- 1 Dephosphorylation of 4EBP1/2 Induces Prenatal Neural Stem Cell Quiescence
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41 antibodies for use in immunostaining. 41 antibodies for use in immunostaining.
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63 **Abstract**
64 A
65 proliferat 64 A limiting factor in the regenerative capacity of the adult brain is the abundance and
65 proliferative ability of neural stem cells (NSCs). Adult NSCs are derived from a subpopulation of
66 embryonic NSCs that temporar 66 embryonic NSCs that temporarily enter quiescence during mid-gestation and remain quiescent until
67 postnatal reactivation. Here we present evidence that the mechanistic/mammalian target of rapamycin 67 bostnatal reactivation. Here we present evidence that the mechanistic/mammalian target of rapamycin
68 (mTOR) pathway regulates quiescence entry in embryonic NSCs of the developing forebrain. Throughout 68 (mTOR) pathway regulates quiescence entry in embryonic NSCs of the developing forebrain. Throughout
69 embryogenesis, two downstream effectors of mTOR, p-4EBP1/2 T37/46 and p-S6 S240/244, were 69 embryogenesis, two downstream effectors of mTOR, p-4EBP1/2 T37/46 and p-S6 S240/244, were
70 mutually exclusive in NSCs, rarely occurring in the same cell. While 4EBP1/2 was phosphorylated in stem 69 69 70 mutually exclusive in NSCs, rarely occurring in the same cell. While 4EBP1/2 was phosphorylated in stem
T1 6 cells undergoing mitosis at the ventricular surface, S6 was phosphorylated in more differentiated cells 71 cells undergoing mitosis at the ventricular surface, S6 was phosphorylated in more differentiated cells
72 migrating away from the ventricle. Phosphorylation of 4EBP1/2, but not S6, was responsive to 72 migrating away from the ventricle. Phosphorylation of 4EBP1/2, but not S6, was responsive to
73 quiescence induction in cultured embryonic NSCs. Further, inhibition of p-4EBP1/2, but not p-S6, was 73 mauiescence induction in cultured embryonic NSCs. Further, inhibition of p-4EBP1/2, but not p-S6, was 74
74 msufficient to induce quiescence. Collectively, this work offers new insight into the regulation of 74 sufficient to induce quiescence. Collectively, this work offers new insight into the regulation of
75 quiescence entry in embryonic NSCs and, thereby, correct patterning of the adult brain. These data 75 quiescence entry in embryonic NSCs and, thereby, correct patterning of the adult brain. These data
76 suggest unique biological functions of specific posttranslational modifications and indicate that the 76 suggest unique biological functions of specific posttranslational modifications and indicate that the
77 preferential inhibition of such modifications may be a useful therapeutic approach in 77 preferential inhibition of such modifications may be a useful therapeutic approach in
78 neurodevelopmental-diseases-where-NSC-numbers,-proliferation,-and-differentiation-are-altered. neurodevelopmental diseases where NSC numbers, proliferation, and differentiation are altered. 79
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88 Introduction
89 The v
90 Dostnatal ma 89 The ventricular-subventricular zone (V-SVZ) is the largest neural stem cell (NSC) niche in the
80 postnatal mammalian brain and cells from this niche produce multiple subtypes of neurons and glia
81 (Alvarez-Buvlla & Ga 91 (Alvarez-Buylla & García-Verdugo, 2002; Bond et al., 2021; Chaker et al., 2016; David-Bercholz et al.,
92 2021; Delgado et al., 2021; Doetsch et al., 1997; Ihrie & Álvarez-Buylla, 2011; Merkle et al., 2004; 92 2021; Delgado et al., 2021; Doetsch et al., 1997; Ihrie & Álvarez-Buylla, 2011; Merkle et al., 2004;
93 Obernier & Alvarez-Buylla, 2019; Radecki & Samanta, 2022; Young et al., 2007). Postnatal adult NSCs, 93 Obernier & Alvarez-Buylla, 2019; Radecki & Samanta, 2022; Young et al., 2007). Postnatal adult NSCs, 94 also termed B1 cells, are derived from a subpopulation of embryonic NSCs, termed pre-B1 cells, that 94 also termed B1 cells, are derived from a subpopulation of embryonic NSCs, termed pre-B1 cells, that
95 enter a transient quiescence during mid- to late- neurogenesis, with the cell cycle slowing down 95 enter a transient quiescence during mid- to late- neurogenesis, with the cell cycle slowing down
96 beginning at embryonic day 13.5 and being completed by embryonic day 15.5. These cells remain 96 beginning at embryonic day 13.5 and being completed by embryonic day 15.5. These cells remain
97 quiescent until reactivation in early adulthood (postnatal days 21 – 28) (Fuentealba et al., 2015; 97 quiescent until reactivation in early adulthood (postnatal days $21 - 28$) (Fuentealba et al., 2015;
98 Furutachi et al., 2015). Disruptions to either the cycling kinetics or total number of quiescent NSC 98 Furutachi et al., 2015). Disruptions to either the cycling kinetics or total number of quiescent NSC
99 – populations in the embrvo alter postnatal neurogenesis in both the V-SVZ and the dentate gyrus. the 99 populations in the embryo alter postnatal neurogenesis in both the V-SVZ and the dentate gyrus, the
100 second major neurogenic niche (Berg et al., 2019; Bond et al., 2021; Hu et al., 2017; Kokovay et al., 999 100 second major neurogenic niche (Berg et al., 2019; Bond et al., 2021; Hu et al., 2017; Kokovay et al.,
101 2012; D. Y. Wang et al., 2020). While prior work has established that the regenerative capacity of the 101 2012; D. Y. Wang et al., 2020). While prior work has established that the regenerative capacity of the
102 adult brain is directly related to pre-B1 cells' transient quiescence entry, little is known about the 102 adult brain is directly related to pre-B1 cells' transient quiescence entry, little is known about the 103
103 dynamics and regulation of this essential process. dynamics and regulation of this essential process.

104 The mechanistic/mammalian target of rapamycin (mTOR) kinase is a principal regulator of cell 105 growth (Laplante & Sabatini, 2012; G. Y. Liu & Sabatini, 2020) that has been shown to regulate the
106 quiescence of other stem cell populations, including in postnatal NSCs (Cho & Hwang, 2012; Nieto-106 quiescence of other stem cell populations, including in postnatal NSCs (Cho & Hwang, 2012; Nieto-
107 González et al., 2019; Rodgers et al., 2014; Rossi et al., 2021; Sousa-Nunes et al., 2011). However, this 106 quiescence of other stem cell populations, including in postnatal NSCs (Cho & Hwang, 2012; Nieto-108 potential relationship has not yet been explored in embryonic NSCs. The two primary downstream

Sous and the al., 2014; Sous al., 2011). However, this is also alleged to allege allege allege allege alleges potential relationship has not yet been explored in embryonic NSCs. The two primary downstream
109 effectors of mTOR, phosphorylated ribosomal S6 kinase (p-S6) and phosphorylated 4E-binding proteins
110 (p-4EBP1/2), have n 110 (p-4EBP1/2), have not been systematically characterized together in embryonic NSCs throughout
111 neurogenesis or in the context of quiescence. Canonically, p-S6 regulates cell size and growth through its neurogenesis or in the context of quiescence. Canonically, p-S6 regulates cell size and growth through its 111 neurogenesis or in the context of quiescence. Canonically, p-S6 regulates cell size and growth through its
Canonically, p-S6 regulates cell size and growth through its and growth through its and growth through its and

function in the ribosome, while p-4EBP1/2 is involved in cap-dependent mRNA translation (Gingras et
113 al., 1999; Magnuson et al., 2012; Montagne et al., 1999; Ruvinsky & Meyuhas, 2006). However, in many
114 studies, eith 114 studies, either p-S6 is used as the sole readout of mTOR activity, or the two proteins are used
115 interchangeably. A growing body of evidence suggests that these two proteins have distinct, non-115 interchangeably. A growing body of evidence suggests that these two proteins have distinct, non-
116 compensatory biological functions that are triggered by phosphorylation (Magnuson et al., 2012). 115 interchangeably. A growing body of evidence suggests that these two proteins have distinct, non-117 Specifically, in postnatal NSCs, p-4EBP1/2 has been shown to regulate selective translation and
2012 Specifically, in postnatal NSCs, p-4EBP1/2 has been shown to regulate selective translation and Specifically, in postnatal NSCs, p-4EBP1/2 has been shown to regulate selective translation and
118 regulation of self-renewal (Hartman et al., 2013). In glioblastoma cell lines, only p-4EBP1/2 was shown
119 to correlate w to correlate with a cell's entry to or exit from the cell cycle (Fan et al., 2017, 2018).

120 **Additionally, p-4EBP1/2 and p-S6 can behave independently in response to different ligands** 121 binding the upstream receptors that can activate this signaling pathway, such as insulin, growth factors,
122 or amino acids (Sparta et al., 2021). Establishing the differences in activity and function between p-S6 122 or amino acids (Sparta et al., 2021). Establishing the differences in activity and function between p-S6
123 and p-4EBP1/2 in embryonic NSCs may have direct relevance for patients with disorders of dysregulated 123 and p-4EBP1/2 in embryonic NSCs may have direct relevance for patients with disorders of dysregulated
124 – mTOR signaling for whom mTOR inhibitors are often prescribed (Cavalheiro et al., 2021; Ebrahimi-124 mTOR signaling for whom mTOR inhibitors are often prescribed (Cavalheiro et al., 2021; Ebrahimi-
125 Fakhari et al., 2021; Franz, 2011; Karalis & Bateup, 2021; Overwater et al., 2019). Three generations of 124 ± 124 mTOR inhibitors are often prescribed (Cavalheiro et al., 2021; Ebrahimi- Δ 126 Fakhari et al., 2021; Franz, 2021; Franz, 2021; Three generations of al., 2021; Three generations of al., 20
126 Fakhari Sunday Stars et al., 2019). The generations of all three generations of all three generations of 128 to have a greater effect on p-S6 than p-4EBP1/2 and on mTOR complex 1 (mTORC1) than complex 2 to have a greater effect on p-S6 than p-4EBP1/2 and on mTOR complex 1 (mTORC1) than complex 2
129 (mTORC2) (Choi et al., 1996; Fan et al., 2018). Second generation Tork inhibitors were designed to have
130 improved potency 130 improved potency against mTORC2 (Feldman et al., 2009), while third generation inhibitors, including
131 RapaLink-1, were designed to have improved potency against p-4EBP1/2 (Rodrik-Outmezguine et al., 131 RapaLink-1, were designed to have improved potency against p-4EBP1/2 (Rodrik-Outmezguine et al.,
132 12016). This array of inhibitors offers an opportunity to separate the biological effects of p-S6 and p-132 2016). This array of inhibitors offers an opportunity to separate the biological effects of p-S6 and p-
133 4EBP1/2 in embryonic NSCs, where they have been untested, and their potential contributions to external in the matrix of the state of t

134 quiescence.

135 Here, the signaling patterns of mTOR targets in embryonic NSCs of the V-SVZ and their potential
136 involvement in pre-B1 cell quiescence were investigated by adapting an established *in vitro* model of
137 reversible 137 reversible quiescence to prenatal cells and quantifying functional effects of modulating downstream
138 targets of mTORC1. p-4EBP1/2 and p-S6 were found to be expressed independently, not coordinately, in 137 reversible quiescence to prenatal cells and quantifying functional effects of modulating downstream 139 distinct populations of NSCs in the embryonic brain. The proliferative ability of an embryonic NSC was
140 dependent upon phosphorylation of 4EBP1/2, as decreases in p-4EBP1/2, but not S6, were sufficient to 140 dependent upon phosphorylation of 4EBP1/2, as decreases in p-4EBP1/2, but not S6, were sufficient to
141 induce quiescence. These results suggest mTOR-dependent phosphorylation of 4EBP1/2 is a key 141 induce quiescence. These results suggest mTOR-dependent phosphorylation of 4EBP1/2 is a key
142 regulatory step in quiescence entry of embryonic pre-B1 NSCs and thus establishment of the postnatal 142 regulatory step in quiescence entry of embryonic pre-B1 NSCs and thus establishment of the postnatal
143 stem cell niche. 142 regulatory step in quiescence entry of embryonic pre-B1 NSCs and thus establishment of the postnatal
143 stem cell niche.

144 Materials and Methods
145 Animals: All procedures involving animals were performed in accordance with animal health, safety, and 146 buellness protocols outlined by both institutional (Institutional Animal Care and Use Committee) and
147 butional (National Institute of Health) governing bodies. Wild type C57 Black 6 mice were obtained from 147 mational (National Institute of Health) governing bodies. Wild type C57 Black 6 mice were obtained from
148 Charles River Laboratories. To collect embryos for cells, timed pregnant embryonic day 15.5 (E15.5) 148 Charles River Laboratories. To collect embryos for cells, timed pregnant embryonic day 15.5 (E15.5)
149 dams were euthanized via Avertin overdose and dissected. The embryos were collected for culture 149 dams were euthanized via Avertin overdose and dissected. The embryos were collected for culture
150 generation as described in the following sections. To collect embryos for tissue sections, timed pregnant 150 generation as described in the following sections. To collect embryos for tissue sections, timed pregnant
151 1513.5, E15.5, and E17.5 dams were euthanized via Avertin overdose and transcardially perfused with 151 E13.5, E15.5, and E17.5 dams were euthanized via Avertin overdose and transcardially perfused with
152 0.9% saline followed by 4% paraformaldehyde solution diluted in 0.2M phosphate buffer. Dissected 152 D.9% saline followed by 4% paraformaldehyde solution diluted in 0.2M phosphate buffer. Dissected
153 embryos were processed further for imaging as described in the following sections. embryos were processed further for imaging as described in the following sections.

154 **iDISCO+ Tissue Clearing:** Whole E13.5 embryos were processed, stained, and cleared according to the 155 immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO+) protocol
156 described in Renier et al., 2014. Briefly, following perfusion of the pregnant dam, the embryos were 156 described in Renier et al., 2014. Briefly, following perfusion of the pregnant dam, the embryos were
157 drop fixed in 4% paraformaldehyde overnight at 4°C, and stored in PBS containing 0.1% sodium azide 157 drop fixed in 4% paraformaldehyde overnight at 4°C, and stored in PBS containing 0.1% sodium azide
158 until staining. The embryos were dehydrated in six methanol washes (20%, 40%, 60%, 80%, and 100% at 157 drop fixed in 4% paraformaldehyde overnight at 4°C, and stored in PBS containing 0.1% sodium azide 159 room temperature and a 100% wash chilled to 4°C), stored overnight in 66% dichloromethane in
160 methanol at 4°C, washed twice with methanol (100%) at room temperature, and bleached overnight 160 methanol at 4°C, washed twice with methanol (100%) at room temperature, and bleached overnight
161 vith 5% hydrogen peroxide in methanol at 4°C. The samples were rehydrated through washes in 161 with 5% hydrogen peroxide in methanol at 4°C. The samples were rehydrated through washes in
162 methanol (80%, 60%, 40%, 20%), 1X PBS, and two washes in buffer containing PBS and 0.2% TritonX-100 162 methanol (80%, 60%, 40%, 20%), 1X PBS, and two washes in buffer containing PBS and 0.2% TritonX-100
163 - one at room temperature and one overnight at 4°C. All washes, except those that lasted overnight, 163 – one at room temperature and one overnight at 4°C. All washes, except those that lasted overnight,
164 – were 1 hour. Samples were incubated for 12 hours at 37°C with a permeabilization solution containing 164 – were 1 hour. Samples were incubated for 12 hours at 37°C with a permeabilization solution containing
165 – 10% PBS/0.2% TritonX-100/20% DMSO/2.3% glycine and incubated for 12 hours at 37°C with blocking 165 10% PBS/0.2% TritonX-100/20% DMSO/2.3% glycine and incubated for 12 hours at 37°C with blocking
166 solution containing 10% PBS/0.2% TritonX-100/6% normal donkey serum/10% DMSO. The samples were solution containing 10% PBS/0.2% TritonX-100/6% normal donkey serum/10% DMSO. The samples were 166 solution containing 10% PBS/0.2% TritonX-100/6% normal donkey serum/10% DMSO. The samples were incubated for 24 hours with primary antibodies in buffer containing 10% PBS/0.2% Tween-20/0.1% 10
168 mg/mL Heparin/5% DMSO/3% normal donkey serum at 37°C, washed for 24 hours in buffer containing
169 10% PBS/0.2% Tween-20 169 10% PBS/0.2% Tween-20/0.1% 10 mg/mL Heparin at 37°C, incubated for 24 hours with Alexa Fluor
170 secondary antibodies (Thermo Fisher Scientific) in buffer containing 10% PBS/0.2% Tween-20/0.1% 10 170 secondary antibodies (Thermo Fisher Scientific) in buffer containing 10% PBS/0.2% Tween-20/0.1% 10
171 mg/mL Heparin/3% normal donkey serum at 37°C, and washed for an additional 24 hours in buffer 171 mg/mL Heparin/3% normal donkey serum at 37°C, and washed for an additional 24 hours in buffer
172 containing 10% PBS/0.2% Tween-20/0.1% 10 mg/mL Heparin at 37°C. Antibodies are listed in Table 2. 172 containing 10% PBS/0.2% Tween-20/0.1% 10 mg/mL Heparin at 37°C. Antibodies are listed in Table 2.
173 The samples were dehydrated through six hour-long washes in methanol (20%, 40%, 60%, 80%, 100%, 173 The samples were dehydrated through six hour-long washes in methanol (20%, 40%, 60%, 80%, 100%,
174 100%), incubated for 3 hours in 66% dichloromethane in methanol, washed twice in dichloromethane 174 100%), incubated for 3 hours in 66% dichloromethane in methanol, washed twice in dichloromethane
175 16 15 minutes, and cleared and stored in dibenzyl ether until imaging at 4X and 15X via SmartSPIM 175 100 115 100 15 minutes, and cleared and stored in dibenzyl ether until imaging at 4X and 15X via SmartSPIM
176 1. (LifeCanvas Technologies) light sheet fluorescence microscope. Imaris Software version 9.5.1 was used 176 (LifeCanvas Technologies) light sheet fluorescence microscope. Imaris Software version 9.5.1 was used
177 for image and video reconstruction. 177 for image and video reconstruction.

178 Immunostaining of Mouse Brains: The heads were removed from E13.5, E15.5, and E17.5 embryos, 179 drop fixed in 4% paraformaldehyde 2 hours (for 10 µm thick sections) or overnight (for 50 µm thick
180 sections) at 4°C, and then sunk in 30% sucrose solution. Fixed heads were embedded into optimal 180 sections) at 4°C, and then sunk in 30% sucrose solution. Fixed heads were embedded into optimal
181 cutting temperature compound (OCT) (Tissue-Tek, Sakura, 4583) before cryosectioning and mounting on 181 cutting temperature compound (OCT) (Tissue-Tek, Sakura, 4583) before cryosectioning and mounting on
182 Color Frost Plus microscope slides (Thermo Fisher Scientific, 12-550-16). Slides with OCT-embedded 182 Color Frost Plus microscope slides (Thermo Fisher Scientific, 12-550-16). Slides with OCT-embedded
183 – embryonic brain slices were removed from the freezer and allowed to acclimate to room temperature 183 embryonic brain slices were removed from the freezer and allowed to acclimate to room temperature
184 for 20 minutes in the chemical hood. Slides were washed three times in 1X PBS for 5 minutes, incubated 184 for 20 minutes in the chemical hood. Slides were washed three times in 1X PBS for 5 minutes, incubated
185 in blocking solution containing PBS/1% normal donkey serum/1% BSA/0.1% Triton X-100 for 30 minutes 185 in blocking solution containing PBS/1% normal donkey serum/1% BSA/0.1% Triton X-100 for 30 minutes
186 at room temperature. Primary antibodies and primary-secondary antibody conjugates were applied to 186 at room temperature. Primary antibodies and primary-secondary antibody conjugates were applied to
187 the slides overnight at 4°C. Antibodies are listed in Table 2. Slides were washed again three times in 1X 187 the slides overnight at 4°C. Antibodies are listed in Table 2. Slides were washed again three times in 1X
188 PBS for 5 minutes and Alexa Fluor secondary antibodies (Thermo Fisher Scientific) were applied to the 188 PBS for 5 minutes and Alexa Fluor secondary antibodies (Thermo Fisher Scientific) were applied to the
189 Slides for approximately 1 hour at room temperature. Slides were washed one time with 1X PBS for 5 I89 slides for approximately 1 hour at room temperature. Slides were washed one time with 1X PBS for 5
190 minutes. 42,6-diamidino-2-phenylindole (DAPI) (diluted 1:10,000 in 1X PBS) was applied to the slides for minutes. $4\overline{2}$,6-diamidino-2-phenylindole (DAPI) (diluted 1:10,000 in 1X PBS) was applied to the slides for 190 minutes. 40 , $2-$ phenylindole (DAPI) (diluted 1:10) was applied to the slides for

20 minutes at room temperature. The slides were washed 2 final times in 1X PBS for 5 minutes and
192 insed with ddH20. Mowiol or Fluoromount-G (Electron Microscopy Sciences, 1798425) were used to
193 mount coverslips (Fish mount coverslips (Fisher Scientific, 12-544-18P) and the slides were allowed to dry overnight. Slides
194 were imaged on an LSM 710 Confocal Microscope (Zeiss) at specified magnifications and z-stacks at the
195 Vanderbilt 194 were imaged on an LSM 710 Confocal Microscope (Zeiss) at specified magnifications and z-stacks at the
195 Vanderbilt Cell Imaging Shared Resource and Zen Blue software (Zeiss) was used for image acquisition
196 and rec 195 Vanderbilt Cell Imaging Shared Resource and Zen Blue software (Zeiss) was used for image acquisition
196 and reconstruction.

197 Human Induced Pluripotent Stem Cell (hiPSC) Cell Culture: Two hiPSC lines (1) GM25256s from the 196 and reconstruction. 198 Coriell Institute and (2) 77s from the Sahin lab (Sundberg et al., 2018) were cultured as previously
199 described (Armstrong et al., 2017; Chalkley et al., 2022; Snow et al., 2020). In brief, iPSCs were grown as
200 c 198 colonies on Matrigel (Corning, 354277) coated 6 well plates in mTeSR1 medium (StemCell Tech, 85850)
1981 at 37°C and 5% CO₂. Culture media was replaced daily, and the cells were passaged with ReLSR 199 described (Corning, 354277) coated 6 well plates in mTeSR1 medium (StemCell Tech, 85850)
201 at 37°C and 5% CO₂. Culture media was replaced daily, and the cells were passaged with ReLSR
202 (StemCell Tech. 05872) upo 201 at 37°C and 5% CO₂. Culture media was replaced daily, and the cells were passaged with ReLSR
202 (StemCell Tech, 05872) upon reaching confluency. 201 at 37°C and 5% CO₂. Culture media was replaced daily, and the cells were passaged with ReLSR
202 (StemCell Tech, 05872) upon reaching confluency.
203 **Cortical Neurosphere Culture**: iPSCs at 70% confluence were incub

203 **Cortical Neurosphere Culture:** iPSCs at 70% controlled Technologies, 07920) for 5 minutes at 37°C to dist 203 **Cortical Neurosphere Culture:** iPSCs at 70% confluence were incubated with Accutase (Stem Cell
204 Technologies, 07920) for 5 minutes at 37°C to dissociate cells. Cells were pelleted via centrifugation for
205 5 minu Technologies, 07920) for 5 minutes at 37°C to dissociate cells. Cells were pelleted via centrifugation for
205 5 minutes at room temperature at 300 x g. 3 million cells were added to one well of an Aggrewell™800
206 (StemC 205 5 minutes at room temperature at 300 x g. 3 minion cells were added to one well of an Aggrewell 300
206 (StemCell Tech, 34815) with neural induction media, containing 1:1 mixture DMEM/F12 GlutaMAX
207 (Gibco, 10565-018 207 (Gibco, 10565-018) and Neurobasal (Gibco, 21103049), 0.5X N2 (Gibco, 17502048), 0.5X B27 with
208 vitamin A (17504044), 2.5 µg/mL insulin (Life Technologies, 12585014), 0.75X Glutamax (Gibco,
209 35050061), 50 µM nones 208 vitamin A (17504044), 2.5 μg/mL insulin (Life Technologies, 12585014), 0.75X Glutamax (Gibco,
209 35050061), 50 μM nonessential amino acids (Sigma, M7145), 50 μM 2-mercaptoethanol (Sigma,
210 M6250), 50 μM nonessentia 208 vitamin A (17504044), 2.5 μg/mL insulin (Life Technologies, 12585014), 0.75X Glutamax (Gibco,
209 35050061), 50 μM nonessential amino acids (Sigma, M7145), 50 μM 2-mercaptoethanol (Sigma,
210 M6250), 50 U/mL penicilli 210 M6250), 50 U/mL penicillin-streptomycin (Gibco, 15140122), 10 μ M SB431542 (Cayman Chemical,
211 13031) and 100 nM LDN-193189 (Tocris, 6053). Cells were allowed to aggregate overnight into a sphere
212 while maintai 210 M6250), 50 U/mL penicillin-streptomycin (Gibco, 15140122), 10 μ M SB431542 (Cayman Chemical,
211 13031) and 100 nM LDN-193189 (Tocris, 6053). Cells were allowed to aggregate overnight into a sphere
212 while maintai 212 while maintained at 37°C and 5% CO₂. Neural induction media was replaced daily for 10 days. After 24 212 while maintained at 37°C and 5% CO₂. Neural induction media was replaced daily for 10 days. After 24 version of the state of hours in culture, the neurospheres were transferred into well plates and maintained in suspension on an
214 orbital shaker (95 rpm).
215 Immunostaining of Neurospheres: All neurospheres were fixed by incubation in 4% paraf

215 Immunostaining of Neu 215 Immunostanting of Neurospheres: All neurospheres were fixed by incubation in 4% paraformaldehyde
216 If or 15 minutes at 4°C. Fixed samples were blocked with blocking buffer containing PBS/1% normal
217 Idiuted in blo 216 for 15 minutes at 4°C. Fixed samples were blocked with blocking buffer containing PBS/1% normal
217 donkey serum/1% BSA/0.1% Triton X-100 for 1 hour at room temperature. Primary antibodies were
218 diluted in blocking 217 donkey serum/1% BSA/0.1% Triton X-100 for 1 hour at room temperature. Primary antibodies were
218 diluted in blocking buffer and then incubated overnight at 4° C. Alexa Fluor secondary antibodies
219 (Thermo Fishe 218 diluted in blocking buffer and then incubated overnight at 4°C. Alexa Fluor secondary antibodies
219 (Thermo Fisher Scientific) were diluted in blocking buffer and then incubated 1 hour at room
220 temperature in the 220 temperature in the dark. Antibodies are listed in Table 2. Hoechst (diluted 1:10,000 in 1X PBS) was
221 applied to the slides for 20 minutes at room temperature. Images were acquired using a Prime 95B
222 camera mounte applied to the slides for 20 minutes at room temperature. Images were acquired using a Prime 95B
222 camera mounted on a Nikon spinning disk confocal microscope using a Plan Apo Lambda 20x objective
223 Lens at the Vanderb 222 camera mounted on a Nikon spinning disk confocal microscope using a Plan Apo Lambda 20x objective
223 lens at the Vanderbilt Nikon Center of Excellence. The software used for image acquisition and
224 ceconstruction we 223 lens at the Vanderbilt Nikon Center of Excellence. The software used for image acquisition and
224 reconstruction were NIS-Elements Viewer (Nikon) and ImageJ (FIJI). 224 reconstruction were NIS-Elements Viewer (Nikon) and ImageJ (FIJI).
225 **Cell Pellet Preparation:** Cultured cells were dissociated using Accutase and pelleted via centrifugation

225 **Cell Pellet Preparation:** Cultured cells were dissociated using Accu
226 for 5 minutes at room temperature at 100 x g prior to fixation **225 Cell Pellet Preparation:** Cultured cells were dissociated using Accutase and pelleted via centrifugation
226 for 5 minutes at room temperature at 100 x *g* prior to fixation with 1.6% paraformaldehyde for 15
227 minut 226 for 5 minutes at room temperature at 100 x g prior to fixation with 1.6% paraformaldehyde for 15
227 minutes, washed with 1x PBS, and re-pelleted via centrifugation for 5 minutes at room temperature at
228 100 x g. Th 228 at 100 x g. The supernatant was removed and replaced with 70% ethanol. The pellet was then paraffin
229 embedded and prepared as 5-7 µm sections. 229 100 embedded and prepared as 5-7 µm sections.
230 10 Quantification of *ex vivo Mouse Imaging Data: A custom Stardist 3D nuclear segmentation model was*

220 **Quantification of ex vivo Mouse Imaging Dand Control Cont** 230 Quantification of ex vivo Mouse Imaging Data: A custom Stardist 3D nuclear segmentation model was
231 trained using 16 expert annotated cropped regions of interest from the dataset using the protocol
232 described at h described at https://github.com/stardist/stardist. Nuclear segmentation model was applied to each
233 image in dataset followed by 3D pixel expansion to segment probable cell bodies. Marker mean intensity
234 was measured 233 image in dataset followed by 3D pixel expansion to segment probable cell bodies. Marker mean intensity
234 was measured and recorded for each nucleus, cytoplasm, and cell body (nucleus + cytoplasm). For each 233 image in dataset followed by 3D pixel expansion to segment probable cell bodies. Marker mean intensity
234 vas measured and recorded for each nucleus, cytoplasm, and cell body (nucleus + cytoplasm). For each 234 was measured and recorded for each nucleus, cytoplasm, and cell body (nucleus + cytoplasm). For each 236 confirmed per staining batch by two independent viewers (LCG and AAB).
237 **Quantification of** *in vitro* **Human Imaging Data:** A custom Stardist 2D nuclear segmentation model was

238 commined per staming batch by two independent viewers (LCG and AAB).
237 **Quantification of** *in vitro* **Human Imaging Data:** A custom Stardist 2D numeration at the state of the state of the trained using 18 expert annot 237 Quantification of *in vitro* Human Imaging Data: A custom Stardist 2D nuclear segmentation model was
238 trained using 18 expert annotated cropped regions of interest from the dataset using the protocol
239 described a described at https://github.com/stardist/stardist. The model was applied to each image in the dataset
240 tollowed by 2D pixel expansion to segment probable cell bodies. Marker mean intensity was measured
241 the protocolo 240 followed by 2D pixel expansion to segment probable cell bodies. Marker mean intensity was measured
241 and recorded for each nucleus, cytoplasm, and cell body (nucleus + cytoplasm). For each measured
242 marker, cells 241 and recorded for each nucleus, cytoplasm, and cell body (nucleus + cytoplasm). For each measured
242 marker, cells were scored as positive or negative by thresholding. Thresholds were set and confirmed
243 per staining and recorded for each nucleus, cytoplasm, and cell body (nucleus + cytoplasm). For each measured
242 marker, cells were scored as positive or negative by thresholding. Thresholds were set and confirmed
243 per staining bat 243 per staining batch by two independent viewers (LCG and AAB).
244 **Primary Cell Cultures:** Mouse embryos were collected at E15.5 from timed pregnant dams. To obtain

243 per staming batch by two independent viewers (LCG and AAB).
244 **Primary Cell Cultures:** Mouse embryos were collected at E15
245 stem cell cultures from the developing V-SVZ, a "top-down" dis 244 Primary Cen Cultures: Mouse embryos were collected at E15.5 from timed pregnant dams. To obtain
245 stem cell cultures from the developing V-SVZ, a "top-down" dissection approach was employed. Briefly,
246 the heads we 246 the heads were separated from the body, the two brain hemispheres were pulled back to either side to
247 steveal the developing cortex which was collected as dorsal NSCs. The developing ganglionic eminences
248 were co 247 the developing cortex which was collected as dorsal NSCs. The developing ganglionic eminences
248 were collected as ventral NSCs. The collected tissue was minced and incubated at 37°C, 5% CO2 with
249 0.25% trypsin-EDT were collected as ventral NSCs. The collected tissue was minced and incubated at 37°C, 5% CO2 with
249 0.25% trypsin-EDTA solution for 20 minutes while rocking. The tissue was then gently dissociated via
250 trituration wi were collected as ventral NSCs. The collected tissue was minced and incubated at 37°C, 5% CO2 with
249 0.25% trypsin-EDTA solution for 20 minutes while rocking. The tissue was then gently dissociated via
250 trituration wi 251 neural stem cells as described in Moghadam et al., 2018: Neurobasal media (ThermoFisher, 21103049);
252 1X B27 supplement without vitamin A (ThermoFisher, 12587010); 20 ng/mL mouse epidermal growth 1251 neural stem cells as described in Moghadam et al., 2018: Neurobasal media (ThermoFisher, 21103049);
252 1X B27 supplement without vitamin A (ThermoFisher, 12587010); 20 ng/mL mouse epidermal growth
253 1 factor (Therm 252 1X B27 supplement without vitamin A (ThermoFisher, 12587010); 20 ng/mL mouse epidermal growth
253 factor (ThermoFisher, 53003018); 10 ng/mL mouse basic fibroblast growth factor (ThermoFisher,
254 PMG0035): 1 U/mL hepar factor (ThermoFisher, 53003018); 10 ng/mL mouse basic fibroblast growth factor (ThermoFisher,
254 PMG0035); 1 U/mL heparin (Sigma, 9041-08-01); 1X GlutaMax (ThermoFisher, 35050061); 1X modified
255 Eagle's medium non-esse 254 PMG0035); 1 U/mL heparin (Sigma, 9041-08-01); 1X GlutaMax (ThermoFisher, 35050061); 1X modified
255 Eagle's medium non-essential amino acids (11140050); 0.1 mM β-mercaptoethanol; 10 μg/mL
256 gentamicin. Cells were fe Eagle's medium non-essential amino acids (11140050); 0.1 mM β -mercaptoethanol; 10 μ g/mL 256 gentamicin. Cells were fed every 2-3 days and passaged upon reaching confluence.
256 gentamicin. Cells were fed every 2-3 days and passaged upon reaching confluence. 256 gentamicin. Cells were fed every 2-3 days and passaged upon reaching confluence.

- 257 Quiescence Induction: To induce quiescence, embryonic NSC media was prepared as above without
258 epidermal growth factor and with the addition of 50 ng/mL mouse bone morphogenetic protein 4 (R&D
259 Systems, 5020-BP-0 259 Systems, 5020-BP-010, stock dissolved in 4 mM hydrochloric acid with 0.1% BSA). Cells were fed every 2-
260 3 days. Systems, 5020-BP-010, stock dissolved in 4 mM hydrochloric acid with 0.1% BSA). Cells were fed every 2-
260 3 days.
Pharmacological modulators: mTOR inhibitors were added to culture media at concentrations listed in 261 Pharma
262 text and
-
- 262 text and figure legends. Initial dissolutions of inhibitors were in DMSO, as specified by manufacturers'
263 instructions, and subsequent dilutions of concentrated stocks were in PBS.
264 Table 1
- 263 instructions, and subsequent dilutions of concentrated stocks were in PBS.
264 Table 1
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276 with PBS and spun at 800 x g for 5 minutes at room temperature. Cells were stained with a cocktail of antibodies against intracellular antigens diluted in PBS/1% BSA for 30-60 minutes at room temperature.
278 Sample 278 Samples were washed with PBS and spun at 800 x g for 5 minutes at room temperature. Samples were
279 analyzed on either a Fortessa 4-laser or 5-laser instrument. Beads (Invitrogen, A10513) stained with a
280 single flu 278 Samples were washed with FBS and spun at 800 x g for 5 minutes at room temperature. Samples were
280 analyzed on either a Fortessa 4-laser or 5-laser instrument. Beads (Invitrogen, A10513) stained with a
280 single flu 280 single fluorophore and unstained cells from the same cell line and treatment conditions were used for
281 compensation and sizing controls. Gating was performed to isolate live, intact, single cells. Signaling
282 valu compensation and sizing controls. Gating was performed to isolate live, intact, single cells. Signaling
282 values for each antigen were determined as both (1) the arcsinh transformed and (2) fold change of
283 each sample 282 values for each antigen were determined as both (1) the arcsinh transformed and (2) fold change of
283 each sample compared to the column minimum for each antigen. The arcsinh scale used has been
284 previously describ each sample compared to the column minimum for each antigen. The arcsinh scale used has been
284 values for each the alle previously described (Irish et al., 2010; Rushing et al., 2019). On the arcsinh scale, a difference corresponds to a nearly 2-fold difference in total protein. All analyses were performed in Cytobank. 285 corresponds to a nearly 2-fold difference in total protein. All analyses were performed in Cytobank.
286 **Cell cycle analysis:** Cultured cells were dissociated for 7-10 minutes using Accutase at 37°C, pelleted by

286 **Cell cycle analysis:** Cultured cells were dissociated for 7-10 minutes using Accutase at 37°C, pellete
287 centrifuging for 5 minutes at room temperature at 100 x g, resuspended in the original spent media 286 Cell cycle analysis: Cultured cells were dissociated for 7-10 minutes using Accutase at 37°C, pelleted by
287 centrifuging for 5 minutes at room temperature at 100 x g , resuspended in the original spent media in 5
2 289 intact cells. Cells were fixed with 1.6% paraformaldehyde, permeabilized with 70% ice cold ethanol, and
290 stored at -20°C until staining. On the day of staining, samples were washed with PBS and spun at 800 x q 291 for 5 minutes at room temperature twice. Cell pellets were resuspended in 1.5 µM Hoechst 33342 (Cell 1990 intact at -20°C until staining. On the day of staining, samples were washed with PBS and spun at 800 x *g*
1991 for 5 minutes at room temperature twice. Cell pellets were resuspended in 1.5 μM Hoechst 33342 (Cell
199 290 stored at -20°C until staming. On the day of staming, samples were washed with PBS and spun at 800 x g
291 for 5 minutes at room temperature twice. Cell pellets were resuspended in 1.5 µM Hoechst 33342 (Cell
292 Signal 292 Signaling Technology, 4082) and incubated for 1 hour at 37°C and vortexed and every 15 minutes during
293 incubation. Samples were analyzed on a BD LSRII 5-laser instrument. Cells in each phase of the cell cycle
294 we 293 incubation. Samples were analyzed on a BD LSRII 5-laser instrument. Cells in each phase of the cell cycle
294 were determined via gating of live, intact, single cells. All analyses were performed in Cytobank. 294 were determined via gating of live, intact, single cells. All analyses were performed in Cytobank.
295 **Quantification and statistical analysis**: The quantification methods used and statistical tests performed

295 **Quantification and statistical analysis:** The quantification methods used and statistical tests per
296 are detailed in each figure and figure legend. GraphPad Prism 9 was used to perform all analyses. 295 Quantification and statistical analysis: The quantification methods used and statistical tests performed
296 are detailed in each figure and figure legend. GraphPad Prism 9 was used to perform all analyses.
297 **Data A**

297 **Data Availability Statement:** All flow cytometry data will be made publicly available on FlowRep upon publication (Spidlen et al., 2012). 297 Data Availability Statement: All flow cytometry data will be made publicly available on FlowRepository
298 upon publication (Spidlen et al., 2012). 298 upon publication (Spidlen et al., 2012).

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311 Experimental Design
312 Mouse Experiments:
313 and female embryos **Mouse Experiments:** The control groups for each experiment are listed in each figure legend. Both male

and female embryos were used for immunohistochemistry experiments. Each embryo counted as a

unique biological replic 314 unique biological replicate (N=1). Embryos used in experiments presented here span at least two litters
315 from each timepoint. Cultures were generated by pooling tissue collected from the indicated region
316 from al 315 from each timepoint. Cultures were generated by pooling tissue collected from the indicated region
316 from all the embryos (both male and female) from 1 or 2 timed pregnant dams, with an average litter
317 size of 7. 316 from all the embryos (both male and female) from 1 or 2 timed pregnant dams, with an average litter
317 size of 7. Cultures were grown up for one passage before cryopreservation. Each thawed cryovial
318 counted as a u size of 7. Cultures were grown up for one passage before cryopreservation. Each thawed cryovial
318 counted as a unique biological replicate (N=1). Cultures used in experiments presented here represent
319 three independen 318 counted as a unique biological replicate (N=1). Cultures used in experiments presented here represent
319 three independent pregnant dam dissections and culture generations. Cultures treated with BMP4 were
320 compared 319 three independent pregnant dam dissections and culture generations. Cultures treated with BMP4 were
320 compared to matched cultures from the same cryovial that were not exposed to BMP4. Cultures treated
321 with a pha 319 three independent pregnant dam dissections and culture generations. Cultures treated with BMP4 were
320 compared to matched cultures from the same cryovial that were not exposed to BMP4. Cultures treated
321 with a pha with a pharmacological inhibitor were compared to matched cultures from the same cryovial treated
322 vith vehicle (1X PBS).
323 **Human Cells:** Neurospheres were differentiated from two unique wild type induced pluripotent

323 **Human Cells:** Neurospheres were differentiated from two unique wild type induced pluripotent stem
324 cell lines. Each differentiation counted as a unique biological replicate (N=1). Individual neurospheres 23 Human Cells: Neuros
324 cell lines. Each differe cell lines. Each differentiation counted as a unique biological replicate (N=1). Individual neurospheres
325 from each biological replicate were considered technical replicates.
326 **Statistical Analysis:** Unless otherwise 325 from each biological replicate were considered technical replicates.
326 **Statistical Analysis:** Unless otherwise indicated, all experiments had an N of at least 3. Exact Ns

326 **Statistical Analysis:** Unless otherwise indicated, all experiments
327 (separate biological replicates) are listed in the figure legends fo (separate biological replicates) are listed in the figure legends for each experiment. D'Agostino and
328 Pearson K-squared tests were performed on each dataset to determine departure from normality. If a
329 dataset faile 329 dataset failed to reach normality (p value < 0.05) or did not have enough replicates to perform the 330 D'Agostino test (N < 8), then a nonparametric Mann-Whitney U test was performed. If the dataset 329 dataset failed to reach normality (p value < 0.05) or did not have enough replicates to perform the
330 D'Agostino test (N < 8), then a nonparametric Mann-Whitney U test was performed. If the dataset
331 D'Agostino te $D'Agostino test (N < 8)$, then a nonparametric Mann-Whitney U test was performed. If the dataset reached normality (D'Agostino test p value > 0.05), a parametric student's t-test was performed.
332 Whether the test was paired or un 331 reached normality (D'Agostino test p value > 0.05), a parametric student's t-test was performed.
332 Whether the test was paired or unpaired is indicated in the figure legends for each comparison. P values 331 reached normality (D'Agostino test p value > 0.05), a parametric student's t-test was performed.
332 Whether the test was paired or unpaired is indicated in the figure legends for each comparison. P values 332 Whether the test was paired or unpaired is indicated in the figure legends for each comparison. P values

334 for each comparison are listed in the figure legends. Graph pad Prism 9 was used to perform all statistical
334 analyses and generate plots. 334 analyses and generate plots.

kesuits
336 High lev
337
²²⁸ Ambrua High levels of p-4EBP1/2, but not p-S6, are present in embryonic NSCs at the ventricle.

To visualize levels of mTORC1 signaling prior to the genesis of pre-B1 cells, whole brains from

embryonic day 13.5 mice were process 338 embryonic day 13.5 mice were processed, cleared, and stained for p-S6 S240/244 and p-4EBP1/2
339 T37/46. p-S6 S240/244 was distributed in multiple cell layers of both the developing cortex and
340 ganglionic eminences 339 T37/46. p-S6 S240/244 was distributed in multiple cell layers of both the developing cortex and
340 ganglionic eminences (Fig 1A). In contrast, p-4EBP1/2 T37/46 was primarily present in cell bodies lining
341 the apica ganglionic eminences (Fig 1A). In contrast, p-4EBP1/2 T37/46 was primarily present in cell bodies lining
341 the apical ventricular surface of developing cortex and was not abundant in cells that were more distant
342 from 341 the apical ventricular surface of developing cortex and was not abundant in cells that were more distant
342 from the ventricle. In the lateral and medial ganglionic eminences, p-4EBP1/2 was similarly enriched at
343 t 341 the apical ventricular surface of developing cortex and was not abundant in cells that were more distant
342 from the ventricle. In the lateral and medial ganglionic eminences, p-4EBP1/2 was similarly enriched at
343 t 343 the ventricular surface and was also present in some cells deeper within the tissue (Fig 1B; full brain
344 videos for p-S6 and p-4EBP1/2 in Extended Fig 1-1). To further investigate these patterns throughout
345 neura 344 videos for p-S6 and p-4EBP1/2 in Extended Fig 1-1). To further investigate these patterns throughout
345 neural development and the genesis of pre-B1 cells, coronal sections of embryonic day 13.5, 15.5, and
346 17.5 mo 345 neural development and the genesis of pre-B1 cells, coronal sections of embryonic day 13.5, 15.5, and
346 17.5 mouse brain were co-stained for both readouts of mTORC1 activity (Fig 1C). At all developmental
347 ages me 346 17.5 mouse brain were co-stained for both readouts of mTORC1 activity (Fig 1C). At all developmental
347 ages measured, the two phosphorylated proteins were largely found in distinct cells. p-4EBP1/2 labeling
348 was l ages measured, the two phosphorylated proteins were largely found in distinct cells. p-4EBP1/2 labeling
348 was limited to cells lining the ventricular surface, many of which appeared to be actively undergoing
349 mitosis was limited to cells lining the ventricular surface, many of which appeared to be actively undergoing
349 mitosis (yellow arrows in Fig 1C). At E13.5 and 15.5, p-S6 was not found in cells lining the ventricular
350 surface 350 surface but was abundant in the subventricular zone and intermediate zones. At E17.5, p-S6 was
351 observed in cells contacting the ventricular surface. The two phosphorylated proteins were rarely found 350 surface but was abundant in the subventricular zone and intermediate zones. At E17.5, p-S6 was
351 observed in cells contacting the ventricular surface. The two phosphorylated proteins were rarely found
352 in the same 351 observed in cells contacting the ventricular surface. The two phosphorylated proteins were rarely found
352 in the same cell (E13: average 2.7% of counted cells [N=3], E15: average 0.52% of counted cells [N=4],
353 E17 352 in the same cell (E13: average 2.7% of counted cells [N=3], E15: average 0.52% of counted cells [N=4],
353 E17: average 0.1% of counted cells [N=3]). The frequency of p-4EBP1/2-positive cells decreased with
354 gestat 353 E17: average 0.1% of counted cells [N=3]). The frequency of p-4EBP1/2-positive cells decreased with
354 gestational age, from an average of 4.10% of cells per field at E13.5 to 0.17% at E17.5, while levels of p-
355 S6 E17: average 0.1% of counted cells [N=3]). The frequency of p-4EBP1/2-positive cells decreased with
354 gestational age, from an average of 4.10% of cells per field at E13.5 to 0.17% at E17.5, while levels of p-
355 S6 exp

356 Co-staining with Ki67, a marker of cycling cells, revealed the
357 dividing: 80.97% of cells positive for p-4EBP1/2 at E13.5 were dou 357 dividing: 80.97% of cells positive for p-4EBP1/2 at E13.5 were doubly positive for Ki67, 68.44% at E15.5,
358 and 33.33% at E17.5; though Ki67 expression also decreased with age (27.02% of total cells at E13.5, 357 dividing: 80.97% of cells positive for p-4EBP1/2 at E13.5 were doubly positive for Ki67, 68.44% at E15.5,
358 and 33.33% at E17.5; though Ki67 expression also decreased with age (27.02% of total cells at E13.5, 358% and 33.333% at E17.5; though Ki67 expression also decreased with age (27.02% of total cells at E13.5, 18.01% at E15.5, 2.54% at E17.5), consistent with prior reports (Hu et al., 2017) and the general
decrease in neurogenesis at this stage (Fuentealba et al., 2015; Furutachi et al., 2015). p-S6 positive cells
variably expre 361 variably expressed Ki67 (31.26% of cells positive for p-S6 at E13.5, 27.93% at E15.5, 3.29% at E17.5).
362 Additional Ki67 staining in the developing V-SVZ at E13.5 from cleared brain is shown in Extended Fig 1-
363 1. 362 Additional Ki67 staining in the developing V-SVZ at E13.5 from cleared brain is shown in Extended Fig 1-
363 1. To assign mTOR activity more precisely to radial glia and transit amplifying cells, tissues were co-
364 s 1. To assign mTOR activity more precisely to radial glia and transit amplifying cells, tissues were co-
364 stained for p-S6 S240/244, p-4EBP1/2 T37/46, and the transcription factor t-box brain protein 2 (Tbr2),
365 which 366 Cells expressing Tbr2 were more likely to express p-S6 than p-4EBP1/2 at both E13.5 (12.99% Tbr2/p-S6 365 which distinguishes the transit-amplifying progeny of cortical radial glia (Englund et al., 2005) (Fig 1E).
366 Cells expressing Tbr2 were more likely to express p-S6 than p-4EBP1/2 at both E13.5 (12.99% Tbr2/p-S6
367 366 Cells expressing Tbr2 were more likely to express p-S6 than p-4EBP1/2 at both E13.5 (12.99% Tbr2/p-S6
367 co-positive cells versus 0.65% Tbr2/p-4EBP1/2 co-positive cells) and E15.5 (34.72% Tbr2/p-S6 co-positive
368 cel Cells expressing Tbr2 were more likely to express p-S6 than p-4EBP1/2 at both E13.5 (12.99% Tbr2/p-S6

co-positive cells versus 0.65% Tbr2/p-4EBP1/2 co-positive cells) and E15.5 (34.72% Tbr2/p-S6 co-positive

cells versus

369 6.65 cells positive for p-4EBP1/2 were often co-positive for Ki67 and appeared to be actively
370 dividing, this suggested that cells with low expression of p-4EBP1/2 could be pre-B1 cells, which have 369 As cells positive for p-4EBP1/2 were often
370 dividing, this suggested that cells with low expression 370 dividing, this suggested that cells with low expression of p-4EBP1/2 could be pre-B1 cells, which have
371 been reported to enter quiescence during prenatal development. Lineage tracing studies have indicated
372 that 371 been reported to enter quiescence during prenatal development. Lineage tracing studies have indicated
372 that pre-B1 cells begin entering quiescence as early as E13.5, with most completing quiescence entry by
373 E15. 373 E15.5 (Fuentealba et al., 2015; Furutachi et al., 2015). E15.5 brains were co-stained with p-S6 S240/244,
374 D-4EBP1/2 T37/46, and vascular cell adhesion molecule 1 (VCAM1), a protein required for the E15.5 (Fuentealba et al., 2015; Furutachi et al., 2015). E15.5 brains were co-stained with p-S6 S240/244,
374 p-4EBP1/2 T37/46, and vascular cell adhesion molecule 1 (VCAM1), a protein required for the
375 maintenance of t p-4EBP1/2 T37/46, and vascular cell adhesion molecule 1 (VCAM1), a protein required for the
375 maintenance of the radial glia stem cell population and entry into quiescence (Hu et al., 2017; Kokovay
376 et al., 2012: D. Y 375 maintenance of the radial glia stem cell population and entry into quiescence (Hu et al., 2017; Kokovay
376 et al., 2012; D. Y. Wang et al., 2020) (Fig 1F). VCAM1 was found infrequently in the developing cortex,
377 bu 376 et al., 2012; D. Y. Wang et al., 2020) (Fig 1F). VCAM1 was found infrequently in the developing cortex,
377 but was more abundant in the ganglionic eminences, consistent with previously published findings (Hu
378 et al 378 et al., 2017) (Fig 1D, 20x). At E15.5, VCAM1 was expressed exclusively by cells at the ventricular surface
379 and did not overlap with p-S6. Line scan analysis along the apical ventricular surface showed that cells 378 et al., 2017) (Fig 1D, 20x). At E15.5, VCAM1 was expressed exclusively by cells at the ventricular surface
379 and did not overlap with p-S6. Line scan analysis along the apical ventricular surface showed that cells
38 379 and did not overlap with p-S6. Line scan analysis along the apical ventricular surface showed that cells
380 positive for p-4EBP1/2 and VCAM1 were largely exclusive of each other (Figure 1D). In pixels negative for
381 380 positive for p-4EBP1/2 and VCAM1 were largely exclusive of each other (Figure 1D). In pixels negative for
381 p-4EBP1/2, the average VCAM1 intensity was double that in pixels positive for p-4EBP1/2 (4.22 versus
382 2.0 p-4EBP1/2, the average VCAM1 intensity was double that in pixels positive for p-4EBP1/2 (4.22 versus 382 p-4EBP1, the average VCAM1 intensity was double that in pixels positive for p-4EBP1 (4.22 versus 2.04, N = 3).

382 2.04, N = 3).

384 neural progenitors, neurospheres were differentiated from two separate wild type human induced
385 pluripotent stem cell lines (Sundberg et al., 2018). After 10 days in culture, neurospheres were collected
386 and were pluripotent stem cell lines (Sundberg et al., 2018). After 10 days in culture, neurospheres were collected
386 and were co-stained with p-S6 S240/244, p-4EBP1/2 T37/46, and Ki67 (Fig 1G). As was observed in the
387 mouse. 385 pluripotent stem cell lines (Sundberg et al., 2018). After 10 days in culture, neurospheres were collected
386 and were co-stained with p-S6 S240/244, p-4EBP1/2 T37/46, and Ki67 (Fig 1G). As was observed in the
387 mou 387 mouse, p-S6 was more abundantly expressed than p-4EBP1/2 (21.78% of total cells positive for p-S6
388 versus 4.55% positive for p-4EBP1/2, N = 5 neurospheres) and cells positive for p-4EBP1/2 were often
389 actively un versus 4.55% positive for p-4EBP1/2, N = 5 neurospheres) and cells positive for p-4EBP1/2 were often
389 actively undergoing division and doubly positive for Ki67 (61.72% of p-4EBP1/2 positive cells). Similar to
390 the pa 389 actively undergoing division and doubly positive for Ki67 (61.72% of p-4EBP1/2 positive cells). Similar to
390 the pattern observed in the mouse, only a small percentage of cells (4.00% of total cells) were positive
39 390 the pattern observed in the mouse, only a small percentage of cells (4.00% of total cells) were positive for both p-S6 and p-4EBP1/2. 391 for both p-S6 and p-4EBP1/2.
392 **Exposure to BMP4 induces quiescence in embryonic NSCs** *in vitro***.**
392 **Exposure to BMP4 induces quiescence in embryonic NSCs** *in vitro***.**

Exposure to BMP4 induces quality and provide the BMP4 induces quality of the process of Exposure to BMP4 induces quiescence in embryonic NSCs *in Vitro*.
393 To determine how mTOR activity responds to changes
394 whether cells entering quiescence decrease levels of p-4EBP1/2, a
395 developed and validated. Th 394 whether cells entering quiescence decrease levels of p-4EBP1/2, an *in vitro* model of quiescence was
395 developed and validated. The role of bone morphogenetic protein 4 (BMP4) in directing differentiation
396 and ma 394 whether cells entering quiescence decrease levels of p-4EBP1/2, an *in vitro* model of quiescence was
395 developed and validated. The role of bone morphogenetic protein 4 (BMP4) in directing differentiation
396 and ma 396 and maintenance of stem cells in the V-SVZ and dentate gyrus has been extensively described (Li et al.,
397 1998; Mira et al., 2010). BMP4 has been reported to induce a reversible quiescence *in vitro* in various
398 s 397 1998; Mira et al., 2010). BMP4 has been reported to induce a reversible quiescence *in vitro* in various
398 stem cell populations within 72 hours, including postnatal neural stem cells (Knobloch et al., 2017; Mira
399 398 stem cell populations within 72 hours, including postnatal neural stem cells (Knobloch et al., 2017; Mira
399 et al., 2010; Rossi et al., 2021), but has not been tested in prenatal NSCs.
300 To test its use in prenatal

398 100 578 To test its use in prenatal NSC populations, neural stem cells dissected from the developing
391 dorsal V-SVZ at embryonic day 15.5 were exposed to BMP4 with simultaneous withdrawal of EGF but 399 400 To test its use in prenatal NSC populations, neural stem cells
399 dorsal V-SVZ at embryonic day 15.5 were exposed to BMP4 with simulary 401 dorsal V-SVZ at embryonic day 15.5 were exposed to BMP4 with simultaneous withdrawal of EGF but
402 maintenance of basic fibroblast growth factor (bFGF-2), and markers of cell proliferation and quiescence
403 were quan maintenance of basic fibroblast growth factor (bFGF-2), and markers of cell proliferation and quiescence
403 were quantified using fluorescence microscopy and flow cytometry. After 24 hours in media containing
404 BMP4 and were quantified using fluorescence microscopy and flow cytometry. After 24 hours in media containing
404 BMP4 and bFGF-2, levels of the proliferation marker Ki67 had significantly decreased compared to cells
405 grown in c BMP4 and bFGF-2, levels of the proliferation marker Ki67 had significantly decreased compared to cells
405 grown in control media containing bFGF-2 and epidermal growth factor (EGF) (Fig 2A), with a further
406 decrease se 405 grown in control media containing bFGF-2 and epidermal growth factor (EGF) (Fig 2A), with a further
406 decrease seen by 72 hours and persisting with additional time in BMP4 culture media (time course of 406 decrease seen by 72 hours and persisting with additional time in BMP4 culture media (time course of decrease seen by 72 hours and persisting with additional time in BMP4 culture media (time course of 406 decrease seen by 72 hours and persisting with additional time in BMP4 culture media (time course of α

quiescence entry shown in Extended Fig 2-1). Following extended incubation with BMP4/FGF-2 media
408 for 6 days, Ki67 and p-vimentin levels both remained decreased compared to control (Fig 2B). BMP4-
409 mediated decreases mediated decreases in Ki67 were reversible, as re-exposure to media containing EGF and lacking BMP4
410 for escued levels of Ki67 (Extended Fig 2-2). Within 24 hours of exposure to BMP4/FGF-2 media, levels of
411 VCAM1 als For the measure and actual mediated mediated of the suppose in Measure remember of measures in Kissang Contain
410 mescued levels of Ki67 (Extended Fig 2-2). Within 24 hours of exposure to BMP4/FGF-2 media, levels of
412 m 411 VCAM1 also began to rise compared to control cells and remained high at 72 hours (Fig 2C). To further
412 verify that BMP4 was having the desired effect, the percentage of cells in each stage of the cell cycle was
413 112 verify that BMP4 was having the desired effect, the percentage of cells in each stage of the cell cycle was
113 determined following 24 hours of exposure to BMP4. Cells grown in media containing BMP4/FGF-2 had
114 an i determined following 24 hours of exposure to BMP4. Cells grown in media containing BMP4/FGF-2 had
414 an increased percentage of cells in the G0/G1 phase of the cell cycle compared to cells grown in media
415 containing EG an increased percentage of cells in the G0/G1 phase of the cell cycle compared to cells grown in media
415 containing EGF/FGF-2 (averages of 80.83% vs 58.08%, N=9), and had a decreased percentage of cells in
416 both the S 415 containing EGF/FGF-2 (averages of 80.83% vs 58.08%, N=9), and had a decreased percentage of cells in
416 both the S (7.03% vs. 21.70%) and G2/M (8.98% vs 17.31%) phases of the cycle compared to control cells
417 (Fig 2 both the S (7.03% vs. 21.70%) and G2/M (8.98% vs 17.31%) phases of the cycle compared to control cells
417 (Fig 2D; gates used to determine percent of cells in each phase of the cell cycle shown in Extended Fig 2-
418 3). both the S (7.03% vs. 21.70%) and G2/M (8.98% vs 17.31%) phases of the cycle compared to control cells
417 (Fig 2D; gates used to determine percent of cells in each phase of the cell cycle shown in Extended Fig 2-
418 3).

419 BMP4 has also been shown to direct NSCs into neuronal or astrocytic lineages depending on the
420 time of expression (Katada et al., 2021); these effects that are directly antagonized and suppressed by 419
 420 tim 421 EGF and FGF-2 to maintain stemness and self-renewal capacities (Lillien & Raphael, 2000; Sun et al.,
422 2011). To ensure that the removal of EGF and exposure to BMP4 did not differentiation as reported for 421 EGF and FGF-2 to maintain stemness and self-renewal capacities (Lillien & Raphael, 2000; Sun et al.,
422 2011). To ensure that the removal of EGF and exposure to BMP4 did not differentiation as reported for
423 media c 422 2011). To ensure that the removal of EGF and exposure to BMP4 did not differentiation as reported for
423 media containing only BMP4 without FGF-2, levels of Sox2, a transcription factor expressed by neural
424 stem an media containing only BMP4 without FGF-2, levels of Sox2, a transcription factor expressed by neural
424 stem and progenitor cells, but not transit amplifying cells, were measured following acute (72 hours, Fig
425 2E) and 424 stem and progenitor cells, but not transit amplifying cells, were measured following acute (72 hours, Fig
425 2E) and prolonged (6 days, Fig 2F) exposure to BMP4. At 72 hours of BMP4 exposure, levels of Sox2
426 remain 225 2E) and prolonged (6 days, Fig 2F) exposure to BMP4. At 72 hours of BMP4 exposure, levels of Sox2
426 remained unchanged compared to cells grown in EGF/FGF-2 media. At 6 days in culture with BMP4, per-
427 cell Sox2 ex 427 cell Sox2 expression remained high and comparable to cells grown in EGF/FGF-2 media. Further, cells
428 cultured with BMP4 did not begin to express markers of differentiation, including TUJ1 or GFAP 429 Featended Fig 2-3). Taken together, these data indicate that consistent with published models. BMP4 in 428 cultured with BMP4 did not begin to express markers of differentiation, including TUJ1 or GFAP
429 (Extended Fig 2-3). Taken together, these data indicate that consistent with published models, BMP4 in 429 (Extended Fig 2-3). Taken together, these data indicate that consistent with published models, BMP4 in $\frac{1}{2}$ 9 – 3). Taken together, these data indicates that consistent with published models, BMP4 indicates in

430 combination with FGF-2 efficiently maddes reversible quiescence in embryonic NSCs *in vitro* within 24
431 hours without inducing differentiation.
432 p-4EBP1/2 signaling decreases in embryonic NSCs following quiesc

hours without inducing differentiation.
 **1432 p-4EBP1/2 signaling decreases in embryonic NSCs following quiescence induction.

1433 To investigate whether low levels of p-4EBP1/2 were directly correlated with quiescence** 434 stem cells dissected from the developing cortex at embryonic day 15.5 were cultured in media
435 containing BMP4 and FGF-2 for acute (24 hours) or extended (72 hours) time periods and readouts of 435 containing BMP4 and FGF-2 for acute (24 hours) or extended (72 hours) time periods and readouts of
436 the mTOR pathway were measured. Two different phosphorylated sites on S6 were measured: 436 the mTOR pathway were measured. Two different phosphorylated sites on S6 were measured:
437 S235/236, a residue phosphorylated by both the mTOR and MAPK/ERK pathways, and S240/244, a 436 the mTOR pathway were measured. Two different phosphorylated sites on S6 were measured:
437 S235/236, a residue phosphorylated by both the mTOR and MAPK/ERK pathways, and S240/244, a
438 residue exclusively phosphoryla 5235/236, a residue phosphorylated by both the mTOR and MAPK/ERK pathways, and S240/244, a
438 the mathway (Magnuson et al., 2012; Roux et al., 2007). At
439 both 24 and 72 hours in BMP4/FGF-2 media. neither of these reado residue exclusively phosphorylated by the mTOR pathway (Magnuson et al., 2012; Roux et al., 2007). At
439 both 24 and 72 hours in BMP4/FGF-2 media, neither of these readouts showed significant change
440 relative to EGF/FG both 24 and 72 hours in BMP4/FGF-2 media, neither of these readouts showed significant change
440 relative to EGF/FGF-2 media (Figs 3B and Fig 3C), a pattern that was persisted after further prolonged
441 exposure to BMP4/ both 24 and 72 hours in BMP4/FGF-2 media, neither of these readouts showed significant change

440 relative to EGF/FGF-2 media (Figs 3B and Fig 3C), a pattern that was persisted after further prolonged

441 exposure to BMP exposure to BMP4/FGF-2 media (6 days, Fig 3D). In contrast, after 24 hours in media containing BMP4,
levels of p-4EBP1/2 decreased compared to controls (Fig 3E), with a further decrease seen after 72
hours. To determine wh expose of the BMP4. The SMP of the BMP4 exposure to BMP4
442 exposure to BMP4 hours. To determine whether the decrease in p-4EBP1/2 remained after prolonged exposure to BMP4
444 and corresponded to a decrease in proliferat hours. To determine whether the decrease in p-4EBP1/2 remained after prolonged exposure to BMP4
444 and corresponded to a decrease in proliferation, cells were cultured for 6 days and co-stained for KI67
445 and p-4EBP1/2 and corresponded to a decrease in proliferation, cells were cultured for 6 days and co-stained for KI67
and p-4EBP1/2 (Fig 3F). Levels of both Ki67 and p-4EBP1/2 decreased upon exposure to BMP4,
446 compared to cells grown and p-4EBP1/2 (Fig 3F). Levels of both Ki67 and p-4EBP1/2 decreased upon exposure to BMP4,
446 compared to cells grown in EGF/FGF-2 media. At both 24 and 72 hours of BMP4 exposure, levels of an
447 additional mTOR target. 2446 compared to cells grown in EGF/FGF-2 media. At both 24 and 72 hours of BMP4 exposure, levels of an
additional mTOR target, p-STAT3 S727 (Dodd et al., 2015; Yokogami et al., 2000), also decreased
448 compared to the EG additional mTOR target, p-STAT3 S727 (Dodd et al., 2015; Yokogami et al., 2000), also decreased
448 compared to the EGF/FGF-2 media condition (Fig 3A). 2009 additional manufactory of the EGF/FGF-2 media condition (Fig 3A).
2013 - Additional manufactures of al., 2015; Youtogami et al., 2015; Youtogami et al., 2014
2000 - Decreases in mTOR-dependent phosphorylation of S6 ar

EGF/FGF-2 media computer (Fig 3A).
449 **Decreases in mTOR-dependent phosphorylation of S6**
450 **Next, inhibition of these downstream compor** Becreases in mitch dependent phosphorylation of 35 are not summer to induce quiescence.

A50 Next, inhibition of these downstream components of mTOR signaling was tested for summit in inducing quiescence. Rapamycin, a firs 451 in inducing quiescence. Rapamycin, a first generation mTOR inhibitor, has frequently been reported to
452 inhibit the phosphorylation of S6 more effectively than that of 4EBP1/2 due to its specific binding 451 in inducing quiescence. Rapamycin, a first generation mTOR inhibitor, has frequently been reported to
452 inhibit the phosphorylation of S6 more effectively than that of 4EBP1/2 due to its specific binding $\frac{1}{2}$ in the phosphorylation of S6 more effectively than that of 4EBP1/2 due to its specific binding bind

location on mTOR (Choi et al., 1996; Fan et al., 2018). In cultures of embryonic day 15.5 NSCs, 30 nM of

454 Rapamycin decreased levels of p-S6, but did not affect levels of p-4EBP1/2 after 4 hours (Fig 4A). After

455 24 455 24 hours in culture, p-S6 levels recovered and were not significantly different from vehicle treated levels
456 (Extended Fig 4-1). Torkinib (PP242) is a second generation, dual mTORC1 and mTORC2 inhibitor that
457 has 456 (Extended Fig 4-1). Torkinib (PP242) is a second generation, dual mTORC1 and mTORC2 inhibitor that
457 has been reported to inhibit phosphorylation of both S6 and 4EBP1/2 (Feldman et al., 2009), and was
458 tested here has been reported to inhibit phosphorylation of both S6 and 4EBP1/2 (Feldman et al., 2009), and was
458 tested here in a dose response series ranging from 12.5 nM to 400 nM of Torkinib (Fig 4A). At all
459 concentrations t tested here in a dose response series ranging from 12.5 nM to 400 nM of Torkinib (Fig 4A). At all
459 concentrations tested, 4-hour treatment with Torkinib decreased phosphorylation of S6 at both the
460 mTOR-dependent S24 1459 concentrations tested, 4-hour treatment with Torkinib decreased phosphorylation of S6 at both the
1460 mTOR-dependent S240/244 and alternate S235/236 residues. However, phosphorylation of 4EBP1/2
1461 remained unaffec 460 mTOR-dependent S240/244 and alternate S235/236 residues. However, phosphorylation of 4EBP1/2
461 concentrations tested. Following acute (4 hour) and extended (24 hour)
462 treatment with 400 nM Torkinib. levels of p-ST 461 remained unaffected at all concentrations tested. Following acute (4 hour) and extended (24 hour)
462 treatment with 400 nM Torkinib, levels of p-STAT3 S727 similarly did not decrease (Fig 4B). Additional
463 second ge 463 second generation mTOR inhibitors and inhibitors of specific cap-dependent translation components
464 were tested for their ability to decrease levels of p-4EBP1/2 in cultures; however, none had this ability second generation mTOR inhibitors and inhibitors of specific cap-dependent translation components
464 were tested for their ability to decrease levels of p-4EBP1/2 in cultures; however, none had this ability
465 (Extended were tested for their ability to decrease levels of p-4EBP1/2 in cultures; however, none had this ability
465 (Extended Fig 4-1). Torkinib was therefore used as an S6-selective inhibitor to determine whether
466 inhibition (Extended Fig 4-1). Torkinib was therefore used as an S6-selective inhibitor to determine whether
466 inhibition of phosphorylation of S6, but not 4EBP1/2, would be sufficient to induce quiescence. While
467 levels of p-S6 inhibition of phosphorylation of S6, but not 4EBP1/2, would be sufficient to induce quiescence. While
467 levels of p-S6 S235/236 (Fig 4C) and p-S6 S240/244 (Fig 4D) initially decreased following 4 hour
468 treatment with 167 levels of p-S6 S235/236 (Fig 4C) and p-S6 S240/244 (Fig 4D) initially decreased following 4 hour
168 treatment with Torkinib, by 24 hours, both proteins had overcome the inhibition and were not different
169 compared t traction process (Fig 12) and process (Fig 12) initially decreased following the traction
468 treatment with Torkinib, by 24 hours, both proteins had overcome the inhibition and were not different
469 compared to vehicle t treatment with Torkinib, by 24 hours, both proteins had overcome the inhibition and were not different

compared to vehicle treated cells. Phosphorylation of 4EBP1/2 was unaffected by treatment with

470 Torkinib at both 4 471 While there was a small increase in the percentage of cells in G0/G1 with Torkinib treatment compared
472 to vehicle after 24 hours of treatment (averages of 75.03% Torkinib vs 72.67% vehicle, N=9), there was 472 to vehicle after 24 hours of treatment (averages of 75.03% Torkinib vs 72.67% vehicle, N=9), there was
473 not a significant decrease in the percent of cells in the S (7.73% vs 7.90%) or G2/M phases (16.01% vs. 473 not a significant decrease in the percent of cells in the S (7.73% vs 7.90%) or G2/M phases (16.01% vs.
474 15.06%) of the cycle with treatment (Fig 4G). Taken together, these data indicated inhibition of S6 1423 to vehicle after 24 hours of the percent of cells in the S (7.73% vs 7.90%) or G2/M phases (16.01% vs.
15.06%) of the cycle with treatment (Fig 4G). Taken together, these data indicated inhibition of S6
1475 bhosphorv 474 15.06%) of the cycle with treatment (Fig 4G). Taken together, these data indicated inhibition of S6
475 phosphorylation alone was not sufficient to induce quiescence. Given that levels of p-4EBP1/2 475 phosphorylation alone was not sufficient to induce quiescence. Given that levels of p-4EBP1/2 475 phosphorylation alone was not sufficient to induce $\mathcal{A}^{\mathcal{A}}$

decreased upon entry into quiescence, inhibition of p-4EBP1/2 was next tested for sufficiency to induce

quiescence.
 478 Decreases in mTOR-dependent phosphorylation of 4EBP1/2 are sufficient to induce quiescence.

478 **Decreases ir**
478 **Decreases ir**
479 Rapa Rapalink-1, a third generation mTOR inhibitor synthesized from Rapamycin and an ATP-
480 competitive inhibitor of mTOR, MLN0128 (Rodrik-Outmezguine et al., 2016), has previously been shown
481 to inhibit phosphorylation of 482 hours of treatment with 10 nM RapaLink, E15.5 NSCs were analyzed for readouts of the mTOR pathway. to inhibit phosphorylation of 4EBP1/2 in mouse brain tissue (Zhang et al., 2022). Following 24 and 72
182 bours of treatment with 10 nM RapaLink, E15.5 NSCs were analyzed for readouts of the mTOR pathway.
183 Levels of p-S 483 Levels of p-STAT3 S727 were unaffected compared to vehicle at 24 hours, but after 72 hours of
484 treatment with RapaLink, the values were slightly decreased compared to vehicle (Fig 5A). Levels of both 483 Levels of p-STAT3 S727 were unaffected compared to vehicle at 24 hours, but after 72 hours of
484 treatment with RapaLink, the values were slightly decreased compared to vehicle (Fig 5A). Levels of both
485 p-S6 S235/2 1932 Levels of permission and the sumplement of the terms of a 24 hours, and at 24 hours of both
1843 treatment with RapaLink, the values were slightly decreased compared to vehicle (Fig 5A). Levels of both
185 p-S6 S235/2 p-S6 S235/236 (Fig 5B) and p-S6 S240/244 (Fig 5C) decreased following 24 hours of treatment with
486 RapaLink and stayed low compared to vehicle after 72 hours of treatment, contrasting with the transient
487 effects of To 486 RapaLink and stayed low compared to vehicle after 72 hours of treatment, contrasting with the transient
487 effects of Torkinib. Levels of p-4EBP1/2 decreased compared to vehicle following 24 hours of treatment
488 wit 487 effects of Torkinib. Levels of p-4EBP1/2 decreased compared to vehicle following 24 hours of treatment
488 with RapaLink and remained decreased after 72 hours (Fig 5D). After 24 hours of treatment with
489 RapaLink. le with RapaLink and remained decreased after 72 hours (Fig 5D). After 24 hours of treatment with

489 RapaLink, levels of Ki67 were significantly decreased compared to vehicle and remained so at 72 hours

490 (Fig 5E). After RapaLink, levels of Ki67 were significantly decreased compared to vehicle and remained so at 72 hours
489 KapaLink, levels of Ki67 were significantly decreased compared to vehicle and remained so at 72 hours
491 GO/G1 phas 490 (Fig 5E). After 24 hours of treatment, RapaLink treated cells had an increased percentage of cells in the
491 G0/G1 phases of the cell cycle (averages of 78.63% with RapaLink vs. 67.42% with vehicle, N=7) and
492 decre 491 G0/G1 phases of the cell cycle (averages of 78.63% with RapaLink vs. 67.42% with vehicle, N=7) and
492 decreased percentages of cells in the S (12.27% vs. 18.71%) and G2/M phases (5.98% vs. 11.50%) of the
493 cell cycl decreased percentages of cells in the S (12.27% vs. 18.71%) and G2/M phases (5.98% vs. 11.50%) of the
cell cycle compared to vehicle treated cells. Taken together, these data indicate that inhibition of
494 4EBP1/2 is suff cell cycle compared to vehicle treated cells. Taken together, these data indicate that inhibition of
494 4EBP1/2 is sufficient to induce quiescence in cultured embryonic NSCs.
Quiescence entry and mTOR response do not dif 494 4EBP1/2 is sufficient to induce quiescence in cultured embryonic NSCs.
495 Quiescence entry and mTOR response do not differ by dorsoventral position.

494 4EBP1/2 is sufficient to induce quiescence in cultured embryonic NSCs. 497 phosphorylation of mTORC1 targets than their dorsal counterparts (Rushing et al., 2019). To determine
498 vhether embryonic cells also had differential mTOR signaling based on cellular positioning. NSCs from 497 phosphorylation of mTORC1 targets than their dorsal counterparts (Rushing et al., 2019). To determine
498 whether embryonic cells also had differential mTOR signaling based on cellular positioning, NSCs from whether embryonic cells also had differential mTOR signaling based on cellular positioning, NSCs from
498 whether embryonic cells also had differential mTOR signaling based on cellular positioning, NSCs from \sim 98 whether embryonic cells also had differential model on cellular positioning, NSCs from cellular positioning, \sim

499 the developing cortex (dorsal NSCs) and cells from the ganglionic eminences (ventral NSCs) and
1990 to cultured separately and the various mTOR readouts were assessed before and after quiescence
1991 induction. In cult 501 induction. In cultures maintained in media containing EGF/FGF-2, no uniform differences between
502 dorsal and ventral NSCs in any of the readouts were observed across multiple studies. Following 72
503 hours of treatm induction. In cultures maintained in media containing EGF/FGF-2, no uniform differences between
502 dorsal and ventral NSCs in any of the readouts were observed across multiple studies. Following 72
503 hours of treatment 503 hours of treatment with BMP4, Ki67 was decreased, and VCAM1 increased, in ventral NSCs grown in
504 media containing BMP4/FGF-2 compared to controls (Fig 6A and 6B). These data suggest that exposure
505 to BMP4 had a c 504 media containing BMP4/FGF-2 compared to controls (Fig 6A and 6B). These data suggest that exposure
505 to BMP4 had a comparable effect inducing quiescence in both ventral NSCs and dorsal NSCs. Following
506 72 hours of 505 to BMP4 had a comparable effect inducing quiescence in both ventral NSCs and dorsal NSCs. Following
506 72 hours of BMP4 exposure, median levels of p-STAT3 S727 (Fig 6C) and p-4EBP1/2 T37/46 (Fig 6F) both
507 decreased 506 72 hours of BMP4 exposure, median levels of p-STAT3 S727 (Fig 6C) and p-4EBP1/2 T37/46 (Fig 6F) both
507 decreased compared to ventral cells grown in EGF/FGF-2 media as they did in dorsal cells exposed to
508 BMP4. How 507 decreased compared to ventral cells grown in EGF/FGF-2 media as they did in dorsal cells exposed to
508 BMP4. However, as in dorsal NSCs, in ventral NSCs, levels of p-S6 S235/236 (Fig 6D) and p-S6 S240/244
509 (Fig 6E) 508 BMP4. However, as in dorsal NSCs, in ventral NSCs, levels of p-S6 S235/236 (Fig 6D) and p-S6 S240/244
509 (Fig 6E) did not change following 72 hours in culture with BMP4. 509 (Fig 6E) did not change following 72 hours in culture with BMP4.
510 510
 511

510
511

512 Discussion
513 In
514 temporary In both the ventricular-subventricular zone and the dentate gyrus of the hippocampus,
514 temporary quiescence of a subset of embryonic neural stem cells determines the capacity of the adult
515 neurogenic niche through pr 515 neurogenic niche through preservation of self-renewing stem cells that later activate in the adult.
516 Lineage tracing work in rodents has shown extensive consequences of altering prenatal quiescence
517 entry in both 516 Lineage tracing work in rodents has shown extensive consequences of altering prenatal quiescence
517 entry in both stem cell niches, including premature depletion of the postnatal stem cell population and
518 developme 517 entry in both stem cell niches, including premature depletion of the postnatal stem cell population and
518 developmental and learning defects (D. Y. Wang et al., 2020; Hu et al., 2017; Kokovay et al., 2012). This
519 518 developmental and learning defects (D. Y. Wang et al., 2020; Hu et al., 2017; Kokovay et al., 2012). This
519 quiescence entry is thus essential to produce adult neural stem cells and support adult neurogenesis in
520 518 developmental and learning defects (D. Y. Wang et al., 2020; Hu et al., 2017; Kokovay et al., 2012). This
519 quiescence entry is thus essential to produce adult neural stem cells and support adult neurogenesis in
520 520 both the V-SVZ and dentate gyrus. However, the mechanisms regulating the initiation of this essential
521 process remain poorly understood (Urbán, 2022; Urbán et al., 2019).
522 mTOR has been shown to regulate the bala

522 both TOR has been shown to regulate the balance of activation and quiescence in other stem cell
523 bopulations (Cho & Hwang, 2012; Nieto-González et al., 2019; Rodgers et al., 2014). In postnatal NSCs, 522 mTOR has been shown to regulate the balance of activation
523 populations (Cho & Hwang, 2012; Nieto-González et al., 2019; Rodg 523 populations (Cho & Hwang, 2012; Nieto-González et al., 2019; Rodgers et al., 2014). In postnatal NSCs,
524 mTOR also regulates preferential translation of specific mRNA transcripts as stem cells are activated to
525 di 524 mTOR also regulates preferential translation of specific mRNA transcripts as stem cells are activated to
525 divide – that is, upon exit from quiescence (Baser et al., 2019; Rossi et al., 2021). The data shown here
526 525 divide – that is, upon exit from quiescence (Baser et al., 2019; Rossi et al., 2021). The data shown here
526 illuminate a role for this kinase in embryonic NSC quiescence. While the sufficiency of changes in p-
527 4E 526 illuminate a role for this kinase in embryonic NSC quiescence. While the sufficiency of changes in p-
527 4EBP1/2 for initiation of quiescence entry is demonstrated here, the necessity and sufficiency of
528 increased 529 PI3K/Akt signaling upstream of mTOR have been proposed as a regulator of NSC quiescence exit (Chell & 4EBP1/2 for initiation of quiescence entry is demonstrated here, the necessity and sufficiency of
increased p-4EBP1/2 for quiescence exit in these cells remain to be explored. Nutrient sensing and
PI3K/Akt signaling upstre 530 Brand, 2010). Whether mTOR-mediated phosphorylation of 4EBP1/2 is a regulator of both quiescence
531 entry and exit or how it may work coordinately with other signaling pathways well known to be involved Brand, 2010). Whether mTOR-mediated phosphorylation of 4EBP1/2 is a regulator of both quiescence

entry and exit or how it may work coordinately with other signaling pathways well known to be involved

in quiescence, such 531 entry and exit or how it may work coordinately with other signaling pathways well known to be involved
532 in quiescence, such as Notch signaling, is an area of ongoing study.

in quiescence, such as Notch signaling, is an area of ongoing study.
533 These data demonstrate that two primary downstream effectors of mTORC1, p-S6 S240/244 and
534 p-4EBP1/2 T37/46, have distinct patterns of expression 533 These data demonstrate that two primary downstream effectively.
534 p-4EBP1/2 T37/46, have distinct patterns of expression throughout 533 These data demonstrate that two primary downstream effectors of mTORC1, p-S6 S240/244 and $\frac{1}{2}$ p-4EBP1/2 T37, have distinct patterns of expression throughout neurogenesis and rarely appear in $\frac{1}{2}$

the same cell (fewer than 1% of cells at E15 and E17). Cells positive for p-4EBP1/2 line the most apical
portion of the developing telencephalon, the ventricular zone, and their abundance decreases with age,
while cells po 537 while cells positive for p-S6 predominate in the subventricular zone, intermediate zone, and cortical
538 plate and their abundance increases with age. The finding that p-4EBP1/2 and p-S6 were so rarely
539 expressed i plate and their abundance increases with age. The finding that p-4EBP1/2 and p-S6 were so rarely
539 expressed in the same cell indicates that though the mTOR pathway is active in both cells, through a yet
540 unidentified expressed in the same cell indicates that though the mTOR pathway is active in both cells, through a yet
540 unidentified regulatory mechanism, only one signaling effector is being phosphorylated. While it has
541 been rep 540 unidentified regulatory mechanism, only one signaling effector is being phosphorylated. While it has
541 been reported that different ligands (such as amino acids, insulin, and growth factors) activating
542 different 542 different upstream receptors (including the epidermal growth factor receptor, the fibroblast growth
543 factor receptor, and the insulin receptor) can result in various downstream signaling responses (Sparta 542 different upstream receptors (including the epidermal growth factor receptor, the fibroblast growth
543 factor receptor, and the insulin receptor) can result in various downstream signaling responses (Sparta
544 et al. 543 factor receptor, and the insulin receptor) can result in various downstream signaling responses (Sparta et al., 2021), it remains to be explored whether this type of mechanism is responsible for the differing patterns 544 et al., 2021), it remains to be explored whether this type of mechanism is responsible for the differing
545 patterns of phosphorylation of these two key mTORC1 effectors in NSCs. et al., 2021), it remains to be explored whether this type of mechanism is responsible for the differing

patterns of phosphorylation of these two key mTORC1 effectors in NSCs.

The use of BMP4 in embryonic NSC cultures re

546 The use of BMP4 in embryonic NSC cultures resulted in decrease
547 expression of VCAM1, an increased percentage of cells in the G0/G expression of VCAM1, an increased percentage of cells in the G0/G1 phase of the cell cycle and
548 decreased percentages of cells in the S and G2/M phases of the cell cycle. Cells exposed to BMP4 did not
549 enter senescen Expression of VCAME₂, an increased percentage of VCAM phases of the cell cycle. Cells exposed to BMP4 did not
549 enter senescence (data not shown). Upon quiescence entry, levels of p-4EBP1/2, but not p-S6,
550 decreased 549 enter senescence (data not shown). Upon quiescence entry, levels of p-4EBP1/2, but not p-S6,
550 decreased in cultures derived from the developing dorsal region. The wider variance seen in S6 signaling
551 may reflect enter senescence (data not shown). Upon quiescence entry, levels of p-4EBP1/2, but not p-S6,
550 decreased in cultures derived from the developing dorsal region. The wider variance seen in S6 signaling
551 may reflect its 551 may reflect its role in additional biological processes, such as regulating cell size (Hartman et al., 2013;
552 Magnuson et al., 2012; Montagne et al., 1999; Ruvinsky & Meyuhas, 2006). In concordance with the
553 effe Magnuson et al., 2012; Montagne et al., 1999; Ruvinsky & Meyuhas, 2006). In concordance with the
553 effects of BMP4, inhibition of p-4EBP1/2, but not p-S6, was sufficient to induce quiescence entry.
554 Importantly, while effects of BMP4, inhibition of p-4EBP1/2, but not p-S6, was sufficient to induce quiescence entry.
554 Importantly, while levels of another mTOR downstream effector, p-STAT3, decreased in both dorsal and
555 ventral NSCs e 1993 554 Importantly, while levels of another mTOR downstream effector, p-STAT3, decreased in both dorsal and
1955 ventral NSCs exposed to BMP4, it did not decrease upon entry into quiescence after 24 hours with
1956 RapaL For the personal MSCs exposed to BMP4, it did not decrease upon entry into quiescence after 24 hours with
556 RapaLink treatment while cells had already begun to exit the cell cycle and quiesce. The opposing
557 relationsh 556 RapaLink treatment while cells had already begun to exit the cell cycle and quiesce. The opposing
557 relationships of BMP and STAT3 signaling through mTOR to influence stem cell fate have been previously
558 described relationships of BMP and STAT3 signaling through mTOR to influence stem cell fate have been previously described (Rajan et al., 2003). The data here indicate the inhibition of phosphorylation of STAT3 is not 558 described (Rajan et al., 2003). The data here indicate the inhibition of phosphorylation of STAT3 is not

necessary for quiescence entry and may be a secondary consequence, whereas mTOR-dependent

560 phosphorylation of 4EBP1/2 is a regulator of embryonic NSC quiescence entry.

561 While p-4EBP1/2 positive cells were nearly al 561 box 1980 box 1980 phosphoryland compared to 4EBP1/2 positive cells were nearly always dividing and co-exp
562 cells co-expressed Ki67 only 30% of the time, on average. This pattern m 562 cells co-expressed Ki67 only 30% of the time, on average. This pattern may offer insight into the
563 independent biological functions that activation of each signaling protein triggers and the consequences
564 on stem independent biological functions that activation of each signaling protein triggers and the consequences
564 on stem cell proliferation, self-renewal, and differentiation those functions have. It has been widely
565 report 565 ime reported that as stem cells differentiate and migrate tangentially away from the ventricular surface,
1966 itranslation is suppressed. This suppression of translation, and regulation of the process by mTOR, has 565 reported that as stem cells differentiate and migrate tangentially away from the ventricular surface,
566 translation is suppressed. This suppression of translation, and regulation of the process by mTOR, has
567 been 566 translation is suppressed. This suppression of translation, and regulation of the process by mTOR, has
567 been hypothesized to be a mechanism of regulating stem cell fate (reviewed in R. Wang & Amoyel,
568 2022). mTOR 567 been hypothesized to be a mechanism of regulating stem cell fate (reviewed in R. Wang & Amoyel,
568 2022). mTOR-mediated translation of specific transcripts – or lack thereof – during key periods of
569 neurogenesis ha 568 2022). mTOR-mediated translation of specific transcripts – or lack thereof – during key periods of
569 neurogenesis has been shown to regulate cellular differentiation and neuronal subtype specification
570 (Harnett et 568 2022). mTOR-mediated translation of specific transcripts – or lack thereof – during key periods of 570 (Harnett et al., 2022; reviewed in Statoulla et al., 2021). The data presented here support this
571 hypothesis, as p-4EBP1/2, a key regulator of translation, decreases with increasing distance from the
572 ventricular (Harnett et al., 2022; reviewed in Statoulla et al., 2021). The data presented here support this
571 hypothesis, as p-4EBP1/2, a key regulator of translation, decreases with increasing distance from the
572 ventricular sur 573 the role of p-S6 in stem cell differentiation to be explored in future studies, as p-S6 is markedly absent at
574 the ventricular surface but its abundance increases with increasing distance from the ventricular 573 the role of p-S6 in stem cell differentiation to be explored in future studies, as p-S6 is markedly absent at
574 the ventricular surface but its abundance increases with increasing distance from the ventricular
575 su 574 the ventricular surface but its abundance increases with increasing distance from the ventricular surface.

575 surface.
576 An important implication of these data is that each downstream effector of mTOR should be
577 investigated independent of the other signaling molecules in each cell type. mTOR signaling has been 576
577 investiga In important improvement in the state is that each downstream effect of milion increasing
577 Investigated independent of the other signaling molecules in each cell type. mTOR signaling has been
578 Implicated as a propose 578 implicated as a proposed regulatory mechanism in multiple aspects of neural development and a variety
579 of diseases of the nervous system (Andrews et al., 2020; Avet-Rochex et al., 2014; Costa-Mattioli &
580 Monteggi 579 of diseases of the nervous system (Andrews et al., 2020; Avet-Rochex et al., 2014; Costa-Mattioli &
580 Monteggia, 2013; D'Gama et al., 2017; Hartman et al., 2013; Ka et al., 2014; Lee, 2015; Licausi &
581 Hartman. 201 580 Monteggia, 2013; D'Gama et al., 2017; Hartman et al., 2013; Ka et al., 2014; Lee, 2015; Licausi & Hartman, 2018; D. Liu et al., 2018; Mahoney et al., 2016; Maierbrugger et al., 2020; Musah et al., 2020; Paliouras et al 580 Monteggia, 2013; D'Gama et al., 2017; Hartman et al., 2013; Ka et al., 2014; Lee, 2015; Licausi &
581 Hartman, 2018; D. Liu et al., 2018; Mahoney et al., 2016; Maierbrugger et al., 2020; Musah et al., 2020;
582 Paliour Paliouras et al., 2012; Rushing et al., 2019; Tee et al., 2016; Tyler et al., 2009; Wahl et al., 2014; Zeng et 582 Paliouras et al., 2012; Rushing et al., 2019; Tee et al., 2016; Tyler et al., 2009; Wahl et al., 2014; Zeng et al., 2009). Often, however, only a single residue on p-S6 – either S235/236 or S240/244 – is reported as

a representative readout of total mTOR kinase activity. The data here demonstrate that the multiple

signaling effec 585 signaling effectors of mTORC1 behave independently in tissue, *in vitro*, and in response to different
586 pharmacological modulators. More broadly, as multiple generations of mTOR inhibitors enter clinical
587 trials. 585 signaling effectors of inforced behave independently in tissue, in viro, and in response to different
586 pharmacological modulators. More broadly, as multiple generations of mTOR inhibitors enter clinical
587 trials, 587 trials, the use of agents that more effectively inhibit phosphorylation of both S6 and 4EBP1/2 are likely
588 to have broader effects on normal neural development, and cortical hyperplasias, than their
589 predecessors 587 trials, the use of agents that more effectively inhibit phosphorylation of both S6 and 4EBP1/2 are likely

590 Three different generations of mTOR inhibitors were tested here for their ability to inhibit
591 phosphorylation of 4EBP1/2. While the inhibitors tested have all been reported to decrease p-4EBP1/2 -
590 Three
591 phosphorylatic 591 phosphorylation of 4EBP1/2. While the inhibitors tested have all been reported to decrease p-4EBP1/2
592 levels in cell lines and *in vitro* assays, only the third-generation bivalent inhibitor, RapaLink-1, was able
59 1292 levels in cell lines and *in vitro* assays, only the third-generation bivalent inhibitor, RapaLink-1, was able
593 to decrease levels of p-4EBP1/2 in embryonic neural stem cell cultures. Rapamycin, a first-generation
 to decrease levels of p-4EBP1/2 in embryonic neural stem cell cultures. Rapamycin, a first-generation
594 inhibitor, multiple second-generation "Tork" inhibitors, and a eukaryotic initiation factor inhibitor all
595 failed 594 inhibitor, multiple second-generation "Tork" inhibitors, and a eukaryotic initiation factor inhibitor all
595 failed to decrease levels of p-4EBP1/2. The data presented here may indicate cell type-specific
596 mechanis 595 failed to decrease levels of p-4EBP1/2. The data presented here may indicate cell type-specific
596 mechanisms regulating mTOR signaling and susceptibility to inhibition. This finding has potential
597 implications for mechanisms regulating mTOR signaling and susceptibility to inhibition. This finding has potential
597 implications for clinical use, where mTOR inhibitors are often prescribed for a variety of diseases. First
598 generatio 597 implications for clinical use, where mTOR inhibitors are often prescribed for a variety of diseases. First
598 generation mTOR inhibitors (rapalogs) are often prescribed for pediatric patients with "mTORopathies,"
599 597 implications for clinical use, where mTOR inhibitors are often prescribed for a variety of diseases. First
598 generation mTOR inhibitors (rapalogs) are often prescribed for pediatric patients with "mTORopathies,"
599 599 a debilitating class of neurodevelopmental disorders. Patients with tuberous sclerosis complex, one such
500 mTORopathy wherein patients have tumors throughout the entire body, are regularly prescribed the
501 apalog e 599 a debilitation of the entire body, are regularly prescribed the
591 a depending class of neurodes of neurons of the entire body, are regularly prescribed the
502 a 2020: Franz. 2011: Karalis & Bateup. 2021: Overwater e 601 rapalog everolimus to control seizures and limit brain tumor growth (Cavalheiro et al., 2021; Feliciano,
602 2020; Franz, 2011; Karalis & Bateup, 2021; Overwater et al., 2019). This work may indicate that only the
603 602 2020; Franz, 2011; Karalis & Bateup, 2021; Overwater et al., 2019). This work may indicate that only the
603 56 "arm" of the mTOR pathway is inhibited by rapalog treatment and may offer insight as to why such
604 treat 603 56 "arm" of the mTOR pathway is inhibited by rapalog treatment and may offer insight as to why such
604 treatments are not cytotoxic, but merely cytostatic. These data support the testing of improved mTOR
605 inhibitor treatments are not cytotoxic, but merely cytostatic. These data support the testing of improved mTOR
605 inhibitors that more effectively inhibit phosphorylation of 4EBP1/2 in neural stem cells, but also raise
606 concern inhibitors that more effectively inhibit phosphorylation of 4EBP1/2 in neural stem cells, but also raise for the more effectively inhibitors that more effectively in neural stem cells, but also related that the concerning that the concerning may incur additional side effects. 606 concern that this targeting may incur additional side effects.

For a meurospheres derived from human iPSCs presented here suggest the independent phosphorylations of
609 S6 and 4EBP1/2 also occur in human cells. Outer radial glial cells, a cell type unique to the human brain
610 hypot 56 and 4EBP1/2 also occur in human cells. Outer radial glial cells, a cell type unique to the human brain
610 hypothesized to be a cell of origin in disease, have been reported to have increased mTOR activity, as
611 measu 610 by hypothesized to be a cell of origin in disease, have been reported to have increased mTOR activity, as
611 measured by p-S6, compared to other types of cells (Andrews et al., 2020; Nowakowski et al., 2017).
612 Embr measured by p-S6, compared to other types of cells (Andrews et al., 2020; Nowakowski et al., 2017).
612 Embryonic neural stem cells have been hypothesized to be the cell of origin for some of the brain tumor
613 types foun Embryonic neural stem cells have been hypothesized to be the cell of origin for some of the brain tumor
613 types found in tuberous sclerosis complex (Blair et al., 2018; Eichmüller et al., 2022; Hang et al., 2017;
614 Hew types found in tuberous sclerosis complex (Blair et al., 2018; Eichmüller et al., 2022; Hang et al., 2017;
614 Hewer & Vajtai, 2015; Rushing et al., 2019). However, more work is needed to determine whether there
615 are ch types found in tuberous sclerosis complex (Blair et al., 2018; Eichmüller et al., 2022; Hang et al., 2017;
614 Hewer & Vajtai, 2015; Rushing et al., 2019). However, more work is needed to determine whether there
615 are ch Fig. 1914 Here is vajus, 2019, Harming et al., 2019, Horsett, Micro With the Herena is accumum more.

Fig. 3016 Future studies may investigate how consequences of altered quiescence entry, particularly in the

fig. 2017 Co Figures in the changes in most dependent phosphorylation of 4EBP12 upon quiescence entry, manufactured in the
615 Future studies may investigate how consequences of altered quiescence entry, particularly in the
617 context 617 context of disease, may affect a stem cell's lineage and fate. 617 context of disease, may affect a stem cell's lineage and fate.

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2 **Figure 1: High levels of p-4EBP1/2, but not p-S6, are present in embryonic NSCs at the ventricle.** (A)

3 iDISCO+-tissue cleared whole brain from an E13.5 mouse embryo (15X) showing phosphorylation of S6 at

4 the serine 240/244 residues diffuse throughout the ventricular-subventricular zone tissue in both the 5 developing cortex (inset, top) and lateral ganglionic eminence (inset, bottom). Scale bar information is 6 listed on each figure. (B) iDISCO+-tissue cleared whole brain from an E13.5 mouse embryo (15X) showing 7 phosphorylation of 4EBP1 at the threonine 37/46 residues limited to the cells immediately lining the 8 ventricular surface and not present deeper into the subventricular zone in both the developing cortex 9 (inset, top) and lateral ganglionic eminence (inset, bottom). Scale bar information is listed on each figure. (C) Staining of embryonic mouse brain for the downstream effectors of mTOR at E13.5, E15.5, and E17.5. 20X (top) representative images of the developing ventricular-subventricular zone with single slice of 63X z-stack (bottom) of the inset region showing p-S6 S240/244 (green), p-4EBP1 T37/46 (white), Ki67 (red), and DAPI (blue). Yellow arrows in the 63X representative images mark dividing cells with phosphorylated 4EBP1. Scale bars for all 20X images = 50 µm. Scale bars for all 63X images = 10 µm. (N for E13.5 = 3, N for E15.5 = 4, N for E17.5 = 3) (D) Quantification of the percent of shown in (C) across developmental time points. The percent of all cells positive for p-S6 S240/244 (green) and cells co-positive for p-S6 and Ki67 (red) (top). (2 way ANOVA with Tukey's multiple comparisons test: percent of p-S6 positive cells: E13.5 versus E15.5 p = 0.0501, E15.5 versus E17.5 p = 0.0071, E13.5 versus E17.5 p = 0.5377; percent of p-S6 and Ki67 positive cells: E13.5 versus E15.5 p = 0.6704, E15.5 versus E17.5 p = 0.9697, E13.5 versus E17.5 p = 0.5301). The percent of all cells positive for p-4EBP1 T37/46 (green) and cells co-positive for p-4EBP1 and Ki67 (red) (bottom). (2 way ANOVA with Tukey's multiple comparisons test: percent of p-4EBP1 positive cells: E13.5 versus E15.5 p = 0.0022, E15.5 versus E17.5 p = 0.0023, E13.5 versus E17.5 p < 0.0001; 23 percent of p-4EBP1 and Ki67 positive cells: E13.5 versus E15.5 p = 0.0056, E15.5 versus E17.5 p = 0.0184, E13.5 versus E17.5 p < 0.0001). Error bars represent standard deviation. Plots showing statistics with significance shown in Extended Figure 1-1. (E) Staining of E15.5 mouse brain for the downstream effectors of mTOR and marker of intermediate progenitor cells. 5X representative images (top) of the developing ventricular-subventricular zone with single slice of 63X z-stack (bottom) of the inset region showing Tbr2

 (green) colocalizing with p-S6 S240/244 (white, left) but not with p-4EBP1 T37/46 (white, right), and DAPI (blue). Scale bars for 5X images = 200 µm. Scale bars for 63X images = 10 µm. (F) Staining of E15.5 mouse brain for the downstream effectors of mTOR and protein required for radial glia maintenance and quiescence entry. 20X representative image (top left) of the developing ventricular-subventricular zone with single slice of 63X z-stack (bottom left) of the inset region showing p-S6 S240/244 (green), p-4EBP1 T37/46 (white), VCAM1 (magenta), and DAPI (blue). Line trace reporting pixel intensity for p-4EBP1 T37/46 (black) and VCAM1 (magenta) across distance of yellow dashed line shown in maximum projection image of the z stack for the inset region (top right). Maximum projection image of the z stack for the inset region (bottom middle). Quantification of the median fluorescence intensity of VCAM1 in pixels positive for p- 4EBP1 (black) versus pixels negative for p-4EBP1 (magenta) (bottom right). Error bars represent standard deviation. (N = 3, paired two-tailed t test p value = 0.0175). Scale bars for 20X image = 50 µm. Scale bars 39 for 63X images = 10 μ m. (G) Staining of a wild type day 10 neurosphere derived from human induced pluripotent stem cells for the downstream effectors of mTOR. 40X (left) representative image of sphere within organoid with 100X image of the inset region (right) showing a dividing cell expressing p-S6 S240/244 (green), p-4EBP1 T37/46 (white), Ki67 (red), and Hoechst (blue). Scale bars for both images = 50 µm. (N = 2 representing 2 unique differentiations of 2 independent sets of wild type iPSC lines.) (H) Quantification of the percent of shown in (G) of day 10 cortical organoids. The percent of all cells positive for p-S6 S240/244 (green) and cells co-positive for p-S6 and Ki67 (red) (left). The percent of all cells positive for p-4EBP1 T37/46 (green) and cells co-positive for p-4EBP1 and Ki67 (red) (right). Error bars represent standard deviation.

 Figure 2: Exposure to BMP4 induces quiescence in embryonic NSCs *in vitro***.** Quantification of proliferation and quiescence markers in E15.5 NSC cultures grown for 24 (left in plots) and 72 (right in plots) hours with media containing EGF/FGF (blue) or BMP4/FGF (red). For all plots, the Y axis depicts the arcsinh transformed ratio versus column minimum for a particular antigen. A difference of 0.4 corresponds to an approximately 2-fold difference of total protein. Representative histograms of 24 (top) and 72 hours (bottom). For all plots, error bars contact the maximum and minimum values. For Ki67 and

 Figure 3: p-4EBP1/2 signaling decreases in embryonic NSCs following quiescence induction. Quantification of effectors downstream of mTOR in E15.5 NSC cultures grown for 24 (left in plots) and 72 (right in plots) hours with media containing EGF/FGF (blue) or BMP4/FGF (red). For all plots, the Y axis depicts the arcsinh transformed ratio versus column minimum for a particular antigen. A difference of 0.4 corresponds to an approximately 2-fold difference of total protein. Representative histograms of 24 (top)

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 Figure 4: Decreases in mTOR-dependent phosphorylation of S6 are not sufficient to induce quiescence. (A) Representative histograms downstream effectors of mTOR from a dose-response experiment comparing E15.5 NSCs untreated (blue) to E15.5 NSCs treated with DMSO (0.06%, black), Rapamycin (30 nM, orange), or Torkinib (12.5 – 400 nM, green). Quantification of proliferation markers and effectors downstream of mTOR in E15.5 NSC cultures treated for 4 (left in plots) and 24 (right in plots) hours with media vehicle (1X PBS, blue) or 400 nM Torkinib (green). For all plots, the Y axis depicts the arcsinh transformed ratio versus column minimum for a particular antigen. A difference of 0.4 corresponds to an approximately 2-fold difference of total protein. Representative histograms of 4 (top) and 24 hours (bottom). For all plots, error bars contact the maximum and minimum values. For all antigens, at 4 hours, N = 12; at 24 hours, N =9. (B) Quantification of levels of p-STAT3 S727 (4 hours: unpaired two-tailed t-test 104 $p = 0.3123$; 24 hours: unpaired two-tailed t-test $p = 0.6878$). (C) Quantification of levels of p-S6 S235/236 (4 hours: unpaired two-tailed t-test p < 0.0001; 24 hours: Mann-Whitney test p = 0.5457). (D) Quantification of levels of p-S6 S240/244 (4 hours: unpaired two-tailed t-test p < 0.0001; 24 hours: unpaired two-tailed t-test p = 0.8272). (E) Quantification of levels of p-4EBP1 T37/46 (4 hours: unpaired two-tailed t-test p = 0.5915; 24 hours: unpaired two-tailed t-test p = 0.8275). (F) Quantification of levels of Ki67 (4 hours: unpaired two-tailed t-test p = 0.8409; 24 hours: unpaired two-tailed t-test p = 0.4361). (G) Percent of E15.5 NSC cultures treated for 24 hours 400 nM Torkinib in each phase of the cell cycle. (N

- 111 = 9; G0/G1 phase unpaired two-tailed t-test $p = 0.0005$, S phase unpaired two-tailed t-test $p = 0.7134$,
- 112 G2/M unpaired two-tailed t-test $p = 0.1695$).

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 Figure 5: Decreases in mTOR-dependent phosphorylation of 4EBP1/2 are sufficient to induce quiescence. Quantification of proliferation markers and effectors downstream of mTOR in E15.5 NSC cultures treated for 24 (left in plots) and 72 (right in plots) hours with media vehicle (1X PBS, blue) or 10 nM RapaLink (yellow). For all plots, the Y axis depicts the arcsinh transformed ratio versus column minimum for a particular antigen. A difference of 0.4 corresponds to an approximately 2-fold difference of total protein. Representative histograms of 24 (top) and 72 hours (bottom). For all plots, error bars 123 contact the maximum and minimum values. For all antigens, at 24 hours $N = 17$; at 72 hours $N = 15$. (A) Quantification of levels of p-STAT3 S727 (24 hours: unpaired two-tailed t-test p = 0.4550; 72 hours: unpaired two-tailed t-test p = 0.0330). (B) Quantification of levels of p-S6 S235/236 (24 hours: Mann- Whitney test p < 0.0001; 72 hours unpaired two-tailed t-test p < 0.0001). (C) Quantification of levels of p- S6 S240/244 (24 hours: Mann-Whitney test p = 0.0009; 72 hours: unpaired two-tailed t-test p < 0.0001). (D) Quantification of levels of p-4EBP1 T37/46 (24 hours: Mann-Whitney test p < 0.0001; 72 hours: unpaired two-tailed t-test p < 0.0001). (E) Quantification of levels of Ki67 (24 hours: unpaired two-tailed t-test p = 0.0433; 72 hours: Mann-Whitney test p < 0.0001). (F) Percent of E15.5 NSC cultures treated for

