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RIT1 GTPase Regulates Sox2 Transcriptional Activity and Hippocampal Neurogenesis*

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Adult neurogenesis, the process of generating mature neurons from neuronal progenitor cells, makes critical contributions to neural circuitry and brain function in both healthy and disease states. Neurogenesis is a highly regulated process in which diverse environmental and physiological stimuli are relayed to resident neural stem cell populations to control the transcription of genes involved in self-renewal and differentiation. Understanding the molecular mechanisms governing neurogenesis is necessary for the development of translational strategies to harness this process for neuronal repair. Here we report that the Ras-related GTPase RIT1 serves to control the sequential proliferation and differentiation of adult hippocampal neural progenitor cells, with *in vivo* expression of active RIT1 driving robust adult neurogenesis. Gene expression profiling analysis demonstrates increased expression of a specific set of transcription factors known to govern adult neurogenesis in response to active RIT1 expression in the hippocampus, including sex-determining region Y-related HMG box 2 (Sox2), a well established regulator of stem cell self-renewal and neurogenesis. In adult hippocampal neuronal precursor cells, RIT1 controls an Akt-dependent signaling cascade, resulting in the stabilization and transcriptional activation of phosphorylated Sox2. This study supports a role for RIT1 in relaying niche-derived signals to neural/stem progenitor cells to control transcription of genes involved in self-renewal and differentiation.

The adult brain harbors germinal cell niches in the subventricular zone of the lateral ventricles and the subgranular zone in the dentate gyrus of the hippocampus (1–3). The activation of these relatively quiescent neural progenitor cell (NPC)² populations and their capacity to differentiate into specialized cells is under rigorous cellular control (4). Transduction of a variety

of extracellular niche stimuli results in the activation of intracellular regulatory mechanisms within NPCs, signaling cascades that include transcription factors, and epigenetic regulators that serve to finely coordinate gene expression during neurogenesis (5). The transcription factor sex-determining region Y-related HMG box 2 (Sox2) is a member of the SOXB1 family of transcription factors with established roles in maintaining stem cell/progenitor cell properties in diverse cellular populations (6, 7). Genetic deletion of Sox2 causes neurodegeneration and impaired neurogenesis in the adult mouse brain, whereas human Sox2 mutations are associated with anophthalmia, a disorder characterized by cognitive disabilities and defects in hippocampal development (8, 9). Although the role of Sox2 in stem cell maintenance within the neurogenic niche has been described previously (10, 11), the molecular mechanisms that control Sox2 activation in response to appropriate neurogenic cues remain poorly characterized.

ES cell self-renewal and pluripotency are regulated by a core group of transcription factors, including Sox2 (12–14). Although Sox2 is not highly expressed in ES cells, its protein levels are under stringent control. For example, moderate increases in Sox2 lead to differentiation of ES cells primarily into neural ectodermal cells (15), whereas reduced levels of Sox2 trigger differentiation toward the trophectoderm cell fate (16). Furthermore, Sox2 has a critical role in lineage specification (17), and Sox2 proteins levels are differentially regulated in distinct cell lineages during early development. Although Sox2 expression is under rigid transcriptional control (14), additional post-transcriptional mechanisms have recently been reported. In embryonic stem cells, Sox2 stabilization and transcriptional activation are controlled by a balance of site-specific methylation and phosphorylation (18, 19). However, it is unclear whether a similar regulatory cascade operates in NPCs, and the molecular mechanisms that regulate Sox2 activity in the neurogenic niche remain to be identified.

RIT1 is member of the Ras-related family of small GTP-binding proteins, a group of structurally related and evolutionarily conserved proteins that share the ability to undergo guanine nucleotide-dependent conformational change (20, 21). Functioning with their allied regulatory and effector protein networks, Ras-related GTPases serve as critical cellular biotimers, coupling diverse cellular stimuli to the spatial and temporal regulation of signal transduction pathways that contribute to almost every aspect of cellular physiology. RIT1 is widely expressed, including throughout the human and mouse brain

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² The abbreviations used are: NPC, neural progenitor/stem cell; Dox, doxycycline; DTG, double transgenic; HNPC, hippocampal neural progenitor cell; DCX, doublecortin; EV, empty vector; DG, dentate gyrus; CA, constitutively active; HBSS, Hank's Balanced Salt Solution.

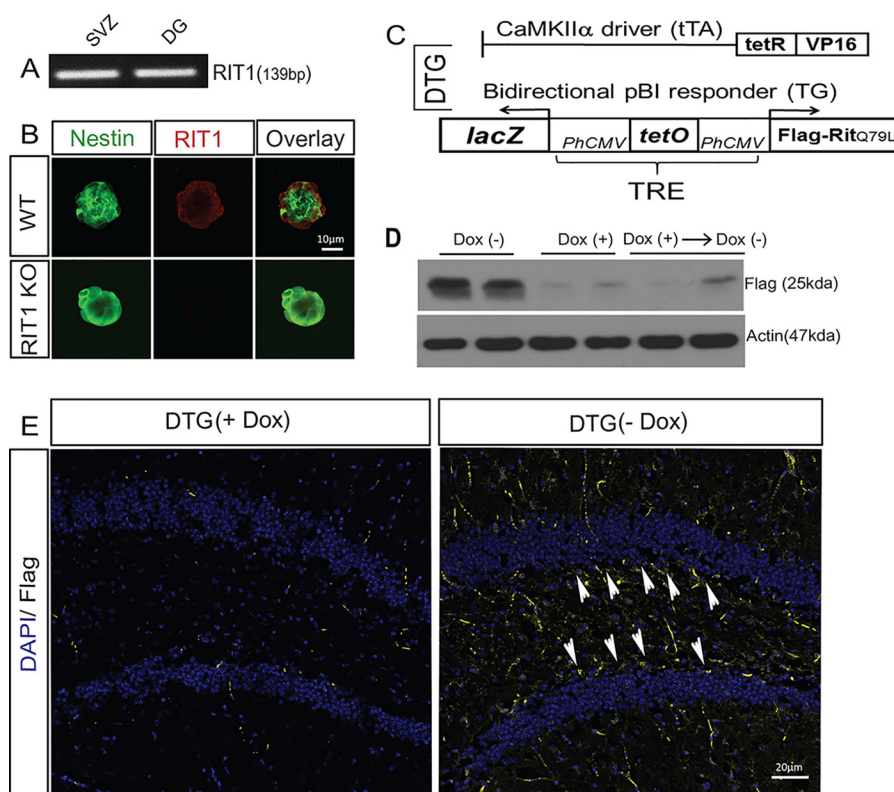


FIGURE 1. Generation of the conditional *RIT1* mouse model. *A*, semiquantitative RT-PCR demonstrates *RIT1* expression in the adult mouse subventricular zone (SVZ) and DG ($n = 4$). *B*, representative confocal images of *RIT1* protein expression in WT and *RIT1*^{-/-} cultured neurospheres. *C*, schematic representing the binary transgenic system regulated by Dox to inducibly overexpress *RIT1*. *D*, representative Western blotting analysis showing FLAG-*RIT1*^{Q79L} protein expression in brain extracts from 4-month-old DTG mice \pm Dox versus WT control. *E*, representative immunohistochemistry for FLAG-*RIT1*^{Q79L} (yellow) in the dentate gyrus of 4-month-old DTG mice on Dox (+) or 3 weeks after removal of the Dox diet (-). Arrowheads, FLAG-expressing cells. Nuclei (DAPI) are shown in blue (magnification $\times 20$).

(22–24). At the molecular level, we have previously described roles for *RIT1* in the regulation of axonal and dendritic growth (23), activation of Akt (25–27), and control of cAMP response element-binding protein transcriptional activity (27). More recently, we identified a role for *RIT1* in the survival of adult-born hippocampal neurons following traumatic brain injury (28). Following cortical contusion, *RIT1* deficiency resulted in a significant delay in injury-induced hippocampal neurogenesis, suggesting that *RIT1* might be an integral component of a signaling pathway involved in neural progenitor activation (28). To generate a deeper understanding *RIT1* function in the CNS, we developed a conditional mouse model allowing doxycycline-regulated expression of activated *RIT1*. Here we report that active *RIT1* expression drives robust hippocampal neurogenesis through activation of a pro-neural transcriptional program. *RIT1* signaling controls the transcriptional activity of Sox2 in neural progenitor cells, supporting a key role for *RIT1* in the dynamic regulation of adult neurogenesis.

Results

***RIT1* Is Expressed in the Dentate Gyrus**—*RIT1* loss sensitizes immature hippocampal neurons to stress-dependent apoptosis and blunts hippocampal neural progenitor cell activation following traumatic brain injury (23). Consistent with a role for *RIT1* in regulating adult neurogenesis, semiquantitative RT-PCR analysis confirmed *RIT1* expression in both neurogenic niches of the CNS (Fig. 1*A*), the subgranular zone in the dentate

gyrus of the hippocampus, and the subventricular zone of the lateral ventricles. This was validated using confocal laser-scanning imaging of *RIT1* protein expression in WT and *RIT1*^{-/-} subgranular zone neurospheres (Fig. 1*B*). To model *RIT1* activation and examine its functional effect on neurogenesis, we generated a line of constitutively active *RIT1*-overexpressing transgenic mice using a neuron-specific binary tetracycline/doxycycline (Dox)-regulated system in which double transgenic (DTG) mice express FLAG-tagged *RIT1*^{Q79L} when Dox is removed from the diet (29) (Fig. 1*C*). Western blotting confirmed Dox-regulated expression of the transgene (Fig. 1*D*). Moderate *in vivo* overexpression of active *RIT1* was observed in the dentate gyrus of young adult DTG mice 3 weeks after removal of the Dox diet (Fig. 1*E*).

***RIT1* Signaling Induces Pro-neural Gene Expression**—Cell cycle regulators, transcription factors, and epigenetic control proteins are key regulators of adult neurogenesis (30). Because *RIT1* is known to control a variety of transcription factors (21, 27, 31), we performed a pathway-focused PCR array analysis of the dentate gyrus from DTG mice 3 weeks after removal of doxycycline from the diet to investigate the expression of genes known to regulate neurogenesis and neural stem cell differentiation. As seen in Fig. 2*A*, active *RIT1* expression stimulates the expression of Sox2 ($p < 0.05$) along with a collection of pro-neural genes, including Ngn2 ($p < 0.01$), Ascl1 ($p < 0.05$), and NeuroD1 ($p < 0.05$). These results were independently verified

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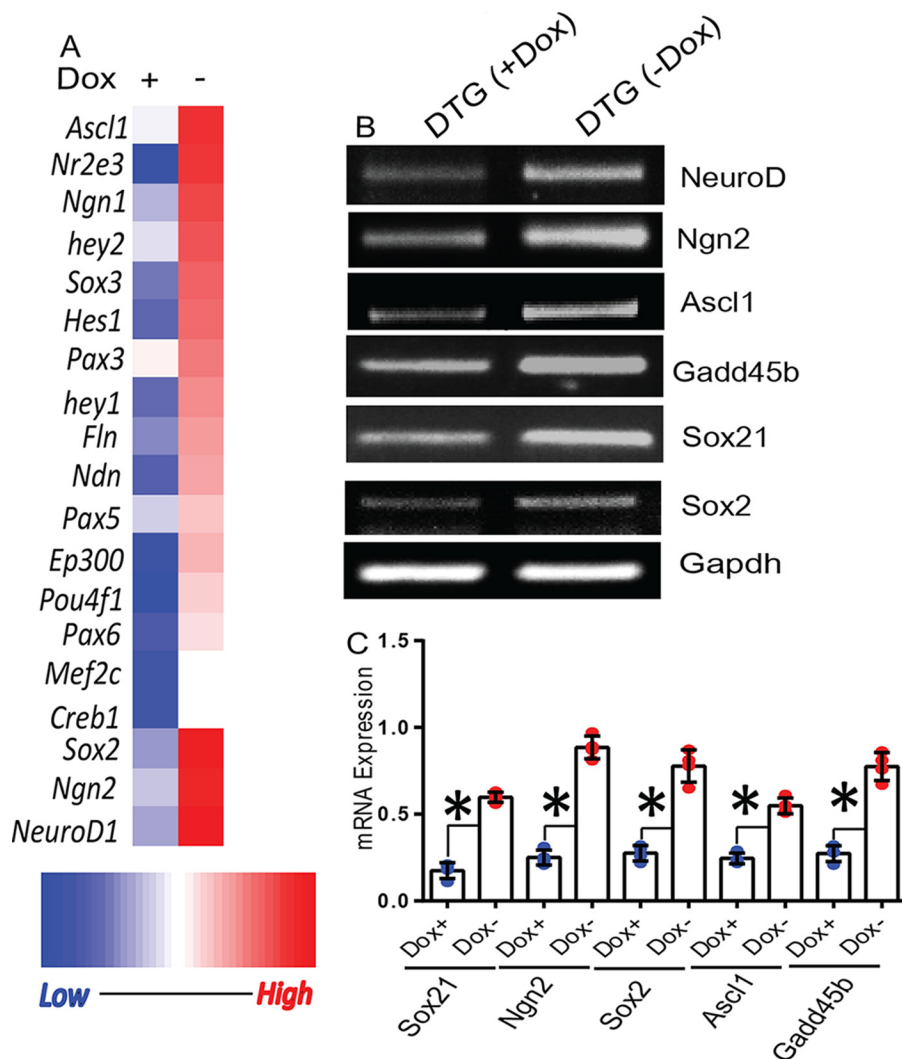


FIGURE 2. **Pro-neural gene expression profile associated with RIT1 expression.** *A*, total RNA prepared from the dentate gyrus of DTG mice \pm Dox diet was subjected to PCR array analysis for neurogenic transcription factors, and the data were plotted as a heat map. *B*, semiquantitative RT-PCR validation of the array data and downstream targets of Sox2 (Sox21 and Gadd45b). *C*, quantification of RT-PCR data. Results are presented as mean \pm S.E. calculated from three separate experiments. *, $p < 0.05$.

using semiquantitative RT-PCR (Fig. 2, *B* and *C*). Because Sox2 has been shown to directly bind the promoters of NeuroD1 and Ngn2 to enable activation of the neuronal differentiation program in response to appropriate neurogenic stimuli (32), we also examined the expression of more widely known targets of Sox2 transcription, e.g. Sox21 and Gadd45b. As seen in Fig. 2*C*, both Sox21 and Gadd45b levels increased in RIT1^{Q79L}-overexpressing DTG mice. Taken together, these data suggest that constitutively active RIT1 signaling leads to activation of Sox2 and expression of pro-neural genes in the dentate gyrus.

Active RIT1 Stabilizes Sox2 Protein Levels in Vivo and in Vitro—To determine whether RIT1-dependent pro-neural gene induction involves Sox2 activation, we next examined whether RIT1 signaling regulates Sox2 protein levels *in vivo* and *in vitro*. Immunohistochemical analysis using laser-scanning confocal microscopy showed a prominent increase in Sox2 protein levels ($p < 0.01$) in the dentate gyrus of DTG mice following Dox withdrawal (Fig. 3, *A* and *D*). This result was confirmed by immunoblotting (Fig. 3*C*) Transient transfection of primary hippocampal neural progenitor cells (HNPCs) with

a vector expressing active RIT1 also resulted in elevated Sox2 levels relative to empty vector ($p < 0.05$) (Fig. 3, *B* and *E*). Together with the gene expression analysis, these data suggest that RIT1 regulates Sox2 function in the hippocampus.

Active RIT1 Promotes HNPC Expansion—Sox2 plays important roles in maintaining neural stem cell/progenitor cell properties, including their capacity to proliferate and self-renewal (10, 11). Because RIT1 signaling was capable of directing a pro-neural transcriptional program, including Sox2 activation, we reasoned that it might also regulate HNPC proliferation. We performed immunostaining to identify proliferating (Nestin⁺/Ki67⁺) hippocampal neural stem cells. As seen in Fig. 4, *A* and *C*, expression of activated RIT1 leads to robust amplification of Nestin⁺/Ki67⁺ cells within the dentate gyrus. These results were further confirmed by transfecting HNPCs with a vector expressing active RIT1 and resulted in increased proliferation, as monitored by the number of Nestin⁺/Ki67⁺ cells (Fig. 4, *B*, and *D*; $p < 0.05$). RIT1-dependent HNPC expansion was accompanied by increased Sox2 transcriptional activity, monitored using a luciferase reporter assay in transfected

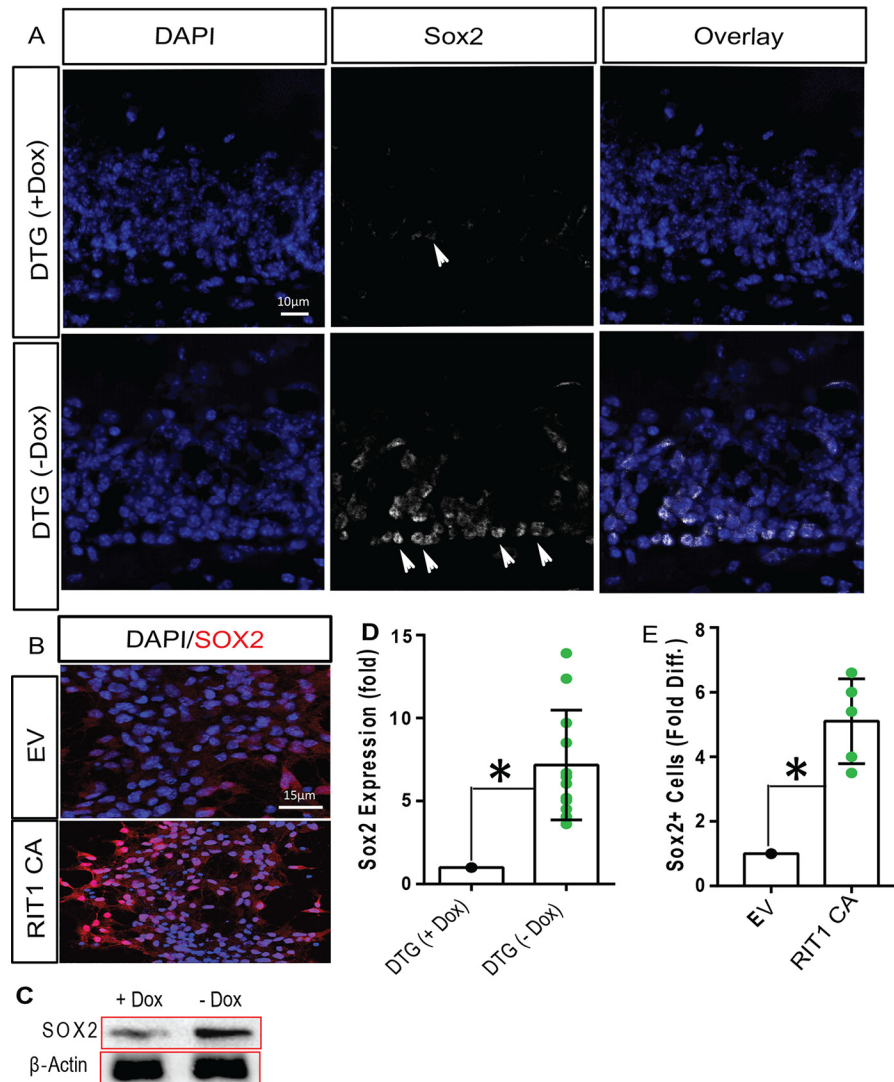


FIGURE 3. RIT1 overexpression increases Sox2. *A*, immunofluorescence for Sox2 in *white* (white arrowheads, magnification $\times 20$), showing that protein expression is greatly increased in the DG of DTG mice after Dox removal ($n = 6/\text{group}$). Nuclei are shown in blue. *B*, immunofluorescence of Sox2 (red) in HNPCs following transfection with FLAG-RIT1^{Q79L} (CA) or EV control. Nuclei are shown in blue ($n = 3$). *C*, immunoblot analysis of endogenous Sox2 in the DG of DTG mice before and after Dox removal ($n = 3/\text{group}$). Note that Sox2 levels increase upon expression of active RIT1. *D* and *E*, quantitation of Sox2 expression and Sox2-expressing cells. Results are presented as mean \pm S.E. calculated from three separate experiments. *, $p < 0.05$.

HNPCs (Fig. 4*E*, $p < 0.01$). Cumulatively, these data demonstrate that RIT1 regulates HNPC proliferation in parallel with Sox2 transcriptional activation.

RIT1-Sox2 Signaling Regulates Adult Hippocampal Neurogenesis—To examine the *in vivo* effect of active RIT1 expression on hippocampal neurogenesis, 3-month-old DTG mice were shifted to a Dox-free diet for 3 weeks, and the number of immature doublecortin-positive (DCX⁺) neuroblasts was assessed by immunostaining. As seen in Fig. 5*A*, expression of activated RIT1 results in a marked increase in DCX⁺ neuroblasts compared with DTG mice under Dox suppression ($p < 0.01$) (Fig. 5, *A* and *B*). Consistent with an ability of Rit to promote adult hippocampal neurogenesis, there were significant increases in both Tbr2⁺ (Fig. 5*C*, $p < 0.05$) and NeuroD1⁺ intermediate neural progenitors (Fig. 5, *D* and *E*; $p < 0.05$) in DTG mice following Dox withdrawal. These data strongly suggest that RIT1 signaling regulates hippocampal neurogenesis with an accompanying expansion of immediate neural precursor cells.

RIT1-mediated Sox2 Activation Involves Akt Signaling—Our previous studies have shown that RIT1 activates a p38/mTORC2/Akt signaling cascade to promote cell survival in response to oxidative stress (26). Because Akt is known to phosphorylate Sox2 at Thr¹¹⁸, increasing both protein stability and transcriptional activity (18), we next asked whether RIT1 controls an Akt/Sox2 signaling cascade in HNPCs. Expression of activated RIT1 increased the number of HNPCs expressing activated Akt ($p < 0.01$), monitored by anti-phospho-Akt Ser⁴⁷³ immunostaining (Fig. 6, *A* and *B*). In agreement with the increase in active Akt, there was an 8-fold increase in the number of phospho-Sox2-Thr¹¹⁸⁺ nuclei in HNPCs transfected with active RIT1 compared with empty vector (EV) controls (Fig. 7, *A* and *C*; $p < 0.01$). Pharmacological inhibitor studies suggest that RIT1-dependent Sox2 activation requires Akt signaling because treatment with the Akt inhibitor triciribine resulted in a marked decrease in the number of phospho-Sox2-Thr¹¹⁸⁺ nuclei in RIT1^{Q79L}-overexpressing HNPCs (Fig. 7, *A*

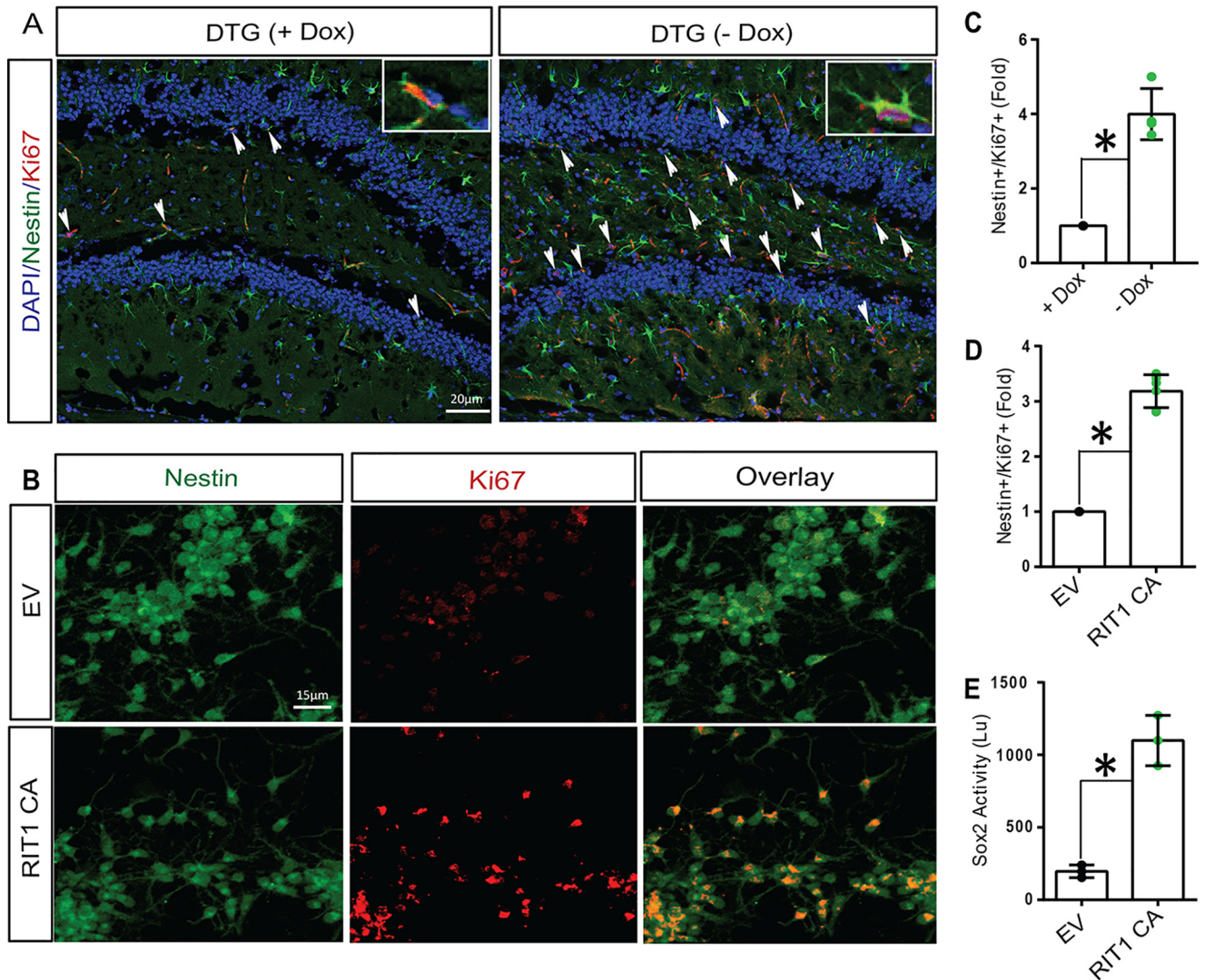


FIGURE 4. Neuronal RIT1 expression increases HNPC proliferation and Sox2 transcriptional activity. *A* and *C*, representative immunofluorescent images (*A*) and quantification (*C*) of the DG from DTG mice immunostained for Nestin (green) and Ki67 (red) to label proliferating neuronal stem cells. Nuclei (DAPI) are shown in blue. *B*, HNPCs were transfected with Myc-tagged RIT1^{Q79L} (CA) or EV, and proliferation was assessed by immunohistochemical detection of Nestin (green) and Ki67 (red) co-labeled cells. *D*, quantification of HNPC proliferation (Nestin⁺/Ki67⁺) as mean \pm S.E. from three independent experiments. *E*, HNPCs were transfected as in *A* in the presence of a Sox2 luciferase reporter construct. Luciferase activity was evaluated 48 h post-transfection as described under "Experimental Procedures." Results are presented as mean \pm S.E. for three independent experiments repeated in triplicate. *, $p < 0.05$.

and *C*). Moreover, Akt inhibition blocked the RIT1^{Q79L}-mediated increase in Tuj1-immunoreactive neurons in transfected HNPCs (Fig. 7, *B* and *D*; $p < 0.05$). Taken together, these data suggest that the RIT1-mediated increase in hippocampal neurogenesis requires Akt-dependent Sox2 activation.

Discussion

NSC proliferation and differentiation are regulated by a variety of extracellular niche signals (33). Transcriptional cascades play fundamental roles in NSC regulation and are dynamically regulated by a large number of synergistic and antagonistic niche signals to ensure a ready supply of progenitors to meet the demand for new neurons, oligodendrocytes, and astrocytes (34). Although the core transcription factor Sox2 is a master regulator of neural stem cell biology (7), playing a critical role in neurogenesis within the adult brain (32), the molecular mechanisms that control Sox2-dependent neuronal differentiation remain incompletely characterized.

Here, using a conditional mouse overexpression model (DTG mice), we identify a role for the RIT1 GTPase in Sox2 regulation and the control of neural progenitor/stem cells. We observed prominent expression of a set of pro-neural transcription factors, including Sox2, in the dentate gyrus of mice expressing active RIT1 (Fig. 2). Because Sox2 has recently been shown to bind to the promoters of poised pro-neural genes in NPCs to enable an appropriate neuronal differentiation (32, 35) program, including the neurogenic genes *Ngn2* and *NeuroD1*, we reasoned that RIT1-dependent neurogenic gene expression might rely on Sox2 activation. Indeed, active RIT1 expression both increases Sox2 protein levels (Fig. 3) and stimulates Sox2 transcriptional activation (Fig. 4). Presumably, RIT1-dependent Sox2 regulation provides a novel molecular mechanism for the control of hippocampal neurogenesis in response to select neurogenic stimuli. In keeping with this hypothesis, active RIT1 expression drives robust NPC expansion within the dentate gyrus (Fig. 4). Moreover, although RIT1 deficiency

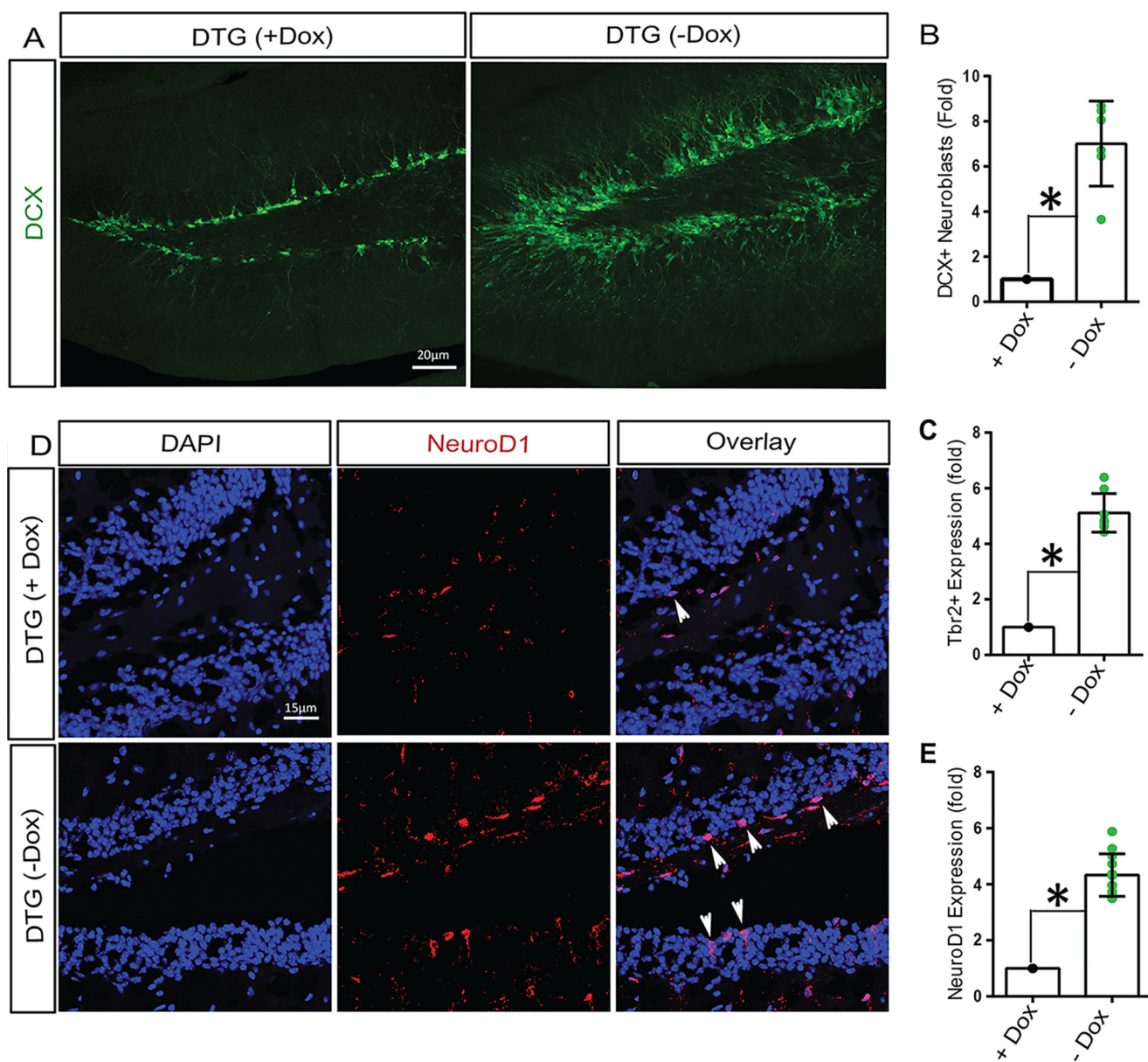


FIGURE 5. **RIT1 induces hippocampal neurogenesis.** *A*, immunofluorescence detection of DCX (green) expression in the DG of DTG mice ($n = 6/\text{group}$). *B*, quantification of DCX⁺ neuroblasts presented as mean \pm S.E. *, $p < 0.05$. *C*, quantification of Tbr2⁺ intermediate neural precursors in the DG of DTG \pm Dox diet mice for 3 weeks ($n = 6/\text{group}$). *, $p < 0.05$. *D*, immunofluorescence detection of NeuroD1 (red, arrowheads), showing that expression is greatly increased in the DG of DTG mice 3 weeks after removal from the Dox diet ($n = 6/\text{group}$) compared with littermates remaining under Dox suppression. Nuclei are shown in blue (magnification $\times 20$). *E*, quantification of NeuroD1⁺ neuroblasts ($n = 6/\text{treatment group}$) as mean \pm S.E. *, $p < 0.05$.

permits normal levels of basal neurogenesis, we have previously noted significant defects in hippocampal neurogenesis following traumatic brain injury (28). Sox2-deficient HNPCs display increased cell death during neuronal differentiation (32), and Sox2-null mice have deficits in hippocampal neurogenesis and augmented neurodegeneration (36, 37), suggesting that RIT1 may be needed for injury-mediated Sox2 activation. Studies are underway to test this hypothesis.

In embryonic stem cells, Sox2 is regulated by competing posttranslational modifications (18, 19). Site-specific methylation promotes Sox2 ubiquitination and degradation. In contrast, Akt phosphorylates Sox2 at Thr¹¹⁸ (18, 38), antagonizing methylation, to stabilize Sox2 levels and induce transcription. As RIT1 was shown to regulate an mTORC2-Akt signaling cas-

cade to promote cellular oxidative stress survival (26), we explored whether RIT1-mediated Sox 2 activation might involve Akt signaling. Active RIT1 signaling results in Akt activation in HNPCs (Fig. 6) and increased levels of total and Sox2-Thr¹¹⁸p⁺ nuclei (Fig. 7), suggesting that RIT1 controls an Akt-Sox2 cascade to promote neurogenesis. In support of this mechanism, pharmacological Akt inhibition blocked active RIT1-dependent Sox2-Thr¹¹⁸ phosphorylation and the generation of Tuj1⁺ neurons in transfected HNPCs (Fig. 7).

The self-renewal and pluripotency of embryonic stem cells is regulated by a core set of transcription factors, including Oct3/Oct4, Sox2, and Nanog (12–14). Recent advances in cell reprogramming have fueled intense interest in the regulation of Sox2 and its role in neural fate determination. Expression of Sox2

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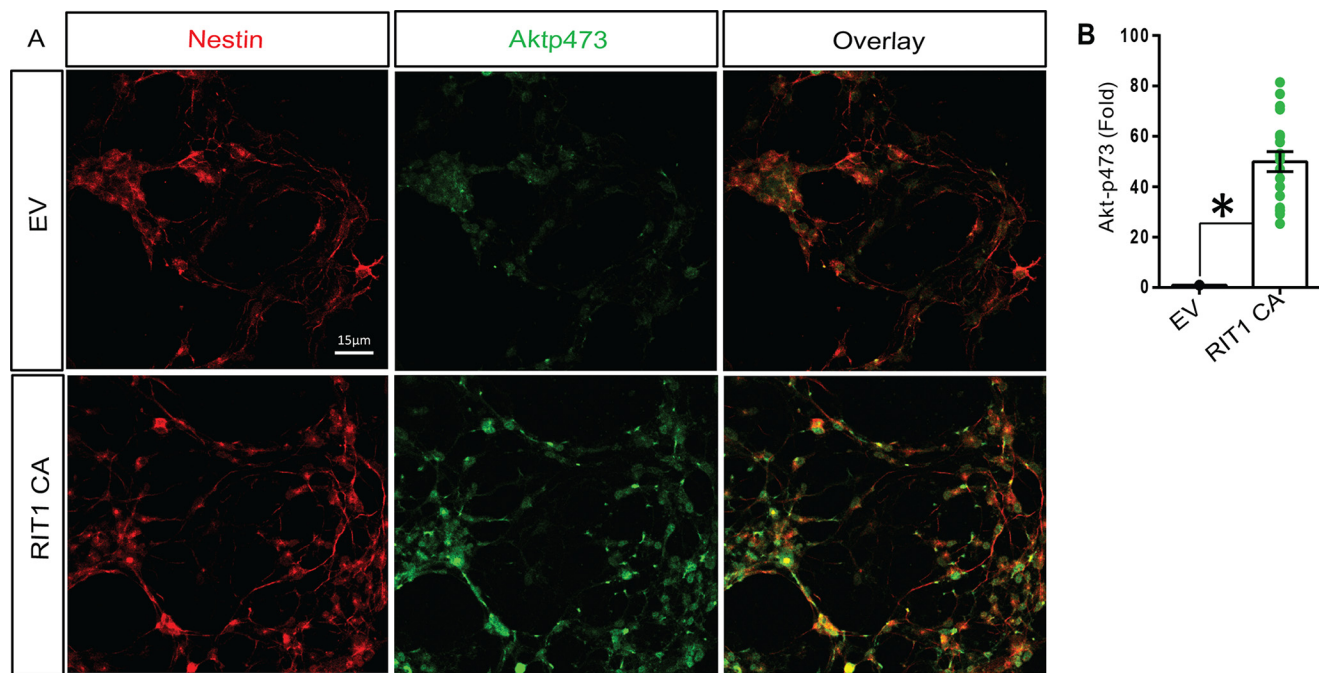


FIGURE 6. **RIT1 regulates Akt in HNPCs.** *A*, HNPCs were transfected with either EV or FLAG-tagged RIT1^{Q97L} (CA), and immunohistochemistry was used to detect Nestin (red) and active Akt (green, phospho-Akt (Ser⁴⁷³)) (magnification $\times 20$). *B*, quantification of HNPC proliferation as mean \pm S.E. from three independent experiments. *, $p < 0.05$.

alone or as part of a core set of transcription factors has been shown to drive somatic cell reprogramming (39–44). In the adult CNS, retroviral delivery of Sox2 has been found to directly or indirectly reprogram resident astrocytes into induced neurons in the injured adult cerebral cortex (45–47). Importantly, there is a cellular progression for Sox2-mediated conversion of adult astrocytes to neurons, involving the neural commitment of progenitors (46). These induced adult neuroblasts are capable of *in vivo* proliferation and generate mature neurons when supplied with neurotrophic factors or following small-molecule treatment (46, 47), which may provide a novel strategy for neuronal regenerative therapy. Because RIT1 controls neurogenesis and stimulates a Sox2/pro-neurogenic transcriptional program, it will be important in future to examine whether active RIT1 might be used for resident astroglial cell reprogramming.

In summary, these studies extend our understanding of Sox2 regulation, identifying RIT1-Akt-Sox2 cascade signaling as a mechanism governing NPC proliferation and neurogenesis. Because RIT1 is known to couple diverse neuronal mitogens and cellular stress stimuli to transcriptional activation, an intriguing possibility is that RIT1-mediated Sox2 activation plays a role in neurogenic niche sensing, serving to link select environmental or physiological stimuli to neural stem cell self-renewal and differentiation. Growing literature indicates that Sox2-dependent reprogramming strategies allow the conversion of glial cells into neurons, allowing the possibility of regenerative cell therapy for repair of the damaged brain (45–49). Identification of the cellular stimuli that activate RIT1 or inducible delivery of active RIT1 (50) might provide a novel therapeutic repair strategy, especially during states of crisis such as the aftermath of traumatic brain injury.

Experimental Procedures

Materials—The following materials were used: DMEM and F12 nutrient mixture (Gibco), triciribine (Millipore), Jetprime transfection reagent with buffer (Polyplus), doxycycline, fetal bovine serum (Gibco), N2 supplement (Thermo Fisher), B27 supplement (Thermo Fisher), RT-PCR buffer, RT Aid enzyme and ribonuclease inhibitor (Thermo Fisher), RT² Profiler neurogenesis PCR array system (SA Biosciences), and SYBR Green with reference control (Bio-Rad).

Animals—All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee in accordance with guidelines established by the National Institutes of Health in the Guide for the Care and Use of Laboratory Animals. Animals were housed up to 5 mice/cage in the University of Kentucky Medical Center vivarium with a 14:10-h light/dark photoperiod and were provided food and water *ad libitum*. DTG mice were produced by transgenesis to overexpress RIT1^{Q79L} in a subset of CNS cells using a regulated binary system based on the tetracycline transactivator protein and the Tet operator (pBI Tet responder, Clontech). Active RIT1 was engineered to contain three copies of the FLAG tag peptide at the N terminus so that it could be distinguished from the endogenous protein. A fragment from the pBI-FLAG-RIT1^{Q79L} vector was released and microinjected into the pronuclei of oocytes (University of Cincinnati Gene Targeting Core, Cincinnati, OH) from C57BL/6 mice. Mice expressing the tTA gene under the control of the Ca²⁺-calmodulin-dependent kinase II α promoter, CaMKII-tTA (Tet-Off), were obtained from The Jackson Laboratory (003010) (51), permitting co-expression of LacZ and FLAG-tagged RIT1^{Q79L} within the CNS, including the dentate gyrus (29). From the prenatal period to the time of study initiation, DTG

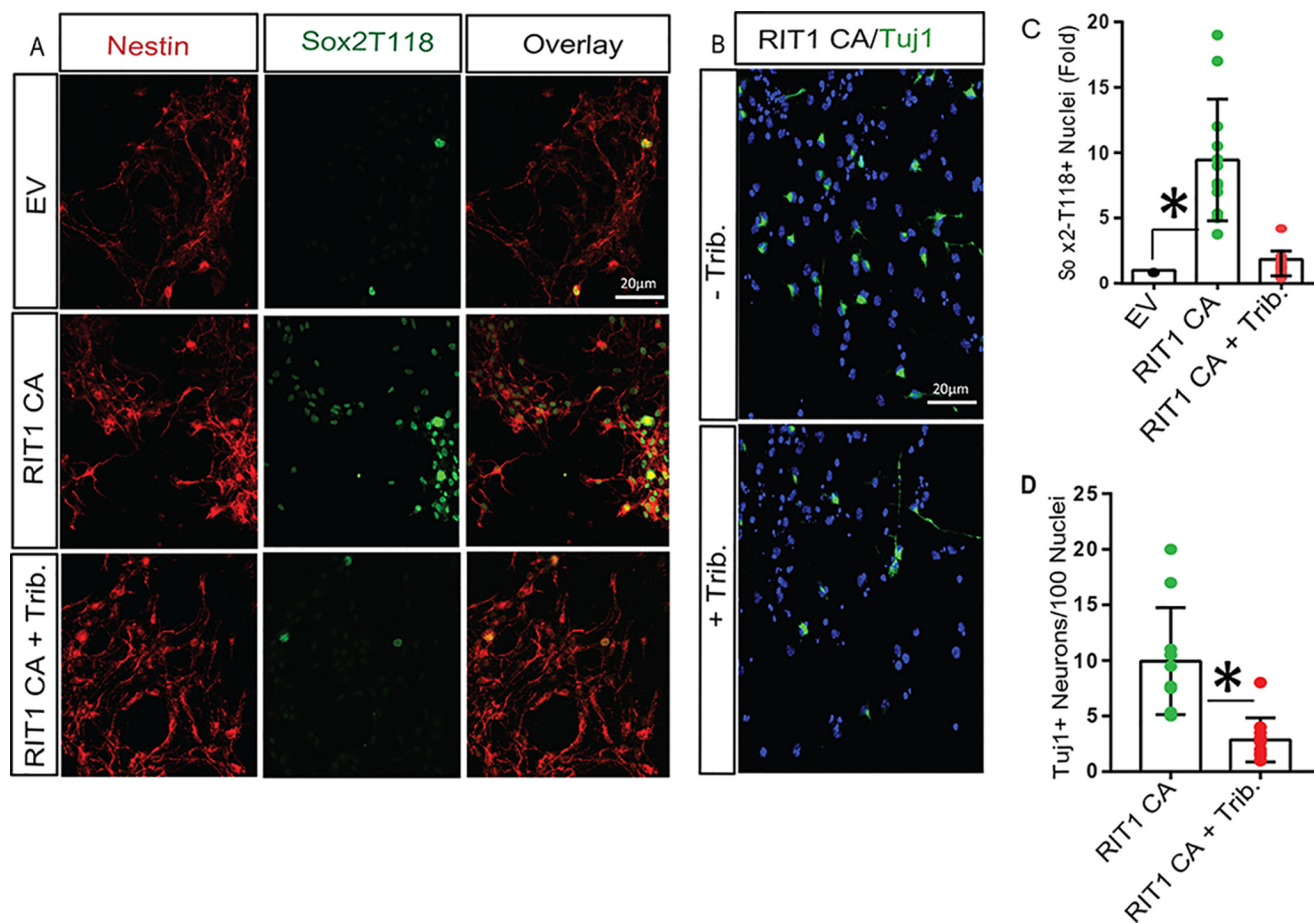


FIGURE 7. RIT1-mediated Sox2 activation requires Akt. A, HNPCs were transfected with either EV or activated RIT1 (CA) and treated with or without Akt inhibitor (trichiribine, 10 μ M) for 12 h, and immunofluorescence was used to detect Nestin (red) and cellular levels of Sox2-Thr¹¹⁸ phosphorylation (green). B, HNPCs expressing FLAG-tagged RIT1^{Q79L} were treated with Akt inhibitor and grown in complete neuronal differentiation medium for 6 days. Neuronal differentiation was assessed by immunofluorescence detection of Tuj1⁺ neurons (magnification \times 20). C and D, quantification of phospho-Sox2⁺ and Tuj1⁺ neurons, respectively, as mean \pm S.E. from three independent experiments. *, $p < 0.05$.

transgenic mice (and their dams) were given dietary supplementation of Dox, an analog of tetracycline, in their chow (200 mg/kg) to suppress FLAG-tagged RIT1^{Q79L} expression. tTA littermates also received Dox-supplemented food. Selective neuronal FLAG-RIT1 expression was achieved by switching mice to a normal chow diet (–Dox) for 3 weeks.

Isolation and Passage of HNPCs—HNPCs were isolated from wild-type mice as described previously (52). Briefly, mice were euthanized and immediately sterilized using 70% ethanol. The brain was quickly dissected and immersed in dissection buffer (HBSS (1 \times) with no Ca²⁺ or Mg²⁺ and 1 \times antibiotic solution (1 \times antibiotic-antimycotic, Gibco)). Using a stereomicroscope, the dentate gyrus (DG) was dissected and placed in dissection buffer on ice. Typically, four to five hippocampi were pooled. After several washes with HBSS (1 \times), the tissue was incubated with enzymatic digestion solution (0.25% trypsin in 1 \times HBSS with activated papain) at 37 $^{\circ}$ C for 30–45 min with frequent shaking. Following digestion, trypsin activity was quenched by repeated washing with 5–10 ml of DMEM, and prewarmed culture medium (N2 containing DMEM/F12 (1:1) with antibiotics, EGF (20 ng/ml), and FGF2 (10 ng/ml)) was added. The tissues were triturated using fire polished Pasteur pipettes three to four times to release hippocampal NPCs. Approximately, 50 \times 10⁴ cells were plated in 12-well plates for 4–5 days. Neurospheres

are evident from day 3 onward. For passaging purposes, the neurospheres from the respective groups were pooled and mechanically dissociated before replating for further culture. All cells used in this study were passaged at least twice. Neurospheres were counted using a grid on a microscope.

Cell Transfections and Treatments—For HNPC transfections, fresh neurospheres were passaged, and \sim 10⁵ cells were plated on poly-D-lysine-coated 12/18-mm coverglasses after appropriate sterilization using nitric acid and repeated autoclaving (53). Cells were transfected 72 h using Jetprime transfection reagent after the initial plating, which is necessary for HNPCs to regain contact. All DNA constructs used in this study have been described previously (27, 28, 54, 55). Jetprime transfection reagent has been shown previously to mediate efficient transfection of murine neuronal stem cells (53). Briefly, pCMV10-Myc RIT1^{Q79L} or empty pCMV10 (1 μ g) vector was mixed with 2 μ l/ μ g Jetprime reagent according to the protocol of the manufacturer. For transfection, 200 μ l of culture medium was collected and mixed with the transfection mixture and distributed in a dropwise method with frequent stirring. The cells were incubated for 6–7 h and washed with Dulbecco's phosphate-buffered saline (1 \times , Corning), and fresh complete medium was added. Cells were allowed to recover for 48 h. To monitor the transfection efficiency, we performed RT-PCR for

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RIT1 and confocal laser-scanning microscopy for the tag protein Myc in these cultures. We could always get >75% transfection efficiency in our experiments. For neuronal differentiation assays, cells after treatments were differentiated by addition of 1% FBS and retinoic acid (1 μM) for 4–6 days.

Tissue Collection and Processing—Animals received an overdose of Fatal-plus (65 mg/kg intraperitoneal sodium pentobarbital) and were perfused with 0.98% saline followed by 4% paraformaldehyde (56). Brains were removed from the skull immediately and post-fixed in 4% paraformaldehyde at 4 °C for 2 days. Brains were then washed extensively to remove the excess paraformaldehyde and incubated in increasing concentrations of 10–30% sucrose solution overnight for 3 days at room temperature. Finally, fully immersed brains were cryosectioned. The tissue blocks were embedded in optimal cutting compound (OCT) and snap-frozen. All blocks were allowed to stand at –80 °C for at least 2 days before sectioning using a cryostat.

Immunohistochemistry—Coronal brain sections, 15 and 40 μm , were cut and mounted on positive fixed microfrosted glass slides (Fisher Scientific). General antigen retrieval was performed in citrate buffer (pH 6) (57). BrdU antigen retrieval was performed using warm trypsin (0.25%) containing 2 N HCl. Sections were washed in PBS and incubated in blocking and permeabilizing buffer (1% serum (matching the host of the secondary antibody that was used) and 1% Triton X-100 in PBS) for 10 min at room temperature, followed by extensive washing (in PBS (pH 7.4)). Primary antibodies against RIT1 (14G7) (Santa Cruz Biotechnology), FLAG, Ki67 (rabbit), BrdU (Sigma), Doublecortin (DCX) (Millipore), Sox2 (Abcam), NeuroD1 (donkey) (Santa Cruz), Nestin (Covance), Akt, phospho-Akt^{S473}, β III tubulin, ERK1/2, anti-phospho-ERK1/2 (Cell Signaling), and phospho-Sox2^{T118} (ECM Biosciences) were diluted in blocking serum, incubated overnight with sections at 4 °C, followed by extensive washing with 1 \times PBS (room temperature). Then, secondary antibodies, either conjugated with Alexa 488, Alexa 568, Alexa 594, or phycoerythrin, were applied to the sections for 2 h in the dark, followed by extensive washing with 1 \times PBS (room temperature). The sections were air-dried, mounted with DAPI-containing medium, and imaged 2–3 days later for coverslips to settle. For immunocytochemistry, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized and blocked as described for tissue sections. Imaging was performed using either a Nikon A1 or C2 confocal microscope. All images were acquired using the NIS Elements software package.

PCR Array—The mouse neurogenesis RT² Profiler PCR array (Qiagen) was used for neurogenesis-specific transcription factor screening. Briefly, mice were sacrificed, and total RNA was prepared (Promega SV total RNA kit) from the dentate gyrus of DTG mice (lifetime doxycycline diet (control) or 3 weeks after removal of the Dox diet (–Dox)) and immediately frozen at –70 °C. Aliquots of RNA (500 ng from each group) were reverse-transcribed using a Bio-Rad RT kit. The cDNA was diluted 1:3 in ultrapure water for the final PCR. The cDNA was mixed with SYBR Green into the array plates, and cycling was performed according to the instructions of the manufacturer. A melt curve analysis was performed to check for product integ-

ity. The values were obtained using the ΔCT method and plotted as heat maps.

Semiquantitative Real-time PCR—Briefly, total RNA was prepared from the dissected hippocampi and subventricular zones separately under aseptic conditions from each mouse (56) using a Promega SV total RNA isolation kit. RNA was stored immediately at –70 °C in aliquots. RNA (100 ng/sample) was reverse-transcribed using the iScript first strand synthesis kit (Bio-Rad) with the following gene-specific primers: *Sox2*, 5' AAGCCATGAATGCAGAGGAGGACT3' (forward) and 5' AGCTGCAGG CAGCCGGCGACC3' (reverse); *Ascl1*, 5' CCCCCAACTACTCCAACGAC3' (forward) and 5' GTCCAGCAGCTCTTGTTCCT3' (reverse); *Sox215*, 5' CTCATCCTT-CCTCCCTCCCG3' (forward) and 5' CCAAGCCAGCGGAC-TCAGAGAC3' (reverse); *Gadd45b*, 5' CCTGGCCATAGAC-GAAGAAG3' (forward) and 5' AGCCTCTGCATGCCTGAT-AC3' (reverse); *BDNF*, *NeuroD1*, 5' AAGCCATGAATGCAG AGGAGGACT3' (forward) and 5' AGCTGCAGGCAGCCG-GCGACC3' (reverse); *Neurog2*, 5' TCACGAAG ATCGAGAC-GCTG3' (forward) and 5' CTCCAGGAGGAAGGTGGAGA3' (reverse); and *GAPDH*, 5' TGCACCACCACTGCTTAGC3' (forward) and 5' GGCATGGACTGTGGTCATGAG3' (reverse). PCR products were amplified using DreamTaq Green (Thermo Fisher), resolved on 1–2% agarose gels, and imaged with Gel Logic 112 (Fisher Biotech).

Luciferase Gene Reporter Assays—The Cignal Sox2 reporter (Qiagen, CCS-0038L) contains repeats of the Sox2 promoter and Sox2 binding sites driving firefly luciferase expression. HNPCs were allowed to adhere for 72 h after replating and then transfected with EV or *RIT1* Q79L (RIT1 CA). Approximately 72 h post-transfection, cells were washed with PBS and lysed for 30 min in passive lysis buffer (Promega luciferase kit). The lysate was centrifuged at 12,000 $\times g$ for 30 s at room temperature and put on ice for immediate use or frozen at –80 °C. A 10- to 15- μl aliquot of the lysis supernatant was mixed with 100 μl of detection reagent, and luminescence was recorded using a luminometer with a 10-s time interval. The readings were noted and averaged for statistical purposes.

Statistical Analysis—All data are represented as mean \pm S.E. Statistical analysis was carried out by either non-parametric unpaired one-tailed *t* test or one-way analysis of variance combined with post hoc analysis using Tukey-Kramer multiple comparisons. Comparisons with *p* < 0.05 were considered significant.

Author Contributions—S. M. conducted most of the experiments, analyzed the results, and wrote most of the paper. W. C. generated the DTG transgenic mouse and performed studies examining how active RIT1 alters neurogenesis. D. A. A. conceived the idea for the project, helped to generate the transgenic model, and wrote the manuscript with S. M. All authors analyzed the results and approved the final version of the manuscript.

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RIT1 GTPase Regulates Sox2 Transcriptional Activity and Hippocampal Neurogenesis

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