ets repression as a key mechanism underlying the phenotypic effects of Cic loss in NSCs.

The observation that DNETV5 could abrogate so much of the pro-proliferative and pro-OPC phenotype resulting from Cic loss was unexpected, and together with our expression and ETV5 knockdown experiments, points to a critical role for this particular Ets factor. However, we do not exclude that other Ets factors such as Etv4 may contribute. The dominant-negative construct may not be entirely specific for Etv5; cross-reactivity has been reported with similar approaches, and further experiments would be required to dissect the relative contribution of different Ets genes. Nevertheless, the identification of Ets factors as likely mediators is salient to the development of future therapies. Reports of Ets inhibition through small-molecule or small molecule approaches42-44 lend hope that Etv5 could potentially be inhibited for clinical benefit for ODG.

Finally, though relevant to ODG, our studies do not directly model ODG. Our Cic conditional knockout mice, however, will be a useful tool for future cell type- and stage-specific Cic deletion in the context of other mutations such as of IDH1(R132H)45. We conceptualize IDH1/2 mutations as a first event in ODG, causing epigenetic changes that expand the potential pool of cells vulnerable to transformation, with second-hit loss of Cic serving to dysregulate proliferation, bias cells to oligodendroglial lineage, and delay them in an immature state. Additional studies using mouse models and human ODG samples, however, are needed to further dissect the relationship between Cic and IDH mutations. Although one recent study used single-cell RNA-Seq to examine ODG cell subpopulations and did not detect any differences between ODG cells with and without Cic mutation aside from the increased expression of PEA3 Ets factors, the number of cells analyzed was limiting for resolution of cell type-specific differences, and functional studies had not been performed46. Similar approaches with larger cell numbers might provide further insight into the ODG biology with respect to Cic.

In summary, we show that Cic regulates proliferation, fate decisions, and differentiation in neural stem cells, with loss particularly expanding the OPC population. Furthermore, our findings indicate that disruption of the Cic-Etv axis is central to the biology of ODG.

**Methods**

**Mice.** CD1 outbred mice (Jackson Labs) were used for Cic expression analyses. Cic conditional knockout (Cic-CKO) mice were generated at Taconic Artemis by homologous recombination to flank Cic exons 2-11 with loxp sites. The exons targeted are 2-11 of Cic short form, transcript variant 1, NM_027882.4 (equivalent to exons 3-12 of the Cic long form, NM_001302811.1, Cic transcript variant 4). The targeting vector was generated using clones from the C57BL/6J RPCIB-731 BAC library, and consisted of a 4.9 kb 5’ flanking arm, neomycin resistance cassette (flanked by FRT sites), 5.5 kb loxp-flanked region, a pyruvate resistance cassette (flanked by F3 sites), and 6.0 kb 3’ flanking arm. After homologous recombination in C57BL/6 N Tac ES cells, generation of chimeric animals, and germline transmission, Neo6 and Puro6 cassettes were removed via Flp recombination by breeding with a Flp deleter line. The final Cic-CKO allele carries loxp sites in introns 1 and 11, and single residual FRT and F3 sites. Expression of Cre recombinase results in deletion of exons 2-11 and frameshifting of the remaining Cic-S exons 12-20. Genotyping primers were as follows: Cic-5’ flanking region (273 bp wt, 410 bp KO); Cic-3’-CTTTGTCACTGTCTGCCTTCC-3’ (forward) and 5’-TGGTATACACCCGCTTGG-3’ (reverse); Cic-3’ flanking region (273 bp wt, 410 bp KO); Cic-3’-CTTTGTCACTGTCTGCCTTCC-3’ (forward) and 5’-TGGTATACACCCGCTTGG-3’ (reverse). FoxG1-cre mice (Jackson Labs) were bred to Cic-CKO mice to generate telencephalic Cic.
Fig. 10 Tumorigenicity of ODG cells is reduced by CIC re-expression or ETV5 blockade. a, b Bioluminescence imaging of NOD-SCID mice at 6 weeks post-orthotopic implantation of BT88 oligodendroglialoma cells stably transfected with empty-GFP-luciferase or CIC-GFP-luciferase. Representative images a and luminescence data b from five mice per cohort. Data shown as mean ± SD. Statistical analyses by student’s t test. **p < 0.01. c, e Representative images of brain sections from mice implanted with control-GFP-luciferase BT88 cells or CIC-GFP-luciferase BT88 cells stained with H&E c or GFP e at 6 weeks post implant. Scale bars: 50 μm. d, f Representative images of brain sections from mice implanted with control-GFP-luciferase BT88 cells or DNETV5-GFP stained with H&E d or GFP f at 6 weeks post implant. Scale bars: 50 μm. g Kaplan–Meier survival analysis of mice implanted with BT88 cell transfected with empty-GFP-luciferase or CIC-GFP-luciferase. n = 5 mice per cohort. Statistical analysis by log-rank (Mantel-Cox) test; p = 0.0021. h Kaplan–Meier survival analysis for BT88 cell transfected with empty-GFP or DNETV5-GFP. n = 5 mice per cohort. Source data are provided as a Source Data file. Statistical analysis by log-rank (Mantel-Cox) test; p = 0.0018
knocks out and littermate controls. After killing, dissected brains were processed for
histology, western blotting, or cell culture. Knockout in tissue or cells post-cr was
confirmed by qRT-PCR using primer/probe sets for CIC (Applied Biosystems).

**In vitro electroporation.** In utero electroporation was performed as described
previously using the following plasmids: plKO.1-Cic shRNA (Sigma),
TRCN0000030462; 5'-CCGAGCCGAGAGAGAGACATCATCTTCGGAAT
TATGGTCCTTCTCCCGCTTTTTTG-3', plKO.1-non-targeting shRNA (Sigma);
5'-CTAAGGTTAATGCGCCTGCGAGCGAGGGCGACTTAACCT-
TAGG-3', pCIG2-Cre (which contains Cre-ires-GFP), pCIC-ETV5 (which
contains ETV5-ires-mCherry), Super piggyBac Transposase (Systems Biosciences,
SB), and piggyBac cargo vector PBSR1B1 (SB) into which cDNA were cloned for
Turbo-Cre and Etv5. The DNETV5 consists of an
contains Etv5-IRES-mCherry), Super piggyBac Transposase (Systems Biosciences,
SB), and piggyBac cargo vector PBSR1B1 (SB) into which cDNA were cloned for
Turbo-Cre and Etv5. The DNETV5 consists of an

**Immunostaining.** Tissue was fixed overnight in 4% PFA, cryoprotected in 30%
sucrose/PBS, and embedded in Tissue-Tek O.C.T. (Sakura Finetek) prior to cutting
6 µm cryosections on SuperFrost Plus slides (VWR). Cultured cells grown on
coverslips were fixed for 20 min in 4% PFA then rinsed in PBS. Permeabilization
was performed with 1 x TBST (Tris-buffed saline: 25 mTs Tris, 0.1% NaCl, 0.1%
Triton X-100) for 15 min at room temperature (RT). Three percent goat or horse
serum in TBST x 30 min was used for block. Primary antibodies applied for 1 h at
RT, followed 4 h in 4% BSA. Alexa Fluor secondary antibodies were applied at 1:500
dilution for 1 h at RT. Nuclei were counterstained in 4'-6-diamidino-2phenylindole
(DAPI, Santa Cruz) and mounted with FluorSave Reagent (Calbiochem). For
immunostaining of tissue sections when two or more primary antibodies were from
the same host species and for comparison of cell type specific CIC expression,
the Opal 4-color IHC kit (Perkin Elmer) was used for manufacturer’s protocol.

**Western blotting.** Cells/tissue was lysed in RIPA buffer (100 mTris Cl pH 8,
1 mTe sodium deoxy-

**Antibodies.** Primary antibodies directed against the following were used: CIC
(Rabbit Polyclonal, 1:500, 1:1000 WB; A301-204, Bethyl), CIC (Rabbit
Polyclonal, 1:100 IF; PA4-46018; Thermo), Olig2 (Mouse Monoclonal, 1:1250,
1:10000 WB; AB11635, Millipore), Olig2 (Rabbit (Polyclonal, 1:500, 1:1000
WB; A5431, Sigma), ETV4 (Rabbit Polyclonal, 1:500 IF; A2062, Cell Signaling);
ETV5 (Polyclonal Rabbit, 1:500, 1:1000 WB, AB15894, Millipore), Neuron-specific beta-III Tubulin
(Mouse Monoclonal, 1:1000, 1:1000 WB, MAB1195, clone TuJ1, R&D), GFAP
(Mouse Monoclonal, 1:1000, 1:1000 WB, MAB360, clone G5, Millipore), GAP43
(Rat Monoclonal, 1:1000, 1:1000 WB, 9G8, Millipore), Tubulin beta chain).

**Trypan assay.** 150,000 cells were seeded per T25 flask in mNSC media and
counted after 72 h on a TC20 cell counter (Biorad) using Trypan blue stain

**Neural stem cell culture.** The VZ of E15 brains were dissected, and tissue was
dissociated to single cells using Accumax (EMD Millipore). Cells were grown at
time of harvesting. EdU (50 µg/ml in phosphate-buffered saline; PBS) was injected
then followed by electrical pulses (6 x 43 V, 950 ms interval) using platinum tweezers
stereos (7 mm, Protec) using a BTX square wave generator (Harvard Apparatus).
Post-procedure, embryos were allowed to develop until the time of harvesting.
EdU (50 µg/ml in phosphate-buffered saline; PBS) was added intraperitoneally into
the pregnant dam 30 min prior to killing.

**Transfection of cultured cells.** In all, 1-4 x 106 dissociated cells were
suspended in 100 µL of Amaxa Mouse NSC Nucleofector Solution (VPG-1004,
Lonza) with 5 µg of plasmid DNA. Nucleofection was performed with a Nucleo-
ector II Device (Amaxa) using the A-033 program. Cells were returned to mouse
neural stem cell media for further expansion/selection. For siRNA-mediated Etv5
knockdown, cells were transfected with 30 nM Etv5 or Etv4 ON-TARGET
Plus SMARTpool siRNAs (Dharmacon) using Lipofectamine 3000 reagent
per manufacturer’s protocol. Cells were assayed 48 h hours post transfection.

**Trypan assay.** In total, 150,000 cells were seeded per T25 flask in mNSC media
and counted after 72 h on a TC20 cell counter (Biorad) using Trypan blue stain

**Neural colony-forming cell assay.** Cells in semi-solid media were prepared using
the Neurocult NCFC Assay Kit (Stem Cell Technologies) per manufacturer’s
protocol. Cells were plated at a density of 1650 cells/ml in mNSC media on
plates coated with CTS CELStart (Thermo). After 20 h, cells were fixed in 4%
paraformaldehyde (PFA) and immunostained for Ki67. Plates were scored as
symmetric proliferative if both daughter nuclei were Ki67+ and asymmetric if one nucleus was Ki67+ and
the other Ki67–.

**Lineage-directed differentiation of NSCs.** To promote neuronal differentiation,
NSCs were seeded in mNSC proliferation media (Stem Cell Technologies) on
coverslips coated with Poly-l-Ornithine and Laminin. After 24 h, media was
replaced with Neurobasal Media, 2% B-27, 20 mGluMaxA-M (Thermo). After
more 4 days dibutyryl cAMP (Sigma) was added daily to a final concentration
of 0.5 mM. At the time of plating, NSCs were cultured on coverslips coated with
Poly-l-Ornithine and Laminin. After 24 h, media was replaced with Neurobasal media, 2% B-27, GlutaMAX-I, 30 ng/mL, 3.3,5-Tri-iodo-
-thyronine sodium (Sigma). To promote astrocyte differentiation, NSCs were
seeded on coverslips coated with Gelrex (Thermo). After 24 h, media was replaced
with DMEM, 1% N2 Supplement, 2 mGluMaxA-M, 1% FBS. The cells were
maintained in the respective differentiation media for 10 days prior to assays
of lineage and differentiation.
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**Intracranial xenografts and bioluminescence imaging.** BT88 ODG cells were obtained from the Dr. S. Weiss, University of Calgary. A total of 1 × 10^6 BT88 cells stably transfected with CIC-GFP-Luciferase or DNETV5-GFP or respective empty vectors were stereotactically implanted into the right striata of 6- to 8-week-old NOD/SCID mice. Six weeks post implantation, tumor burden was measured either by bioluminescence imaging using IVIS Spectrum In Vivo Imaging System (Xenogen) and/or by histology from killed animals. Mice were anesthetized under isoflurane and intraperitoneal injection of Xenolight D-Luciferin (Perkin Elmer) was administered at a dose of 150 mg/kg body weight. Acquisition of bioluminescence images was performed 10 min post injection. Analysis was performed using Living Image Software by measurement of photon counts (photons/cm^2) with a region of interest drawn around the bioluminescence signal.

**Statistics.** Data are represented as mean ± SD from at least three biologic replicates for experiments. Comparisons between experimental and control samples were made using two-tailed t test or, when there were more than two groups, using ANOVA. Tukey’s procedure was applied post hoc to correct for multiple comparisons during multiple pairwise analyses (e.g., differential Cic expression among cell types). Bonferroni post hoc correction was applied for statistical analysis of data from NanoString assays. Survival curves were analyzed by log-rank (Mantel–Cox) test. Statistical analyses were performed using Prism software (Graphpad).

**Study approval.** Animal use was approved by the University of Calgary Animal Care Committee (protocol AC16-0266) in compliance with the Guidelines of the Canadian Council of Animal Care. Collection and generation of the glioma cell lines were approved by the Health Research Ethics Board of Alberta (protocols HREBA.CC-16-0762 and HREBBA.CC-16-0154).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files. All biological materials used in this study are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization, J.A.C., S.T.A.; methodology, J.A.C., C.S., S.T.A.; investigation, S.T.A., A.D.R., R.D., L.F., M.J.C., M.A., W.W., S.O.L., M.D.B.; acquisition, analyses, and interpretation of data, J.A.C., S.T.A., A.D.R.; writing—original draft, J.A.C., S.T.A.; writing—review & editing, J.A.C., C.S., J.G.C., S.M.R., M.A.M., W.W., R.D., S.O.L.; funding acquisition, J.A.C., J.G.C., C.S.; resources, L.A., M.A., C.S.; supervision, J.A.C., C.S., J.G.C., S.M.R.

Additional information

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