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1 **NCAM Regulates Temporal Specification of Neural Progenitor Cells via Profilin2**
2 **during Corticogenesis**

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26 **Running title:** NCAM regulates temporal NPC specification

27

28 **Summary**

29 The role of NCAM in corticogenesis is incompletely understood. The authors demonstrate
30 that NCAM controls NPC proliferation and fate decision through profilin2-dependent
31 regulation of actin polymerization. This finding sheds new light on NCAM's functions in
32 neurodevelopmental and mental disorders.

33

34 **Abstract**

35 The development of cerebral cortex requires spatially and temporally orchestrated
36 proliferation, migration and differentiation of neural progenitor cells (NPCs). The molecular
37 mechanisms underlying cortical development are, however, not fully understood. The neural
38 cell adhesion molecule (NCAM) has been suggested to play a role in corticogenesis. Here we
39 show that NCAM is dynamically expressed in the developing cortex. NCAM expression in
40 NPCs is highest in the neurogenic period and declines during the gliogenic period. In mice
41 bearing an NPC-specific NCAM deletion, proliferation of NPCs is reduced, and production of
42 cortical neurons is delayed, while formation of cortical glia is advanced. Mechanistically,
43 NCAM enhances actin polymerization in NPCs by interacting with actin-associated protein
44 profilin2. NCAM-dependent regulation of NPCs is blocked by mutations in the profilin2
45 binding site. Thus, NCAM plays an essential role in NPC proliferation and fate decision during
46 cortical development by regulating profilin2-dependent actin polymerization.

47

48 **Keywords:** NCAM, profilin2, cerebral cortex, neural progenitor cell, actin

49 **Introduction**

50 The development of the mammalian cerebral cortex requires spatially and temporally
51 orchestrated proliferation, migration, and differentiation of neural progenitor cells (NPCs)
52 (Greig et al., 2013). Radial glial cells (RGCs) in the ventricular zone (VZ) contribute to the
53 generation of cortical layers directly or indirectly through intermediate progenitor cells (IPCs)
54 (Gal et al., 2006; Haubensak et al., 2004). Cortical neurons are generated in a defined temporal
55 sequence, in which neurons in deeper layers are generated first. Following neurogenesis,
56 astrocytes appear shortly before birth, whereas oligodendrocytes emerge postnatally in
57 mammals (Kohwi and Doe, 2013). Both intrinsic and extrinsic factors contribute to this
58 developmental sequence. In humans, disturbance of this highly elaborate process leads to
59 neurodevelopmental defects ranging between devastating malformations and relatively mild
60 abnormalities causing neurological diseases such as epilepsy, schizophrenia and autism
61 spectrum disorders (Gaspard and Vanderhaeghen, 2011).

62 The neural cell adhesion molecule (NCAM) is a membrane-bound cell recognition
63 molecule of the immunoglobulin superfamily. NCAM contributes to the nervous system
64 development by influencing neuronal migration, neurite outgrowth, synapse formation, and
65 synaptic plasticity (Sytnyk et al., 2017). Alternative splicing of NCAM transcripts generates
66 three major isoforms: NCAM180, -140, and -120. NCAM180 and NCAM140 are
67 transmembrane isoforms bearing an intracellular domain, which is longer in NCAM180.
68 NCAM120 is anchored to the membrane via a glycosylphosphatidylinositol linkage (Sytnyk et
69 al., 2017). Soluble extracellular NCAM fragments can be produced by NCAM ectodomain
70 shedding (Hubschmann et al., 2005; Secher, 2010). NCAM knockout mice display an abnormal
71 brain structure as well as learning and behavioral abnormalities (Brandewiede et al., 2014;
72 Bukalo et al., 2004; Stork et al., 1999; Wood et al., 1998). Moreover, single nucleotide
73 polymorphisms in the *NCAM* gene and/or abnormal polysialylation or proteolysis of NCAM

74 protein alter NCAM function in neurodevelopmental, neuropsychiatric, and neurodegenerative
75 disorders in humans (Brenneman and Maness, 2010; Hidese et al., 2017; Purcell et al., 2001;
76 Wang et al., 2012), suggesting a crucial role of NCAM in cortical development.

77 NCAM plays a role in regulation of neurogenesis. Recombinant soluble NCAM reduces
78 hippocampal NPC proliferation by heterophilic binding to an unknown cell surface receptor
79 (Amoureux et al., 2000; Shin et al., 2002). Soluble NCAM and overexpression of NCAM140
80 in NPCs promote differentiation of NPCs into the neuronal lineage (Amoureux et al., 2000;
81 Kim and Son, 2006; Kim et al., 2005; Klein et al., 2014), while ectopic expression of
82 NCAM140 in RGCs increases cell proliferation *in vivo* (Boutin et al., 2009). However, it is
83 unknown whether NCAM is an intrinsic modulator of NPC proliferation and differentiation.

84 Regulation of the cell cycle plays a crucial role in controlling temporal and spatial
85 production of neural cells (Dehay and Kennedy, 2007; Politis et al., 2008). Cell cycle
86 progression is modulated by the actin cytoskeleton which regulates cell rounding and rigidity
87 for proper positioning and spindle orientation during mitosis (Heng and Koh, 2010; Kunda and
88 Baum, 2009). Actin cytoskeleton reorganization during mitosis is controlled by actin-binding
89 proteins, among which profilins are essential for cytokinesis (Suetsugu et al., 1999). Profilins
90 are a conserved family of small proteins that facilitate the addition of actin monomers to the
91 fast growing end of actin filaments by accelerating the ADP-ATP nucleotide exchange (Witke,
92 2004). Among the four profilin subtypes, profilin2 is most expressed in the central nervous
93 system (Di Nardo et al., 2000) where it contributes to maintaining spine density and dendritic
94 complexity (Michaelsen et al., 2010). Profilin2 also stabilizes spine structure, controls
95 presynaptic vesicular exocytosis (Pilo-Boyl et al., 2007), and is required for synaptic plasticity
96 (Chakraborty et al., 2014). However, the role of profilins in cortical development is so far
97 unknown.

98 **Results**

99 **NCAM is dynamically expressed in NPCs during cortical development**

100 We first examined the NCAM expression profile. NCAM levels, particularly of the
101 NCAM180 and NCAM140 isoforms, steadily increased in the developing mouse cortex (**Fig.**
102 **S1A-F**). To further analyze the expression of NCAM in distinct cell types, coronal cortical
103 sections at different embryonic stages (embryonic day 12 (E12) to postnatal day 0 (P0)) were
104 co-immunostained for NCAM and either Sox2 (NPCs) or Tuj1 (neurons), respectively. NCAM
105 was expressed in both NPCs (**Fig. 1A**) and neurons (**Fig. 1B**) in the developing cortex.
106 Quantification of NCAM immunofluorescence intensities revealed that NCAM was
107 predominantly expressed by NPCs in the ventricular zone/subventricular zone (VZ/SVZ), and
108 by neurons in the intermediate zone (IZ), cortical plate (CP), and marginal zone (MZ) during
109 the early neurogenic period (E12 to E14). Interestingly, NCAM immunofluorescence intensity
110 in VZ/SVZ in relation to that in the total dorsal brain (**Fig. 1C**) as well as average NCAM
111 immunofluorescence density in VZ/SVZ (**Fig. 1D**) decreased from E16 onward reaching the
112 lowest level at E18 and P0 (gliogenic period). In contrast, NCAM immunoreactivity did not
113 change in the IZ and MZ and even increased in the CP at E18 and P0 compared to E16,
114 indicating that NCAM levels are maintained during these developmental stages in areas
115 enriched in cortical neurons. (**Fig. 1A-D** and **Fig. S1H**). These results indicate that NCAM is
116 expressed in NPCs in the early neurogenic period, whereas NCAM expression in NPCs declines
117 during the gliogenic period.

118

119 **NCAM deficiency results in transiently reduced NPC proliferation in the developing** 120 **cerebral cortex**

121 We next examined the role of NCAM in NPCs by generating NCAM conditional knockout
122 (cKO) mice via crossing NCAM-floxed mice with Nestin-cre mice to ablate NCAM expression

123 in NPCs. NCAM was not detectable in NPCs (**Fig. S1H**) and other brain cell populations (data
124 not shown) in NCAM cKO mice. We then examined whether NPC pool was affected by NCAM
125 deficiency. Numbers of Pax6⁺ RGCs were reduced in the VZ in NCAM cKO mice at E12 and
126 E14, but not at E16 and E18 compared to control littermates (**Fig. 2A, B**). Numbers of Tbr2⁺
127 IPCs, which mainly localize in SVZ, were also decreased in NCAM cKO mice at E12, but not
128 at E14 and E16 (**Fig. 2E, F**). These results indicate that NCAM deficiency leads to transient
129 reduction of NPC numbers during cortical development.

130 The total number of BrdU⁺ cells (**Fig. 2C**) and the percentage of proliferating
131 Pax6⁺BrdU⁺/Pax6⁺ RGCs (**Fig. 2D**) were reduced in NCAM cKO mice compared to control
132 littermates at E12 and E14, but not at E16 and E18, indicating that NCAM regulates RGC
133 proliferation only during earlier developmental stages. Consistently, the numbers of Ki67⁺ (**Fig.**
134 **2G, H**) or PH3⁺ (**Fig. 2I, J**) cells, representing cells in the cell cycle or in mitosis, were also
135 decreased in E12 NCAM cKO mice. This finding suggests that NCAM deficiency results in
136 fewer NPCs in the cell cycle and mitosis at this stage. The decreased proliferation of NCAM
137 cKO NPCs was not due to enhanced apoptosis, as the numbers of cleaved active caspase3⁺
138 NPCs were similar in NCAM cKO and control littermates at E12, E14, and E16 (**Fig. S2**).

139 We also analyzed whether NCAM deficiency affects cell cycle exit and length in the
140 VZ/SVZ. The cell cycle exit index (proportion of BrdU⁺Ki67⁻ cells in BrdU⁺ cell population)
141 was increased at E14 in the VZ/SVZ of NCAM cKO mice compared to control littermates (**Fig.**
142 **2K, L**). S-phase length, estimated by the proportion of BrdU⁺Ki67⁺ cells in the Ki67⁺ cell
143 population, was not altered at E14 (**Fig. 2K, M**). These data indicate that NCAM deficiency
144 results in enhanced cell cycle exit and decreased NPC proliferation, eventually reducing the
145 NPC pool during early neurogenic period.

146

147 **NCAM deficiency delays the generation of cortical neurons**

148 Next, we analyzed whether NCAM regulates the generation of layer-specific neurons at
149 different embryonic stages. The numbers of Tbr1⁺ neurons in layer VI in NCAM cKO cortices
150 were decreased at E12 and E14, increased at E16, and unaltered at E18 (**Fig. 3A, D**). Ctip2⁺
151 neuron numbers in layer V in NCAM cKO mice were decreased at E12 and E14, but unchanged
152 at E16 (**Fig. 3B, E**). The numbers of Cux1⁺ neurons in layers II–IV in NCAM cKO mice were
153 reduced at E16, E18 and P0, but returned to control levels at P7 (**Fig. 3C, F**). Spatial distribution
154 of these cortical neurons was unaltered in NCAM cKO mice (**Fig. S3**), suggesting that the lower
155 numbers of cortical neurons observed in NCAM cKO mice are not caused by a migration defect.

156 Decreased numbers of both deep- and upper-layer cortical neurons at earlier developmental
157 stages, but normal levels at later developmental stages can also be explained by a delayed
158 generation of cortical neurons in NCAM deficient mice. To assess this possibility, we performed
159 birth-dating analysis to examine the generation date of distinct cortical neurons. Pregnant mice
160 were injected with BrdU at E11.5, E14.5, E15.5, or E16.5. The embryos were collected at either
161 E18 (for analysis of Tbr1⁺ and Ctip2⁺ neuron generation) or P2 (for analysis of Cux1⁺ neuron
162 generation). The proportion of cell-specific neurons born at the time of injection (BrdU⁺marker⁺
163 cells/total number of marker⁺ cells) was analyzed. Generation of Tbr1⁺ and Ctip2⁺ neurons was
164 lower at E11.5 (**Fig. 4A, B, D**), but higher at E14.5 (**Fig. 4A, B, E**) in NCAM cKO mice. This
165 inter-genotype difference diminished at E15.5 (**Fig. 4A, B, F**) when deep-layer neuron
166 generation is close to completion. These data aligned well with our findings that the numbers
167 of deep-layer neurons were reduced in NCAM cKO mice at E12 and E14, but were normal by
168 E18 (**Fig. 3D, E**). The generation of upper-layer neurons (Cux1⁺BrdU⁺/Cux1⁺cells) was
169 reduced at E16.5 in NCAM cKO mice (**Fig. 4C, G**), which could explain the decreased numbers
170 of upper-layer neurons from E16 to P0 (**Fig. 3F**). The number of upper-layer neurons in NCAM
171 cKO mice was normal at P7 (**Fig. 3F**), indicating a postnatal “rescue” generation of upper-layer

172 neurons. In summary, these data strongly indicate that NCAM regulates the temporal generation
173 of cortical neurons.

174

175 **NCAM deficiency leads to precocious gliogenesis**

176 We further examined whether NCAM regulates the temporal generation of glial cells. More
177 GFAP⁺ cells were observed in NCAM cKO mice than in control littermates at E18 and P0 (**Fig.**
178 **5A, B**). Interestingly, GFAP⁺ cells were observed in the VZ/SVZ of NCAM cKO mice at E16
179 (**Fig. 5A, C**) while fewer GFAP⁺ cells were observed in control brains at this stage because
180 astrocytes normally do not appear before E18 (Miller and Gauthier, 2007; Molofsky et al.,
181 2012). The result indicates an earlier appearance of astrocytes in NCAM cKO mice. The inter-
182 genotype difference diminished at P7, when astrocyte generation is close to completion (Wang
183 and Bordey, 2008) (**Fig. 5A, B**). Birth-dating analysis of astrocytes by BrdU pulse-labeling at
184 the onset of their generation at E16.5 (Miller and Gauthier, 2007) and calculating the numbers
185 of BrdU⁺GFAP⁺ cells at P2 revealed more BrdU⁺GFAP⁺ cells in NCAM cKO mice (**Fig. 5D,**
186 **E**), indicating an increased generation of astrocytes at E16.5 in NCAM cKO mice. Numbers of
187 Olig2⁺ oligodendrocytes were increased in NCAM cKO brains at E18, but similar to those in
188 control mice at P0 (**Fig. 5F, G**). Birth-dating analysis of oligodendrocytes by BrdU pulse-
189 labeling at E16.5 showed that the percentage of BrdU⁺Olig2⁺ cells was increased in NCAM
190 cKO mice at P2 (**Fig. 5H, I**). Consistent with these results, numbers of cells expressing brain
191 lipid binding protein (BLBP) (**Fig. 5L, M**), which is initially expressed in RGCs and later
192 becomes restricted to astrocytes (Feng et al., 1994; Kurtz et al., 1994), as well as numbers of
193 cells expressing A2B5, a marker of immature glial restricted progenitors (BaracsKay et al.,
194 2007; Dietrich et al., 2002) (**Fig. 5J, K**), were increased in the brains of NCAM cKO mice at
195 E14 and E16, respectively. This increase was not due to an expansion of the RGC pool since
196 the numbers of Pax6⁺ RGCs were reduced at E14 and reached normal levels at E16 (**Fig. 2A,**

197 **B).** Thus, the increase in numbers of BLBP⁺ and A2B5⁺ cells is likely due to an enhanced glial
198 progenitor density, reflecting an earlier glial specification. Taken together, these results suggest
199 that NCAM deficiency in NPCs results in precocious gliogenesis.

200

201 **Profilin2 binds to NCAM**

202 To investigate the molecular mechanisms underlying NCAM-dependent cortical
203 development, yeast two-hybrid screening was performed with NCAM140 as a bait (Li et al.,
204 2013). NCAM140 in NPCs is expressed at higher levels relative to other isoforms
205 (Prodromidou et al., 2014). Among >2x10⁶ clones screened, 26 clones were positive. A BLAST
206 database search (<http://www.ncbi.nlm.nih.gov/BLAST/>) indicated that one clone, encoding full
207 length profilin2, was in the correct open reading frame. To confirm the association between
208 NCAM and profilin2, NCAM was immunoprecipitated from neonatal brain homogenates using
209 polyclonal antibodies recognizing NCAM extracellular domain. Western blot analysis showed
210 that profilin2 was co-immunoprecipitated by NCAM antibodies. Inversely, NCAM120,
211 NCAM140, NCAM180, and soluble NCAM105 were co-immunoprecipitated by profilin2
212 antibodies (**Fig. 6A**). Direct binding was assessed by ELISA, with profilin2 being substrate-
213 coated and probed by the recombinantly expressed intracellular domains of NCAM140 and
214 NCAM180. NCAM140, but notably not NCAM180, bound to profilin2 in a concentration-
215 dependent and saturable manner (**Fig. 6B**). Identification of profilin2-binding sites in NCAM
216 revealed that peptides encoding aa729-750 and aa748-763 bound to profilin2 (**Fig. 6C**). These
217 two peptides had three overlapping amino acids: asparagine (N⁷⁴⁸), leucine (L⁷⁴⁹), and cysteine
218 (C⁷⁵⁰). To investigate whether these amino acids mediated the binding to profilin2, binding of
219 profilin2 to a non-mutated ⁷⁴⁵IAVNLCGKA⁷⁵³ peptide comprising the NLC motif and two
220 mutated peptides (with two of these three amino acids changed at a time) was analyzed.
221 Mutation of L⁷⁴⁹ and C⁷⁵⁰ into alanine (A⁷⁴⁹) and serine (S⁷⁵⁰) completely blocked the binding

222 to profilin2 (**Fig. 6D**). Mutation of N⁷⁴⁸ and L⁷⁴⁹ into glutamine (Q⁷⁴⁸) and alanine (A⁷⁴⁹)
223 partially suppressed the interaction, suggesting that N⁷⁴⁸ is not crucial for it (**Fig. 6D**). Mutation
224 of ⁷⁴⁹LC⁷⁵⁰ in the recombinant intracellular domain of NCAM140 to ⁷⁴⁹AS⁷⁵⁰ also abolished its
225 binding to profilin2 as tested by ELISA, confirming that L⁷⁴⁹ and C⁷⁵⁰ are essential for the
226 binding of NCAM140 to profilin2 (**Fig. 6E, F**).

227

228 **Profilin2 exhibits an expression pattern similar to that of NCAM in the developing cortex**

229 To analyze whether NCAM co-localizes with profilin2 in the developing cortex, cortical
230 sections of E12-P0 wild type mice were co-immunostained for NCAM, profilin2, and Sox2
231 (**Fig. 6G**), or Tuj1 (**Fig. 6H**). Profilin2 co-localized with NCAM at all developmental stages
232 analyzed exhibiting an expression pattern similar to that of NCAM (**Fig. 6I, J, S5B**). Although
233 profilin2 levels increased in the whole brain during development (**Fig. S1A, G**), both the
234 average profilin2 immunofluorescence density in VZ/SVZ (**Fig. 6I**) as well as the percentage
235 of profilin2 immunofluorescence intensity in VZ/SVZ to that in the total dorsal brain (**Fig. 6J**)
236 decreased. This was particularly evident in the gliogenic period. Profilin2 protein (**Fig. 6K, L**)
237 and mRNA (**Fig. 6M**) expression were reduced in NCAM cKO versus wild type NPCs,
238 suggesting that NCAM regulates profilin2 expression and further implying the functional
239 relationship between these two proteins.

240

241 **Profilin2 is required for NCAM-dependent NPC proliferation and differentiation**

242 To explore whether NCAM controls the proliferation of NPCs through profilin2, BrdU
243 incorporation was analyzed in cultured NPCs transfected with either profilin2 siRNA
244 (siProfilin2) or control (scrambled) siRNA (NC). NPCs were analyzed after incubation with or
245 without NCAM antibodies recognizing NCAM extracellular domain and triggering
246 downstream signaling of NCAM (Li et al., 2013). Transfection of siProfilin2 suppressed

247 profilin2 (**Fig. S4A, B, C**), but not profilin1 expression (**Fig. S4D**) in Neuro-2a cells and NPCs,
248 and decreased NPC proliferation (**Fig. 7A, C**; siprofilin2-399). Incubation with NCAM
249 antibodies increased proliferation of NPCs transfected with control siRNA, but not of NPCs
250 transfected with siProfilin2 (**Fig. 7A, C**). This indicates that NCAM promotes the proliferation
251 of NPCs through profilin2.

252 To investigate whether profilin2 plays a role in NCAM-dependent differentiation of NPCs,
253 NC- or siProfilin2 (siProfilin2-399)-transfected NPCs were allowed to differentiate in the
254 absence or presence of NCAM antibodies for 5 days. Immunofluorescence analysis showed
255 that the percentages of Tuj1⁺ cells (**Fig. 7B, D**) were decreased, whereas the percentages of
256 GFAP⁺ (**Fig. 7E, G**) and O4⁺ cells (**Fig. 7F, H**) were increased by profilin2 knockdown. These
257 observations indicate that profilin2, similar to NCAM, promotes neuronal differentiation and
258 suppresses differentiation of NPCs into glial cells. Consistent with *in-vivo* results, NCAM
259 antibody treatment increased the percentages of Tuj1⁺ cells (**Fig. 7B, D**) but decreased GFAP⁺
260 (**Fig. 7E, G**) and O4⁺ cell percentages (**Fig. 7F, H**) in NC-transfected NPCs. However, the effect
261 of NCAM antibodies on neuronal and astroglial differentiation was abolished in NPCs
262 transfected with siProfilin2 (**Fig. 7B, D, E, G**), indicating that profilin2 is required for NCAM-
263 dependent regulation of neuronal and astroglial differentiation. Profilin2 knockdown reduced,
264 but did not abolish the suppressing effect of NCAM antibodies on oligodendroglial
265 differentiation (**Fig. 7F, H**). To further confirm that profilin2 is involved in NCAM-regulated
266 NPC differentiation in a cell autonomous manner, NPCs were transfected with shRNA Profilin2
267 (shProfilin2) alone or together with shRNA-resistant profilin2 plasmids encoding GFP under a
268 separate promotor. NPCs were then treated with NCAM antibodies. NCAM antibody-enhanced
269 neurogenesis and -decreased astrogenesis were prevented by profilin2 knockdown, which was
270 rescued by cotransfection with shRNA-resistant profilin2 (**Fig. 7I-K**). Therefore, these results
271 indicate that profilin2 is the downstream effector of NCAM, through which NCAM regulates

272 NPC proliferation and differentiation.

273

274 **NCAM regulates actin cytoskeleton dynamics through profilin2**

275 We further tested whether NCAM regulates NPC proliferation and differentiation through
276 profilin2-mediated actin polymerization. Western blot analysis of total cell lysates showed that
277 the expression of actin did not differ between control and NCAM cKO NPCs. However, the
278 depolymerized actin (G-actin) levels were higher in NCAM cKO NPCs. Proportionally, the
279 polymerized actin (F-actin) levels were reduced in NCAM cKO NPCs (**Fig. 8A, B**), indicating
280 that NCAM promotes actin polymerization. Western blot analysis revealed that NCAM
281 knockdown-induced reduction of the F-/G-actin ratio was rescued by wild type NCAM140, but
282 not mutNCAM (unable to bind profilin2) in mouse embryonic fibroblasts (**Fig. 8C**). To confirm
283 these data, control and NCAM cKO NPCs expressing GFP only, co-expressing wild type
284 NCAM140, or mutNCAM were stained by fluorescent phalloidin and deoxyribonuclease I to
285 visualize F- and G-actin, respectively. Fluorescent microscopy analysis showed that the F-/G-
286 actin ratio was reduced in NCAM cKO NPCs. Lentiviral transduction with wild type
287 NCAM140, but not mutNCAM, increased the F-/G-actin ratio in NCAM cKO NPCs to control
288 levels (**Fig. 8D, E**). The decreased F-/G-actin ratio in NCAM cKO NPCs was also rescued by
289 profilin2 overexpression (**Fig. 8F, G**). These results indicate that NCAM promotes actin
290 polymerization through its binding to profilin2.

291 Actin cytoskeleton is crucial for soma rounding, and for increased rigidity during cell
292 division (Kunda and Baum, 2009). The role of NCAM in cell shape alteration was investigated
293 in dividing cells, and the cell shape index (CSI) of NPCs was calculated in meta- and anaphase
294 at the VZ surface. NCAM deficiency leads to elongated morphology of NPCs and reduced CSI
295 values (**Fig. 8H, I**), indicating that mitotic NPCs in NCAM cKO mice fail to round up, what is
296 believed to lead to the perturbation of cell cycle progression (Heng and Koh, 2010).

297 Immunofluorescence analysis showed reduced NPC proliferation and neurogenesis, and
298 increased astrogenesis in NCAM cKO NPCs (**Fig. 8J-N**). Transduction of NCAM cKO NPCs
299 with wild type NCAM140, but not mutNCAM-expressing lentiviruses, normalized
300 proliferation, neurogenesis and astrogenesis (**Fig. 8J-N**). Hence, NCAM controls the
301 proliferation and differentiation of NPCs through binding to profilin2 via regulation of actin
302 cytoskeleton dynamics.

303 **Discussion**

304 We demonstrated that NCAM is dynamically expressed in the developing cortex with high
305 expression in NPCs during the neurogenic period and lower expression in NPCs in the gliogenic
306 period. Expression of NCAM is required for maintenance of the precise proliferation and the
307 timely generation of cortical neurons and glial cells. Furthermore, profilin2 was identified to be
308 the mediator of NCAM-dependent regulation of cytoskeleton dynamics controlling NPC
309 proliferation and differentiation during cortical development (**Fig. 9**).

310 Previous studies suggested that NCAM can function as both a cell-extrinsic and cell-
311 intrinsic signaling molecule in NPCs (Amoureux et al., 2000; Boutin et al., 2009; Kim and Son,
312 2006; Klein et al., 2014; Prodromidou et al., 2014). The present study confirms these views by
313 showing that specific ablation of NCAM expression in NPCs suppressed proliferation and
314 enhanced cell cycle exit of NPCs at the earlier neurogenic stage, leading to a transiently reduced
315 NPC pool. At later developmental stages, i.e. from E16 onwards, neither proliferation of NPCs
316 nor NPC numbers in NCAM mutant mice were different from that in wild type mice.
317 Interestingly, NCAM expression in NPCs was reduced from E16 onward, showing considerably
318 reduced levels at the gliogenic period. Thus, the loss of NCAM-dependent regulation of NPC
319 proliferation at later developmental stages may be due to the decline in NCAM expression.
320 However, the fact that the transiently decreased proliferation in the earlier neurogenic period
321 does not lead to an ultimately depleted NPC pool at later developmental stages also suggests a
322 compensatory mechanism.

323 During brain development, a number of cell cycle regulators are involved in cell fate
324 specification while key factors for cell fate specification influence cell cycle (Politis et al.,
325 2008). In line with this notion, we observed a delayed generation of both upper- and deep-layer
326 cortical neurons and a precocious gliogenesis in NCAM mutant mice. Furthermore, the
327 transiently increased cell cycle exit upon NCAM deletion is paralleled by increased numbers

328 of glial progenitors during the neurogenic period, suggesting that cells exiting the cell cycle at
329 E14 may adopt a glial fate. Consistently, GFAP⁺ astrocytes were observed in NCAM cKO
330 brains as early as E16, despite the fact that astrocytes normally do not appear in rodent brains
331 until E18 (Miller and Gauthier, 2007). Hence, it is likely that precocious astrogenesis is due to
332 depletion of NCAM in NPCs at early rather than later development stages when NCAM
333 expression in NPCs decreases. Gliogenesis is suppressed during the neurogenic period, and
334 induced after neurons had been generated in sufficient numbers (Sloan and Barres, 2014).
335 However, NCAM cKO mice exhibit a delayed neurogenesis. Thus, it is unlikely that the
336 precocious astrogliogenesis observed in NCAM cKO mice is due to the delayed generation of
337 neurons. Moreover, transfection of plasmids encoding NCAM decreased astrogliogenesis from
338 cultured NCAM cKO NPCs, further confirming a cell-autonomous mechanism. Thus, we
339 propose that NCAM, which is highly expressed in NPCs during the neurogenic period,
340 promotes neurogenesis and suppresses gliogenesis (**Fig. 9**).

341 We provide evidence that NCAM regulates the proliferation and differentiation of NPCs
342 via its binding to profilin2, which interacts directly with NCAM140 intracellular domain.
343 NCAM180, NCAM120 and the soluble extracellular domain of NCAM also co-
344 immunoprecipitate with profilin2, most likely due to their homophilic binding to NCAM140
345 (Soroka et al., 2003). Surprisingly, NCAM180 does not bind directly to profilin2 although
346 NCAM140 and NCAM180 comprise overlapping amino acid sequences in their intracellular
347 domains, suggesting that NCAM180 is conformationally restricted from its interaction with
348 profilin2. NCAM180 accumulates at contacts between cells, and a reduction in its association
349 with the actin filament-remodeling proteins may be important for stabilization of cell contacts
350 (Pollerberg et al., 1986). In contrast, NCAM140 is more involved in dynamic cell interactions.
351 NCAM120 and NCAM140 are the predominant forms of NCAM in NPCs at E14 (**Fig. 6K** and
352 (Prodromidou et al., 2014) whereas NCAM180 and NCAM140 are the major isoforms in

353 neurons (Korshunova et al., 2007). Consistently, NCAM140, but not its mutant with abolished
354 binding to profilin2, rescues abnormal proliferation and differentiation of NPCs caused by
355 NCAM depletion in NPCs. Thus, the combined observations suggest that NCAM-dependent
356 regulation of neuronal development is fine-tuned by the temporally specific expression patterns
357 of NCAM isoforms.

358 Regulation of actin dynamics by profilin1 and -2 require both discrete and cooperative
359 activities. One example is that expression of profilin1 rescues the loss of spines (but not
360 dendritic complexity) caused by profilin2 knockdown (Michaelsen et al., 2010). Indeed,
361 profilin2-deficient neurons show an initial, transient increase in the number of sprouting
362 neurites, which may be due to the compensatory function of profilin1 (Da Silva et al., 2003).
363 In addition, NCAM-depleted NPCs exhibit reduced profilin2 levels. Thus, the transient nature
364 of abnormalities in NPCs observed in NCAM cKO cells may be due to a functional
365 compensation by profilin1. We herein show that profilin2 is required for NCAM-regulated NPC
366 proliferation and differentiation, which depends on binding of NCAM to profilin2 which
367 exhibits an expression profile similar to that of NCAM in NPCs during brain development.
368 Acute profilin2 knockdown in cultured NPCs results in a phenotype being comparable to that
369 of NCAM-deficient NPCs, suggesting that profilin2 and NCAM have similar roles in the
370 developing cerebral cortex. Consistently, profilin2-deficient mice are hyperactive and show
371 increased exploratory behavior (Pilo Boyl et al., 2007), thus partly resembling behavioral
372 abnormalities in NCAM-deficient mice. Profilins bind to various ligands and are involved in
373 distinct cellular processes, such as membrane and vesicle trafficking, endocytosis, and receptor
374 clustering. Profilins are also found in the cell nucleus, where they may be involved in chromatin
375 remodeling and transcription (Witke, 2004). Further research is required for a more holistic
376 understanding of the role of profilin2 during cortical development.

377 NCAM is the major carrier of the linear homopolymer α 2-8-N acetylneuraminic acid

378 (PSA), which plays a prominent role in regulation of migration and differentiation of progenitor
379 cells during postnatal brain development (Angata et al., 2007), as well as in the adult brain
380 (Burgess et al., 2008). We did not detect NCAM-PSA in E14 NPCs (**Fig. 6K**), which is in
381 accordance with previous reports showing that NCAM is not polysialylated during the early
382 phases of neurogenesis in the developing brain (Bonfanti, 2006; Prodromidou et al., 2014; Seki
383 and Arai, 1991). This in turn suggests that NCAM rather than PSA plays a key role at these
384 early development stages.

385 Our observations also indicate that NCAM regulates corticogenesis by modulating actin
386 dynamics. NCAM deficiency leads to reduced actin polymerization, elongated progenitor cell
387 shape, and decreased mitosis (**Fig. 9**). Remodeling of the actin cytoskeleton during mitosis is
388 necessary for formation of rounded cells with increased cortical rigidity (Heng and Koh, 2010;
389 Luxenburg et al., 2011). At the end of mitosis, actin rearranges at cleavage furrows and
390 contributes to formation of the contractile ring, which is crucial for cytokinesis. Another mitotic
391 event requiring actin dynamics is centrosome separation, which depends on the flow of
392 submembrane actin and the myosin network (Heng and Koh, 2010). The modulation of cell
393 shape changes in coordination with cell cycle progression is a pre-requisite for the acquisition
394 of appropriate cell fates and the transformation of proliferating, undifferentiated progenitors
395 into fully differentiated, functional cells (Cremisi et al., 2003). We show that NCAM-dependent
396 actin remodeling promotes neurogenesis, but suppresses gliogenesis.

397 The actin cytoskeleton is also involved in transcription control (Miralles and Visa, 2006).
398 Actin is a component of the transcription apparatus, chromatin-remodeling complexes, and
399 RNA-processing machinery (Miralles and Visa, 2006). Our data show that deficiency in NCAM
400 results in inhibited neurogenesis and enhanced gliogenesis from NPCs, suggesting a role for
401 NCAM in the control of the neurogenic-gliogenic switch. This cell fate programming comprises
402 specific signaling pathways, such as JAK-STAT, Notch, BMP, and MEK-ERK-dependent

403 signaling to activate transcription factors (Miller and Gauthier, 2007). Whether NCAM
404 regulates transcription by modulating actin dynamics is a question for future investigation.

405 In the developing brain, neurons are generated first, while gliogenesis is suppressed during
406 the neurogenic period. Astroglialogenesis is induced later, after neurons had been generated in
407 sufficient numbers (Sloan and Barres, 2014). Astrocytes, in turn, guide appropriate neurite and
408 synapse development (Jacobs and Doering, 2010; Sloan and Barres, 2014). This temporal
409 sequence of coordinated generation of neurons and astrocytes is required for proper
410 establishment of neural circuits. Perturbations in the temporally orchestrated generation of
411 neurons and glia may cause impaired neuronal development and synaptic plasticity, leading to
412 neurodevelopmental disorders, such as Down syndrome, autism spectrum disorders and
413 “RASopathies” (Jacobs and Doering, 2010; Sloan and Barres, 2014; Zdaniuk et al., 2011), the
414 latter including Noonan (Tartaglia et al., 2001), neurofibromatosis-1 (Hegedus et al., 2007),
415 costello (Paquin et al., 2009), and cardiofaciocutaneous syndrome (Urosevic et al., 2011).
416 Despite a broad spectrum of clinical manifestations, these syndromes share some degree of
417 mental impairment and precocious astrogenesis (Sloan and Barres, 2014). In turn, NCAM-
418 deficient mice display hyperactivity, increased aggression, learning deficits, and impaired nest
419 building behaviors (Cremer et al., 1994; Stork et al., 1997; Stork et al., 2000; Stork et al., 1999;
420 Vicente et al., 1997).

421 In the mature brain, NCAM is involved in regulation of the number, structure, and
422 molecular composition of synapses, synaptic vesicle recycling, synaptic plasticity, learning
423 (Bukalo et al., 2004; Puchkov et al., 2011; Shetty et al., 2013; Sytnyk et al., 2006), and behavior
424 (Brandewiede et al., 2014; Kohl et al., 2013; Pillai-Nair et al., 2005; Stork et al., 1999). By
425 regulating actin polymerization (Schluter et al., 1997), profilin2 also plays a role in modulation
426 of synaptic vesicle exocytosis, neuronal excitability (Pilo-Boyl et al., 2007), spine density,
427 dendritic complexity (Da Silva et al., 2003; Michaelsen et al., 2010; Witke et al., 1998),

428 learning, and memory consolidation (Lamprecht et al., 2006). Whether NCAM and profilin2
429 co-operate in regulation of synapse formation and function is, however, unknown and remains
430 a question for further investigation.

431 Our study suggests that abnormalities in temporal NPC fate decision can contribute to the
432 pathophysiology of neurodevelopmental diseases associated with abnormal function of NCAM.
433 Understanding the molecular mechanisms underlying these abnormalities may help to design
434 future strategies aimed at correcting neural differentiation in the affected brain.

435 **Materials and methods**

436 **Antibodies**

437 The following antibodies were used for immunofluorescence analysis: goat anti-Sox2
438 (1:150, Santa Cruz Biotechnology, sc-17320, RRID: AB_2286684), mouse anti-BrdU (1:300,
439 Covance, MMS-139S, RRID: AB_10719257), mouse anti- β III-tubulin (1:500, Tuj1, Sigma-
440 Aldrich, T5076, RRID: AB_532291), mouse anti-profilin2 (1:100, Proteintech, 60094-2-Ig,
441 RRID: AB_2163215), rabbit anti-NCAM (1:300, Alomone, ANR-041, RRID: AB_2756690),
442 rabbit anti-NCAM (1:200, Thermo, 701379, RRID: AB_2532477), rabbit anti-Pax6 (1:300,
443 Covance, PRB-278P, RRID: AB_291612), rabbit anti-Tbr1 (1:300, Abcam, ab3190, RRID:
444 AB_2238610), rabbit anti-Tbr2 (1:300, Abcam, ab23345, RRID: AB_778267), rabbit anti-
445 Olig2 (1:300, Abcam, ab109186, RRID: AB_10861310), rabbit anti-GFAP (1:500, Millipore,
446 MAB360, RRID: AB_11212597), rabbit anti-PH3 (1:300, Millipore, 06-570, RRID:
447 AB_310177), rabbit anti-Ki67 (1:100, Thermo, PA5-19462, RRID: AB_10981523), rabbit anti-
448 Ctip2 (1:300, Abcam, ab28448, RRID: AB_1140055), rabbit anti-Cux1 (1:100, Santa Cruz
449 Biotechnology, sc-13024, RRID: AB_2261231), rabbit anti-BLBP (1:100, Abcam, ab32423,
450 RRID: AB_880078), mouse anti-A2B5 (1:300, Thermo, MA1-90445, RRID: AB_1954783)
451 and rabbit anti-cleaved caspase3 (1:300, Cell Signaling, 9661, RRID: AB_2341188). Rabbit
452 anti-NCAM (Alomone, ANR-041, RRID: AB_2756690) and rabbit anti-profilin2 (Abcam,
453 ab174322, RRID: AB_2783646) antibodies were used for immunoprecipitation and Western
454 blot analysis. Chicken anti-NCAM antibody (Li et al., 2013) was used to assay NPC
455 proliferation and differentiation. Rat anti-NCAM (BD Pharmingen, 556323, RRID:
456 AB_396361) antibody was used for Western blot analysis and ELISA. Mouse anti-actin
457 (Sigma-Aldrich, A5441, RRID: AB_476744) antibody was used for immunofluorescence and
458 Western blot analysis. Mouse anti- γ -tubulin antibody (Sigma-Aldrich, T6557, RRID:
459 AB_477584) was used for Western blot analysis. Non-immune mouse immunoglobulin (IgG)

460 and horseradish peroxidase (HRP)-coupled secondary antibodies were purchased from Sigma-
461 Aldrich. Secondary antibodies conjugated with Alexa fluorophores 488, 555 or 647 were
462 purchased from Invitrogen. Acti-stain™ 670 Fluorescent phalloidin (Cytoskeleton, PHDN1-A)
463 was used for F-actin staining. Alexa Fluor 594 conjugated deoxyribonuclease I (Molecular
464 Probes, D12372) was used for G-actin staining.

465

466 **Mice**

467 Homozygous NCAM-floxed mice (NCAMff^{+/+}; (Bukalo et al., 2004) were crossed with
468 Nestin-cre transgenic mice (Jackson Laboratory, 003771) to generate conditionally NCAM-
469 deficient (NCAMff^{+/+}cre^{+/-}, NCAM cKO) mice and their control littermates (referred to as
470 NCAMff^{+/+}cre^{-/-} or NCAMff^{+/-}cre^{-/-}). Successful mating was verified by the presence of a
471 vaginal plug. Observation date of the plug was considered E0.5. Mice had been backcrossed
472 with C57BL/6J mice for more than 10 generations and were maintained on the C57BL/6J
473 background thereafter. All experimental procedures were in accordance with ARRIVE (Animal
474 Research: Reporting of *In Vivo* Experiments) guidelines and were approved by the Institutional
475 Animal Care and Use Committee of Soochow University.

476

477 **DNA constructs, protein expression and ELISA**

478 Sequences of the intracellular domain of rat NCAM140 (NCAM140ICD) and NCAM180
479 (NCAM180ICD) were subcloned from prokaryotic expression pQE30-NCAM140ICD and
480 pQE30-NCAM180ICD plasmids (Li et al., 2013) into pET29b-His vector (Novagen, 69872).
481 The pET29b-NCAM140ICD was used as a template to produce the mutant NCAM140ICD
482 expression vector, pET29b-muNCAM140ICD, by site-directed mutagenesis with CTG
483 encoding L⁷⁴⁹ mutated into GCG, and TGT encoding C⁷⁵⁰ mutated into TCT. Primers for
484 mutagenesis were TGCATCGCTGTAAACGCGTCTGGCAAAGCTGGG (forward), and

485 CATGAGCAGGCCACACTTGTTTCAGGAAGTAGCAGG (reverse). The pET29b-profilin2
486 was synthesized by Takara (Dalian, China). The profilin2 sequence was subcloned into a
487 pCDH-EF1-MCS-T2A-copGFP vector. The pEX4-siRNA-resistant-profilin2 plasmid was
488 generated by Genepharma (Shanghai, China) using the pEX4-profilin2 as a template for three
489 synonymous mutations on the profilin2 siRNA recognition sequence with
490 CATCACGCCAGTAGAAATA mutated into CATTACTCCAGTTGAAATA. All plasmid
491 constructs were verified by sequencing. Prokaryotic pET29b-NCAM140ICD, pET29b-
492 muNCAM140ICD, pET29b-NCAM180ICD, and pET29b-profilin2 plasmids were transformed
493 and expressed in *E. coli* strain BL21, and corresponding recombinant proteins were purified by
494 Ni-NTA chromatography (Qiagen, 30210).

495 Profilin2 (100 µg/ml) was immobilized overnight at 4°C on a polyvinylchloride surface in
496 96-well ELISA plates (Corning, 2595). Then, the wells were washed three times with PBST
497 (PBS with 0.1% Tween 20, pH 7.4) and blocked with 3% BSA in carbonate buffer (35 mM
498 NaHCO₃ and 15 mM Na₂CO₃, pH 9.6) at 37°C for 2 h. Thereafter, increasing concentrations
499 (0.1-12.5 µM) of recombinant NCAM140ICD, NCAM180ICD or muNCAM140ICD (in
500 PBST) were applied at 37°C for 2 h. After three washes, the wells were incubated with NCAM
501 monoclonal antibodies (0.5 µg/ml) for 1.5 h at 37°C. Following five washes with PBST, HRP-
502 conjugated secondary antibodies were applied for 1 h at 37°C. After five washes, protein
503 binding was analyzed by adding the HRP substrate, tetramethylbenzidine reagent (Pierce,
504 34021). The reaction was terminated with 2 M H₂SO₄ and ODs were measured at 450 nm using
505 a plate reader (Thermo, 51119000). Biotinylated peptides comprising amino acid sequences
506 729-750, 748-763, 756-770, 764-777, 766-810 of mouse NCAM140, ⁷⁴⁵IAV NLC GKA⁷⁵³
507 peptide comprising the profilin2 binding site, and mutated ⁷⁴⁵IAV NAS GKA⁷⁵³ and ⁷⁴⁵IAV
508 QAC GKA⁷⁵³ peptides were synthesized by SciLight Peptide (Beijing, China). All constructs
509 were incubated with substrate-coated profilin2 and detected by HRP-coupled NeutrAvidin

510 (Thermo, 31030).

511

512 **RNA interference**

513 The siProfilin2-399 (CAUCACGCCAGUAGAAAUATT), siProfilin2-527
514 (CAAUGGACAUCCGGACAAATT), siNCAM (GUUGGAGAGUCCAAAUUCUTT) and
515 the NC (UUCUCCGAACGUGUCACGUTT) were synthesized by Genepharma (Shanghai,
516 China). The shProfilin2 was constructed by Genechem (Shanghai, China) by inserting the same
517 target sequence as siProfilin2-399 into the GV102 vector. To confirm siProfilin2 efficacy,
518 Neuro-2a cells (ATCC, CCL-131™) were transfected with siRNA/shRNA using Lipofectamine
519 2000 according to the manufacturer's instructions (Invitrogen, 11668030). Mouse embryonic
520 fibroblasts (MEFs, ATCC, SCRC-1008™) were transfected with siRNA using Lipofectamine
521 2000. The profilin2/NCAM knockdown efficacy was verified by Western blot analysis of cell
522 lysates. Cultured NPCs were transfected with 20 pmol of RNA per cuvette using the Amaxa®
523 Nucleofector system (Lonza, VPG-1004) according to the user's manual.

524

525 **Culture and transfection of NPCs**

526 NPCs were obtained from the telencephalic lateral ventricle walls of E14 embryos and
527 cultured in DMEM/F12 culture medium (Gibco, 11320033) supplemented with 2% B27
528 (Gibco, 17504044), 20 ng/ml basic fibroblast growth factor (bFGF, Peprotech, 96-450-33), and
529 20 ng/ml epidermal growth factor (EGF, Peprotech, 315-09) as described (Ma et al., 2008). For
530 differentiation, NPCs were cultured in DMEM/F12 medium containing 2% B27 and 0.5% fetal
531 calf serum (Gibco) without EGF and bFGF for 5-7 days.

532 For NCAM antibody incubation experiments, NCAM antibodies (10 µg/ml) were added
533 to the culture medium and replenished every 24 h. The medium was changed every 48 h.

534 Transfection of cultured NPCs was performed using the Amaxa® Nucleofector system

535 (Lonza, VPG-1004) according to the user's manual.

536

537 **Lentiviral transduction**

538 Lentivirus constructs containing full-length wild type or mutated mouse NCAM140 genes
539 were generated by Genechem (Shanghai, China) in an Ubi-MCS-3FLAG-SV40-EGFP-IRES-
540 puromycin (GV358) vector comprising ubiquitin, SV40, and CMV promoters. Cultured NPCs
541 and MEFs were transduced with 1×10^8 TU/ml lentivirus following the manufacturer's
542 instructions and thereafter maintained for 2-4 d.

543

544 **BrdU labeling**

545 For analysis of proliferation *in vivo*, pregnant mice were intraperitoneally injected with
546 BrdU (50 mg/kg, Sigma-Aldrich, B5002) at different embryonic stages (E12, E14, E16 and
547 E18) and sacrificed 30 min thereafter (Wu et al., 2017). For determination of the cell cycle exit,
548 BrdU (100 mg/kg) was intraperitoneally injected into pregnant mice, and mice were sacrificed
549 18 h after injection. The cell cycle exit index was calculated as $\text{BrdU}^+\text{Ki67}^-/\text{total BrdU}^+$ cells
550 (the percentage of cells exiting the cell cycle). The length of S-phase of the cell cycle was
551 calculated as $\text{BrdU}^+\text{Ki67}^+/\text{total Ki67}^+$ cells.

552 For pulse-chase labeling of newborn cells, pregnant mice were intraperitoneally injected
553 with BrdU (100 mg/kg) at E11.5, E14.5, E15.5 and sacrificed at E18, or injected with BrdU at
554 E16.5 and sacrificed at P2. Quantification of birth-dated neurons was performed by calculating
555 the percentages of BrdU^+ layer-specific neuronal marker⁺ cells/total layer-specific neuronal
556 marker⁺ (Tbr1⁺, Ctip2⁺ or Cux1⁺) cells. Quantitation of birth-dated oligodendrocytes was
557 performed by calculating the percentage of $\text{Olig2}^+\text{BrdU}^+$ cells/total Olig2^+ cells. Quantification
558 of birth-dated astrocytes was calculated as the numbers of $\text{GFAP}^+\text{BrdU}^+$ cells along the
559 dorsolateral VZ.

560 To investigate NPC proliferation *in vitro*, cells were cultured for 4-5 h in NPC culture
561 medium supplemented with 10 μ M BrdU.

562

563 **Immunohistochemistry and image analysis**

564 Immunohistochemistry was performed as described elsewhere (Wu et al., 2017). Briefly,
565 pregnant mice were sacrificed and the fetuses were removed from the uterus. Fetal brains were
566 fixed in 4% formaldehyde in PBS (pH 7.3) for 24 h at 4 °C followed by sequential dehydration
567 using 15% and 30% sucrose in PBS. Coronal sections (14 μ m thick) were sectioned with a
568 cryostat (Leica CM1950), and washed 3 times with PBS before blocking in 10% donkey serum
569 in 0.1% Triton X-100 in PBS for 1 h at room temperature. Primary antibodies were applied in
570 the blocking solution for 16 h at 4°C, followed by 3 washes in PBS. Secondary antibodies were
571 incubated in the blocking solution for 1 h at room temperature. Sections were then washed, air-
572 dried, and mounted using DAPI Fluoromount-G (Southern Biotech, 0100-20). Fluorescence
573 images were acquired with a Carl Zeiss Microscope Axio Scope A1 (20x objective lenses,
574 acquisition software ZEN 2.6 (blue edition)) or a confocal laser scanning microscope LSM700
575 (20x, 40x, 40x oil or 63x oil objective lenses, acquisition software ZEN 2012) at room
576 temperature. NCAM and profilin2 immunofluorescence intensity was quantified by Image J.
577 Identical telencephalon cortical regions from littermates of control and NCAM cKO mice (five
578 sections per brain) were analyzed. NCAM/profilin2 immunofluorescence intensity was
579 calculated as NCAM/profilin2 immunofluorescence intensity in different cortical regions in
580 relation to the whole dorsal cortices. The average fluorescence density of NCAM/profilin2 was
581 obtained by calculating the fluorescence density within a 250 μ m² area in different cortical
582 layers. Cortical cells were counted in regions as described previously (Cappello et al., 2006;
583 Seuntjens et al., 2009). In brief, Tbr1⁺ and ctip2⁺ cells were counted in the medial brain as they
584 distribute evenly in layer VI and V of the dorsal cortex; Cux1⁺ and Olig2⁺ cells in the lateral

585 brain regions where they appear first; and GFAP⁺ cells in the dorsal pallium adjacent to the VZ
586 where they reside. The total GFAP⁺ intensity was counted in VZ/SVZ because there are few
587 GFAP⁺ cells appearing in the wild type at E16, and the earlier appearing GFAP⁺ cells do not
588 distribute evenly in the VZ/SVZ (**Fig. S5**). Proliferating (i.e., BrdU⁺, Ki67⁺, PH3⁺), Tbr2⁺,
589 Pax6⁺, Tbr1⁺, and Ctip2⁺ cells were counted in 100 μm x 250 μm areas in the dorsal pallium
590 perpendicular to the VZ (red rectangle), and numbers of Cux1⁺ and Olig2⁺ cells were counted
591 in 100 μm x 250 μm areas of the dorsolateral pallium (blue rectangle). Numbers of caspase3⁺
592 cells were determined in the entire hemi-telencephalon cortex. The GFAP expression per unit
593 area (150 μm x 150 μm) was measured in the dorsolateral pallium adjacent to VZ using ImageJ
594 (purple square).

595 For cortical neuron distribution analysis, the maximum migration of neonatal cortical
596 neurons was measured as the vertical distance from VZ to the destination of different layers
597 using ImageJ. The length of the entire cortical layers perpendicular to the VZ was measured
598 using ImageJ and defined as total cortical length. The distribution of layer-specific marker⁺
599 (Tbr1⁺, Ctip2⁺ or Cux1⁺) neuron was quantified by calculating maximum migration distance of
600 each type of neurons/total cortical length (shown by schematic diagram in **Fig. S3**).

601

602 **Yeast two-hybrid screening**

603 Yeast two-hybrid screening was performed with the ProQuest Two-Hybrid system
604 (Invitrogen, 10835) in *Saccharomyces cerevisiae* strain MaV203 following the manufacturer's
605 protocol. The DNA fragment encoding the intracellular domain of mouse NCAM140 was used
606 as bait.

607

608 **Co-immunoprecipitation**

609 Lysates of brains from newborn C57BL/6J mice were prepared using ice-cold lysis buffer

610 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1% sodium deoxycholate, 0.5%
611 SDS, 2 mM EDTA, and protease inhibitor cocktail (Roche, 11697498001)). Lysates were
612 centrifuged for 10 min at 15,000 g and 4°C, cleared with protein A/G-agarose beads (Santa
613 Cruz Biotechnology, sc-2003, RRID: AB_10201400), and incubated with corresponding
614 antibodies or, for negative control, non-immune IgG at 4°C overnight. Antibody/protein
615 complexes were collected by incubating lysates with protein A/G-agarose beads for 3 h at 4°C,
616 pelleting the beads and washing in PBS. Proteins were eluted with 2x SDS sample buffer by
617 boiling the beads for 10 min and subjected to Western blot analysis.

618

619 **Quantitative real-time PCR**

620 Total RNA was extracted from cultured NPCs using Trizol Reagent (Invitrogen,
621 15596018). Reverse transcription reactions were performed with the EasyScript[®] One-Step
622 gDNA Removal and cDNA Synthesis SuperMix kits (Transgen Biotech, AE311-02). PCR
623 primers were: forward 5'-GCCTATACGTTGATGGTGACTG-3', reverse 5'-
624 ACAAAGACCAAGACTCTCCCG-3' for profilin2, forward 5'-
625 GACAGAACCCGAAAAGGGC-3', reverse 5'-GTTGGGGACCGTCTTGACTT-3' for
626 NCAM, forward 5'-AGGTCGGTGTGAACGGATTTG-3', reverse 5'-
627 TGTAGACCATGTAGTTGAGGTCA-3' for GAPDH. The reaction procedure was conducted
628 at 94°C, 15 min (1 cycle); 95°C, 30 s; 55°C, 30 s; 72 °C, 60 s (30 cycles), and 72°C, 8 min (1
629 cycle).

630

631 **Immunocytochemistry and image analysis**

632 Immunocytochemistry was performed as described elsewhere (Ma et al., 2008). Briefly,
633 cells were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized by 0.1% Triton X-
634 100 in PBS for 5 min, and blocked by 10% donkey serum in 0.1% Triton X-100 in PBS for 1 h

635 at room temperature. Cells were incubated with appropriate dilutions of primary antibodies in
636 the blocking solution at 4°C overnight. Cells were then rinsed with PBS, and incubated with
637 corresponding secondary antibodies in the blocking solution for 1 h at room temperature. The
638 culture was rinsed three times with PBS, and counterstained with DAPI Fluoromount-G
639 (Southern Biotech, 0100-20). To detect BrdU in cultured NPCs, cells were treated with 2 N HCl
640 for 10 min at 37°C. The proportion of proliferating NPCs was quantified as the numbers of
641 BrdU⁺ cells divided by the total number of DAPI⁺ cells. To estimate differentiation into
642 neurons, astrocytes and oligodendrocytes, numbers of Tuj1⁺, GFAP⁺ or O4⁺ cells, respectively,
643 were divided by the total number of DAPI⁺ cells. Proliferation and differentiation of profilin2
644 siRNA-transfected NPCs were quantified from random images of areas containing cultured
645 cells. The proportion of target cells was quantified as the numbers of BrdU⁺, Tuj1⁺, GFAP⁺ or
646 O4⁺ cells divided by the total number of DAPI⁺ cells in the same field. Proliferation and
647 differentiation of NPCs transfected with plasmids encoding profilin2, NCAM and mutNCAM
648 were quantified from images of areas captured from top-to-bottom and left-to-right across the
649 entire coverslip. The proportion of target cells was quantified as the numbers of BrdU⁺GFP⁺,
650 Tuj1⁺GFP⁺, GFAP⁺GFP⁺ cells divided by the total number of GFP⁺ cells in the same coverslip.
651 Each experiment was performed in independent triplicates.

652

653 **F-actin and G-actin analysis**

654 F- and G-actin levels were analyzed by Western blot using an F-actin/G-actin *in vivo* assay
655 kit (Cytoskeleton, BK037). Briefly, cultured NPCs were lysed with pre-warmed F-actin
656 stabilization buffer (50 mM PIPES buffer, pH 6.9, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA,
657 5% (v/v) glycerol, 0.1% Nonidet P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercapto-
658 ethanol) at 37°C for 10 min. Samples were centrifuged at 100,000 g for 1 h at 37°C.
659 Supernatants containing G-actin were separated from the pellets containing F-actin and placed

660 on ice. The pellets were resuspended in the same volume as the supernatants using ice-cold
661 water containing 1 μ M cytochalasin D and incubated on ice for 1 h by pipetting up and down
662 every 15 min to dissociate F-actin. Equal amounts of protein from each sample were subjected
663 to Western blot analysis with anti-actin antibody with γ -tubulin serving as a control.

664 To analyze levels of G- and F-actin by microscopy, NPCs were co-stained by phalloidin
665 (for F-actin) and deoxyribonuclease I (for G-actin). GFP-positive cells were outlined, the F-
666 actin and G-actin labelling intensities were measured by Image Pro-plus 6.4 software (Media
667 Cybernetics), and the ratio of F-actin/G-actin was calculated.

668

669 **CSI analysis**

670 The CSI analysis was performed as described elsewhere (Thakar et al., 2009). The coronal
671 cortical sections at E12 were immunostained for actin with DAPI counterstaining to visualize
672 cells and chromatin. Mitotic NPCs at metaphase and anaphase were identified by chromosome
673 morphology at the VZ surface, selected, and analyzed (Haydar et al., 2003; Luxenburg et al.,
674 2011). Cell boundaries were outlined with ImageJ. Cell area and perimeter were determined,
675 and the CSI was calculated as follows: $CSI = 4\pi \cdot \text{area}/(\text{perimeter})^2$. The CSI assumes values
676 between 1 (circular shape) and 0 (elongated, linear morphology) (Thakar et al., 2009).

677

678 **Statistical analysis**

679 Data were collected from at least three independent experiments ($n \geq 3$) or at least three
680 pairs of NCAM cKO mice and control littermates ($n \geq 3$, five slices from each animal). Values
681 are presented as means \pm SEM. Data distribution was checked by Kolmogorov-Smirnov test.
682 Statistical difference was tested by Student's t test, one-way ANOVA, or two-way ANOVA (for
683 normal distribution data), Mann-Whitney or Kruskal-Wallis test (for non-normally distributed
684 data) with appropriate *post-hoc* analysis using SPSS 22.0 software (all two-sided). $P < 0.05$ was

685 considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

686

687 **Online supplemental material**

688 Fig. S1. Expression of NCAM and profilin2 in the developing cerebral cortex.

689 Fig. S2. NCAM deficiency does not lead to increased NPC apoptosis during embryonic
690 development.

691 Fig. S3. NCAM deficiency does not affect the distribution of neonatal cortical neurons in the
692 coronal plane.

693 Fig. S4. Profilin2 expression is downregulated specifically by profilin2 RNA interference.

694 Fig. S5. Schematic diagram showing areas chosen for quantification of cells in imaging
695 analysis.

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934 **Nonstandard abbreviations:**

935 A, alanine; C, cysteine; cKO, conditional knockout; CP, cortical plate; CSI, cell shape index;
936 E, embryonic day; G-actin, globular actin; GFAP, glial fibrillary acidic protein; ICD,
937 intracellular domain; IPCs, intermediate progenitor cells; IZ, intermediate zone; L, leucine;
938 MZ, marginal zone; N, asparagine; NCAM, neural cell adhesion molecule; NPCs, neural
939 progenitor cells; P, postnatal day; Q, glutamine; RGCs, radial glial cells; S, serine; siProfilin2,
940 profilin2 siRNA; SVZ, subventricular zone; VZ, ventricular zone.

941 **Figure legends**

942

943 **Figure 1. NCAM is dynamically expressed in NPCs during cortical development.**

944 **A, B:** Coronal sections of mouse cortices from indicated embryonic stages were co-
945 immunostained for NCAM and Sox2 (A) or Tuj1 (B). Scale bars: 50 μ m. **C:** Percentages of
946 NCAM⁺ immunoreactivity in each layer. **D:** Average immunofluorescence density of NCAM
947 in each layer. n=9 brain slices from 3 mice. Values represent mean \pm SEM. * P <0.05, ** P <0.01;
948 *** P <0.001 (two-sided). One-way ANOVA with Bonferroni corrections (C (IZ, CP and MZ)
949 and D (MZ)), Dunnett's T3 correction (C (VZ/SVZ)), Kruskal-Wallis test with Dunn-
950 Bonferroni correction (D (VZ/SVZ, IZ, and CP)). VZ/SVZ, ventricular zone/subventricular
951 zone. PP, preplate. IZ, intermediate zone. CP, cortical plate. MZ, marginal zone.

952

953 **Figure 2. NCAM deficiency transiently suppresses NPC proliferation *in vivo*.**

954 **A:** Coronal sections of control and NCAM cKO cortices were co-immunostained for BrdU and
955 Pax6 30 min after BrdU injection. **B-D:** Numbers of Pax6⁺ (B), BrdU⁺ (C) cells, and
956 percentages of Pax6⁺BrdU⁺ cells in total Pax6⁺ cell population (D). **E-J:** Coronal sections of
957 control and NCAM cKO cortices were immunostained for Tbr2 (E), Ki67 (G) or PH3 (I) with
958 DAPI counterstaining. Numbers of Tbr2⁺ (F), Ki67⁺ (H), and PH3⁺ (J) cells in the VZ/SVZ. **K:**
959 Coronal sections of E14 control and NCAM cKO cortices were co-immunostained for BrdU
960 and Ki67. **L, M:** Percentages of BrdU⁺Ki67⁻ cells in the total BrdU⁺ cell population (L) and
961 percentages of BrdU⁺Ki67⁺ cells in the total Ki67⁺ cell population (M). Scale bars: 50 μ m. n=15
962 brain slices from 3 mice. Values represent mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 (two-
963 sided). Student's t test or Mann-Whitney test (B (E12), C (E12), and F (E14)).

964

965 **Figure 3. NCAM deficiency reduces numbers of cortical neurons at early, but not later**

966 **developmental stages.**

967 **A-C:** Coronal sections of control and NCAM cKO cortices were immunostained for Tbr1 (A),
968 Ctip2 (B), and Cux1 (C) with DAPI counterstaining. Scale bars: 50 μm . **D-F:** Numbers of Tbr1⁺
969 (D), Ctip2⁺ (E), and Cux1⁺ (F) cells per $2.5 \times 10^4 \mu\text{m}^2$. n=15 brain slices from 3 mice. Values
970 represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-sided). Student's t test or Mann-
971 Whitney test (D(E14)).

972

973 **Figure 4. NCAM deficiency delays the generation of cortical neurons *in vivo*.**

974 **A, B:** Cortical sections of E18 control and NCAM cKO mice were co-immunostained for BrdU
975 and Tbr1 (A) or Ctip2 (B). BrdU was injected at E11.5, E14.5 or E15.5. **C:** Cortical sections of
976 P2 control and NCAM cKO mice were co-immunostained for BrdU and Cux1. BrdU was
977 injected at E16.5. Scale bars: 50 μm . **D-G:** Percentages of BrdU⁺Tbr1⁺, BrdU⁺Ctip2⁺ or
978 BrdU⁺Cux1⁺ cells in total populations of Tbr1⁺, Ctip2⁺, or Cux1⁺ cells after BrdU
979 administration at E11.5 (D), E14.5 (E), E14.5 (F) or E16.5 (G). n=15 brain slices from 3 mice.
980 Values represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ (two-sided). Student's t test or Mann-
981 Whitney test (E, F (BrdU⁺Ctip2⁺ cells)).

982

983 **Figure 5. NCAM deficiency results in precocious gliogenesis.**

984 **A:** Coronal sections of the VZ were immunostained for GFAP with DAPI counterstaining. **B:**
985 Densities of GFAP⁺ cells in the dorsolateral VZ. **C:** Total intensity of GFAP labelling per E16
986 VZ/SVZ. **D:** Coronal sections of the dorsolateral VZ of P2 control and NCAM cKO mice were
987 co-immunostained for BrdU and GFAP. BrdU was injected at E16.5. **E:** Numbers of
988 BrdU⁺GFAP⁺ per $2.0 \times 10^4 \mu\text{m}^2$ in the dorsolateral VZ. **F:** Coronal cortical sections of E18 and
989 P0 control and NCAM cKO mice were immunostained for Olig2 with DAPI counterstaining.
990 **G:** Numbers of Olig2⁺ cells per $2.5 \times 10^4 \mu\text{m}^2$. **H:** Percentages of BrdU⁺Olig2⁺ cells in the total

991 Olig2⁺ cell population. **I:** Coronal sections of the dorsal VZ of P2 control and NCAM cKO
992 mice were co-immunostained for BrdU and Olig2. BrdU was injected at E16.5. **J:** Cortical
993 sections of E16 control and NCAM cKO mice were immunostained for A2B5 with DAPI
994 counterstaining. **K:** Densities of A2B5⁺ cells. **L:** Cortical sections of E14 control and NCAM
995 cKO mice were immunostained for BLBP⁺ with DAPI counterstaining. **M:** Densities of BLBP⁺
996 cells. Scale bars: 50 μm. n=15 brain slices from 3 mice. Values represent mean±SEM. **P*<0.05,
997 ***P*<0.01, ****P*<0.001 (two-sided). Student's t test or Mann-Whitney test (C, H).

998

999 **Figure 6. Profilin2 is a novel binding partner of NCAM.**

1000 **A:** Co-immunoprecipitation analysis of the interaction between NCAM and profilin2 using P0
1001 mouse brain homogenates. **B:** ELISA analysis of the binding of NCAM140ICD or
1002 NCAM180ICD to immobilized profilin2. **C-E:** ELISA analysis of the binding of biotinylated
1003 NCAM140ICD-derived peptides (C), wildtype NCAM140 (aa745-753) peptide and its mutant
1004 variants with ⁷⁴⁹LC⁷⁵⁰ mutated to ⁷⁴⁹AS⁷⁵⁰, or ⁷⁴⁸NL⁷⁴⁹ mutated to ⁷⁴⁸GA⁷⁴⁹ (D), wildtype
1005 NCAM140ICD or mutNCAM140ICD (⁷⁴⁹LC⁷⁵⁰ to ⁷⁴⁹AS⁷⁵⁰ mutation, E), to immobilized
1006 profilin2. n=3 biological replicates. **F:** Schematic diagram of amino acid mutations in
1007 mutNCAM140ICD. **G, H:** Coronal sections of the VZ (G) and the cortex (H) of control mice
1008 were co-immunostained for profilin2, NCAM and Sox2 (G) or Tuj1 (H). Scale bars: 50 μm. **I:**
1009 Average profilin2 immunofluorescence density in each layer. **J:** Percentages of profilin2
1010 immunoreactivity in each layer. n=9 brain slices from 3 mice. **K, L:** Western blot analysis of
1011 levels of NCAM and profilin2 in cultured NPCs derived from E14 control and NCAM cKO
1012 VZ/SVZ (K). The relative levels of profilin2 protein in NCAM cKO NPCs, with the profilin2
1013 levels in control NPCs set to 100% (L), n=4 biological replicates. **M:** qPCR analysis of the
1014 levels of profilin2 mRNA in cultured NPCs derived from E14 control and NCAM cKO brains.
1015 Profilin2 mRNA levels in control NPCs were set to 100%, n=5 biological replicates. Values

1016 represent mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 (two-sided). Two-way ANOVA (B-E);
1017 One-way ANOVA with Bonferroni corrections (J (IZ, CP and MZ), Dunnett's T3 correction (J
1018 (VZ/SVZ)); Kruskal-Wallis test with Dunn-Bonferroni corrections (I); paired t test (L, M).
1019 VZ/SVZ, ventricular zone/subventricular zone. PP, preplate. IZ, intermediate zone. CP, cortical
1020 plate. MZ, marginal zone.

1021

1022 **Figure 7. NCAM enhances NPC proliferation and differentiation through profilin2.**

1023 **A:** Cultured NPCs transfected with siProfilin2 or scrambled siRNA (NC) were incubated with
1024 NCAM antibodies and BrdU. Cells were immunostained for BrdU with DAPI counterstaining.
1025 **B, E, F:** Cultured NPCs transfected with siProfilin2 or NC were incubated with NCAM
1026 antibodies or PBS and cultured in differentiation condition for 5 days. Cells were
1027 immunostained for Tuj1 (B), GFAP (E) or O4 (F), and counterstained with DAPI. **C, D, G, H:**
1028 Percentages of BrdU⁺DAPI⁺ (C), Tuj1⁺DAPI⁺ (D), GFAP⁺DAPI⁺ (G) and O4⁺DAPI⁺ (H) cells
1029 in the total population of DAPI⁺ cells. **I-K:** Cultured NPCs cotransfected with profilin2 shRNA
1030 (shProfilin2) and shProfilin2-resistant plasmids (Res Profilin2), shProfilin2 or control vector
1031 expressing GFP alone (GFP) were incubated with NCAM antibodies or PBS and allowed to
1032 differentiate for 3 days. Cells were immunostained for Tuj1 or GFAP. Percentages of Tuj1⁺GFP⁺
1033 (J), GFAP⁺GFP⁺ cells (K) in the total population of GFP⁺ cells. n=15 microscopic fields from
1034 3 biological replicates. Scale bars: 50 μ m (A, F, I) or 20 μ m (B, E). Values represent
1035 mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 (two-sided); ns: not statistically significant.
1036 Kruskal-Wallis test with Dunn-Bonferroni post hoc correction (C); One-way ANOVA with
1037 Bonferroni corrections (D, G, J, K), or Dunnett's T3 correction (H).

1038

1039 **Figure 8. NCAM enhances NPC proliferation and differentiation through profilin2-**
1040 **regulated actin dynamics.**

1041 **A:** Western blot analysis of F- and G-actin levels in cultured control and NCAM cKO NPCs. γ -
1042 tubulin served as a control and was enriched in the F-actin fraction containing polymerized
1043 tubulin. **B:** Relative levels of G- and F-actin in NCAM cKO NPCs. The levels of G- and F-actin
1044 in control NPCs were set to 100%. n=4 biological replicates. **C:** Cultured MEFs were
1045 cotransfected with NCAM siRNA (siNCAM) or scrambled siRNA (NC), and with lentiviruses
1046 co-expressing GFP and wild type NCAM140 (NCAM) or mutant NCAM140 (mutNCAM).
1047 MEFs cotransfected with NC and lentiviruses expressing GFP only served as control. Western
1048 blot analysis of levels of NCAM, actin and tubulin. Lysis with the F-actin stabilization buffer
1049 solubilizes and releases NCAM to the G-actin fraction. Relative levels of NCAM protein in the
1050 G-actin fraction and the relative ratio of G- and F-actin were quantified. n=3 biological
1051 replicates. **D:** Cultured NCAM cKO NPCs were transduced with lentiviruses co-expressing
1052 GFP and NCAM or mutNCAM. NPCs transduced with lentiviruses expressing GFP only served
1053 as control. NPCs were stained by fluorescent Phalloidin to visualize F-actin, and DNase I to
1054 visualize G-actin. **E, F:** F-actin/G-actin ratios in cells are shown in D and G, respectively. n=54
1055 cells (E), 21 cells (F) from 3 biological replicates. **G:** Cultured NCAM cKO NPCs were
1056 transduced with plasmids co-encoding GFP, or profilin2 and GFP, stained with fluorescent
1057 Phalloidin and DNase I. **H:** Coronal VZ sections of E12 control and NCAM cKO mice were
1058 immunostained for actin with DAPI counterstaining. White dotted lines show examples of cell
1059 boundaries. **I:** The CSI for dividing cells in the VZ. n=40 mitotic cells from 3 mice. **J, K:**
1060 Cultured NCAM cKO NPCs were transduced with lentiviruses co-expressing GFP and NCAM
1061 or mutNCAM, incubated with BrdU, and immunostained for BrdU with DAPI counterstaining
1062 (J). Cultured NPCs differentiated for 5-7 days were immunostained for Tuj1, GFAP with DAPI
1063 counterstaining (K). **L-N:** Percentages of BrdU⁺GFP⁺ (L), Tuj1⁺GFP⁺ (M) and GFAP⁺GFP⁺ (N)
1064 cells in total GFP⁺ cell population. n=32 microscope fields from 3 biological replicates (L). n=5
1065 biological replicates (M and N). Scale bars: 20 μ m (D, G, J, K) or 5 μ m (H). Values represent

1066 mean±SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (two-sided); ns: not statistically significant.
1067 Paired t test (B); Mann-Whitney test (I); one-way ANOVA with Dunnett's T3 correction (C) or
1068 Bonferroni corrections (M, N). Kruskal-Wallis test with Dunn-Bonferroni post hoc
1069 comparisons (E, F, and L).

1070

1071 **Figure 9. The role of NCAM in regulating the temporal generation of neurons and glia in**
1072 **the developing cortex.**

1073 **A:** NCAM expression is high in NPCs at the neurogenic period and declines at the gliogenic
1074 period. The intracellular domain of NCAM interacts with profilin2 and promotes actin
1075 polymerization in NPCs. NCAM-dependent actin regulation is required for rounding of NPCs
1076 during mitosis as well as control of NPC proliferation and temporal differentiation into cortical
1077 neurons and glia. **B:** Ablation of NCAM expression in NPCs results in reduced expression of
1078 profilin2 and loss of its NCAM-dependent regulation, leading to decreased actin polymerization
1079 and reduced rounding of mitotic NPCs. This slows down cell cycle progression, reduces NPC
1080 proliferation at early stage of neural development, delays production of cortical neurons, and
1081 leads to precocious formation of cortical glia.

1082

1083 **Supplementary Information**

1084

1085 **Supplementary figure legends**

1086

1087 **Figure S1. Expression of NCAM and profilin2 in the developing cerebral cortex.**

1088 **A-G:** Western blot analysis of NCAM and profilin2 expression in E12, E14, E16, E18, and P0
1089 mouse cortices. γ -tubulin served as a control. The protein levels in E14, E16, E18, and P0 mouse
1090 cortices were quantified relative to the protein levels in E12 mouse cortices set to 1.0. n=3 or 4
1091 biological replicates (total NCAM and profilin2, respectively). **H:** Coronal sections of control
1092 and NCAM cKO mouse cortices were co-immunostained for NCAM and Sox2 at E12 and E14.
1093 Scale bars: 20 μ m. Values represent mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 (two-sided).
1094 One-way ANOVA with LSD corrections (C, F), with Dunnett's T3 correction (B, D, and E) or
1095 Kruskal-Wallis test with Dunn-Bonferroni post hoc comparisons (G).

1096

1097 **Figure S2. NCAM deficiency does not lead to increased NPC apoptosis during**
1098 **embryonic development.**

1099 **A:** Coronal sections of E12, E14 and E16 control and NCAM cKO cortices were
1100 immunostained for activated, cleaved caspase3 and counterstained with DAPI. **B:** Numbers of
1101 caspase3⁺ cells in the entire hemi-telencephalon cortex. Mean \pm SEM values (n=15 brain slices
1102 from 3 mice). Mann-Whitney test did not reveal statistically significant differences between
1103 groups. Scale bars: 50 μ m. VZ/SVZ, ventricular zone/subventricular zone.

1104

1105 **Figure S3. NCAM deficiency does not affect the distribution of neonatal cortical neurons**
1106 **in the coronal plane.**

1107 **A:** The cortical neuron distribution was analyzed by the maximum migration distance of deep-

1108 (red arrow), or upper- (blue arrow) layer neurons from ventricular zone to cortical surface/total
1109 cortical length (purple arrow). **B-D**: Percentages of the maximum migration distance of Tbr1⁺
1110 (B), Ctip2⁺ (C) and Cux1⁺ (D) neurons in total cortical length. Mean±SEM values (n=15 brain
1111 slices from 3 mice). Student's t test or Mann-Whitney test (B (E12, E14) and D (E18)).

1112

1113 **Figure S4. Profilin2 expression is downregulated specifically by profilin2 RNA**
1114 **interference.**

1115 **A**: Western blot analysis of profilin2 levels in Neuro-2a cells transfected with either profilin2
1116 siRNA (siProfilin2) or scrambled siRNA (NC). **B**: Levels of profilin2 in siProfilin2-transfected
1117 cells relative to those in NC-transfected cells which were set to 1.0. **C, D**: qPCR analysis of the
1118 levels of profilin2 (C) or profilin1 (D) mRNA in cultured NPCs transfected with either profilin2
1119 siRNA (399, 527) or NC. The mRNA levels of profilin2/1 in NC-transfected NPCs were set to
1120 1.0. **E, F**: Western blot analysis of profilin2 levels in Neuro-2a cells transfected with scrambled
1121 shRNA (GFP), profilin2 shRNA (shProfilin2) only, or cotransfected with shProfilin2 and
1122 shRNA-resistant profilin2 (Res Profilin2). The levels of profilin2 protein were quantified
1123 relative to those in GFP-transfected cells set to 1.0. **G**: NCAM levels in brain homogenates
1124 loaded in different quantities (26, 53 78, and 104 µg). Values represent mean±SEM. n=4
1125 biological replicates. **P*<0.05, ***P*<0.01 (two-sided); ns: not statistically significant. Paired t
1126 test (B); one-way ANOVA with Dunnett's T3 correction (C, D) or LSD corrections (F).

1127

1128 **Figure S5. Schematic diagram showing areas chosen for quantification of cells in**
1129 **imaging analysis.**

1130 **A**: Red rectangle indicates the 100 µm x 250 µm area of interest in the DP perpendicular to the
1131 VZ. Blue rectangle indicates the 100 µm x 250 µm areas of interest in the DP. Purple square
1132 indicates the 150 µm x 150 µm areas of interest in DP adjacent to VZ. CM, cortical hem, MP,

1133 medial pallium, DP, dorsal pallium, LP, lateral pallium, and LV, lateral ventricle (see materials
1134 and methods for details). **B**: Average immunofluorescence density of profilin2 in each cortical
1135 layer. n=9 brain slices from 3 mice. Values represent mean±SEM. * $P < 0.05$ (two-sided).
1136 Kruskal-Wallis test with Dunn-Bonferroni post hoc test (CP); one-way ANOVA with
1137 Bonferroni corrections (IZ and MZ). IZ, intermediate zone; CP, cortical plate; MZ, marginal
1138 zone.