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**Sox14 is essential for initiation of interneuron differentiation
in the chick spinal cord**

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21 **Abstract**

22 The neural tube comprises several different types of progenitors and postmitotic neurons that
23 co-ordinately act with each other to play integrated functions. Its development consists of two phases:
24 proliferation of progenitor cells and differentiation into postmitotic neurons. How progenitor cells
25 differentiate into each corresponding neuron is an important question for understanding the mechanisms
26 of neuronal development.

27 Here we introduce one of the Sox transcription factors, Sox14, which plays an essential role in
28 the promotion of neuronal differentiation. Sox14 belongs to the SoxB subclass and its expression starts
29 in the progenitor regions before neuronal differentiation is initiated at the trunk level of the neural tube.
30 After neuronal differentiation is initiated, Sox14 expression gradually becomes confined to the V2a
31 region of the neural tube, where *Chx10* is co-expressed. Overexpression of Sox14 restricts progenitor
32 cell proliferation. Conversely, the blockade of Sox14 expression by the *RNAi* strategy inhibits V2a
33 neuron differentiation and causes expansion of the progenitor domain. We further found that Sox14
34 acted as a transcriptional activator. Taken together, Sox14 acts as a modulator of cell proliferation and
35 an initiator protein for neuronal differentiation in the intermediate region of the neural tube.

36

37 **Abbreviations**

38 HH stage: Hamburger and Hamilton's stage

39 pMN: motor neuron progenitor region

40 FP: floor plate

41 hpt: hours post transfection

42 *sh-Sox14*: *short-hairpin RNA* targeting *Sox14*

43 IdU: 5-Iodo-2'-deoxyuridine

44 Shh: Sonic Hedgehog

45 RT-qPCR: reverse transcription and quantitative polymerase chain reaction

46 TUNEL: TdT-mediated dUTP nick-end labelling

47 **Introduction**

48 The neural tube is the embryonic organ of the central nervous system, and several distinct types
49 of progenitor cells and neurons are arrayed along the dorsal-ventral axis in an orderly manner [1]. The
50 cells of each progenitor domain differentiate into their corresponding neurons, and the combination of
51 different types of neurons exerts integrated functions as a whole [2].

52 Neural tube development is largely divided into two phases: the assignment and proliferation
53 of neural progenitor cells and their differentiation into postmitotic functional neurons [3, 4].

54 During the first phase, at the trunk level of the neural tube, a variety of neural progenitor cells
55 are patterned depending on the positional information provided by the signal molecules, collectively
56 called morphogens. In the ventral part of the neural tube, the assignment of the neural domains is mainly
57 governed by sonic hedgehog (Shh), which is expressed in the notochord and later in the floor plate (FP)
58 cells. The ventral neural tube is consequently divided into six regions: p0-p2, motor neuron progenitor
59 region (pMN), p3, and the FP, along the intermediate region to the ventral, whose assignment is
60 dependent on the concentration of Shh. The cells proliferate to specific numbers in a region-specific
61 manner [5, 6].

62 During the second phase of neural tube development, progenitor cells differentiate into
63 postmitotic neurons. This process involves proneural genes, a group of basic helix-loop-helix (bHLH)
64 transcription factors, including neurogenins, Atoh (atonal homolog), and NeuroDs, whose expression
65 is regulated by the Notch/Delta and retinoic acid signals [7, 8]. Combination of the transcription factors
66 of proneural genes, including position-specific transcription factors, promote differentiation [9, 10].
67 Consequently the transcription factors that characterise each neuronal region start to be expressed and
68 each region is established. For instance, the homeodomain transcription factor Chx10/Vsx2 is expressed
69 and is required for the V2a interneuron. In the absence of Chx10, progenitor cells acquire different
70 neuronal identities of the motor neuron (MN), which is located in the adjacent domain of the neural
71 tube [11, 12]. As such, there are many transcription factors that regulate neuronal differentiation and/or
72 acquisition of specific functions. Important questions are how each transcription factor functions and
73 how the gene regulatory networks are formed among them.

74 Among the transcription factors expressed in specific neuronal regions, we focused on one of
75 the Sry-type transcription factors, Sox14. Sox14 belongs to the SoxB subclass, encompassing Sox1-3,
76 Sox14, and Sox21 [13, 14]. In previous studies, the expression of Sox14 has been shown in the V2a
77 region, accompanied by that of Chx10, Lhx3, and Sox21 in the developing spinal cord area, and this
78 profile is supported by single-cell transcriptomic analysis [15, 16]. Sox14 is also expressed in a part of
79 the diencephalon, called the subcortical visual shell, and is required for normal circadian rhythm [17].
80 In humans, mutations in the *Sox14* gene cause congenital disorders, including deformation of the eyelids
81 and severe mental retardation [18, 19]. However, the functions of Sox14 in the spinal cord are not fully
82 understood.

83 In this study, we used chick embryos to explore the expression and function of Sox14 in neural
84 tube development. Analyses revealed that Sox14 is expressed not only during neuronal differentiation
85 stages, but also in the earlier stages, and it regulates cell cycle progression and neuronal differentiation.
86 Together, we suggest that various transcription factors share functions during differentiation into
87 specific neurons of the V2a region.

88 **Results**

89 **Sox14 expression begins before neuronal differentiation starts, and its expression is gradually** 90 **confined to the intermediate neuronal domains**

91 During analysis of genes that induce domain-specific neuronal differentiation [1], we focused
92 on the Sry-type transcription factor Sox14. Sox14 is reportedly expressed in the V2a ventral neuronal
93 domain [11, 20], and acts downstream of the homeodomain transcription factor Vsx2/Chx10 [11].

94 As most transcription factor expression starts after proneural gene expression is initiated, we
95 attempted to identify the onset of Sox14 expression. We performed *in situ* hybridisation analysis of
96 sections of the neural tube at various embryo stages. As a result, we found that Sox14 is already
97 expressed in the neural tube at HH stage 14 (Fig. 1a) before neuronal differentiation starts. Moreover,
98 higher expression was observed in the intermediate region of the neural tube. Once neuronal
99 differentiation started, Sox14 expression became confined to the intermediate regions (Fig. 1b), and
100 was found in the V2 region at HH stage 22 (Fig. 1c).

101 Next, we investigated when *Chx10* expression began. *Chx10* expression was not observed
102 before the initiation of neuronal differentiation (Fig. 1d). The initial expression was found at the V2a
103 region after neuronalisation started (Fig. 1e), and a stronger expression was found at HH stage 22 (Fig.
104 1f). These findings suggest that *Sox14* has upstream factors other than Chx10 for its initial expression
105 [11].

106 As *Sox14* encodes a member of the SoxB subfamily, the *Sox14* RNA probe for *in situ*
107 hybridisation might have been crossed with other SoxB family members. To examine this possibility
108 and to quantitatively evaluate the expression, we conducted an intermediate neural explant assay using
109 the intermediate region of the neural plate. The explants were treated with different concentrations of
110 Shh, and gene expression was evaluated using RT-qPCR every 12 h. As a result, *Sox14* was already
111 expressed at 12 h in the presence of a low concentration of Shh (Shh^L; see Methods for the definition),
112 and the expression increased continuously (Fig. 1g). In contrast, in the presence of a high concentration
113 of Shh (Shh^H), *Sox14* expression was not induced (Fig. 1g), supporting the finding that *Sox14* is
114 expressed in the intermediate region of the neural tube, as obtained by *in situ* hybridisation (Fig. 1b).

115 The same concentration of Shh was used to induce *Chx10*; however, expression began 24 h
116 after the start of culture, suggesting that the onset of *Chx10* expression was later than that of *Sox14* (Fig.
117 1h). Therefore, *Sox14* and *Chx10* are expressed in the same lineage of cells; however, *Sox14* expression
118 precedes that of *Chx10*.

119 Taken together, the collective expression profiling analyses suggest that the two transcription
120 factors, Sox14 and Chx10, have distinct regulation of their expression.

121

122 **Sox14 inhibits neural progenitor cells and promotes transition from the progenitor to the** 123 **postmitotic states**

124 Next, we attempted to determine the activity of Sox14 in neural tube development. For this
125 purpose, we performed a forced expression of the control vector or the expression plasmid conveying
126 the *Sox14* gene by electroporation in the neural tube, and analysed its effects at 48 h post-transfection
127 (hpt).

128 Results indicated that expression of Sox2, a neural progenitor marker, was reduced in the
129 electroporated cells, while no change was found by electroporation of the control vector (Fig. 2a-b';
130 n=5 for control and n=8 for *Sox14*). We reasoned that the cells might have precociously differentiated
131 into neurons; however, Sox14 overexpression also reduced p27^{KIP1} expression, suggesting that neuronal
132 maturation was also perturbed by *Sox14* overexpression (Fig. 2c-d'). Therefore, we hypothesised that
133 the initial stage of the neurons was induced by Sox14, and investigated the expression of p57^{KIP2}, which
134 appears immediately after neuronal birth [21, 22], using *in situ* hybridisation. As a result, we found the
135 expressing area expanded into the progenitor regions, as well as the upregulated expression level (Fig.
136 2e-f'; n=5 for control and n=6/8 for *Sox14*).

137 To quantify the induction of genes involved in neurogenesis [9, 10], we analysed the expression
138 of genes involved in neurogenesis using RT-qPCR in explants electroporated with *Sox14*. We observed
139 selective expression of p57^{KIP2} through the overexpression of Sox14, whereas other proneural genes,
140 including *Ngn1*, *Ngn2*, and *NeuroD4*, remained unchanged in their expression (Fig. 2g).

141 Taken together, Sox14 induces p57^{KIP2}-expressing early-stage neurons.

142

143 **Sox14 restricts proliferation of neural progenitor cells**

144 We found that the cell number tended to be reduced by Sox14 overexpression (Fig.
145 2b,b',d,d',f,f'), and supposed that this may be due to the blockade of cell proliferation or programmed
146 cell death. To test these possibilities, we performed a 5-Iodo-2'-deoxyuridine (IdU) incorporation assay
147 to label S-phase cells at 24 hpt of control or *Sox14*. As a result, the number of cells in the S-phase was
148 greatly reduced in the Sox14-electroporated side (Fig. 3a-c; n=5 for control, n=6 for *Sox14*). In addition,
149 immunofluorescence with phospho-histone 3 (pHH3) to identify the M-phase cells revealed a
150 dramatically reduced number of pHH3-positive cells in the electroporated side (Fig. 3d-f; n=5 for
151 control, n=6 for *Sox14*).

152 Next, we investigated whether programmed cell death occurs in the *Sox14*-electroporated cells
153 and performed a TdT-mediated dUTP nick-end labelling (TUNEL) assay, which detects fragmented
154 genomic DNA. However, we did not find a significant increase in the positive signals in the *Sox14*-
155 electroporated side (Fig. 3g-h'; j; n=5 for control, n=6 for *Sox14*), while *PtchΔ*-overexpressing neural
156 cells showed positive signals (Fig. 3i,i'; j; n=7) [23, 24], suggesting that the experiment *per se* was
157 successful.

158 These findings indicate that cell cycle progression was blocked by Sox14.

159

160 **Sox14 is required for neuronal differentiation**

161 To reveal the essential roles of Sox14 in neural tube development, we attempted to disrupt
162 *Sox14* expression using an RNA interference (RNAi) strategy [25]. We designed the *sh-RNA* construct
163 targeting chick Sox14 (*sh-Sox14*) and electroporated either the control vector (*sh-control*) or the
164 plasmid conveying *sh-Sox14* into the neural tube. At 48 hpt, inhibition of *Sox14* expression by *sh-Sox14*
165 was confirmed, while *sh-control* electroporation maintained expression (Fig. 4a-b'; none
166 downregulated in control (n=5), 100% downregulated in *sh-Sox14* (n=6)). We further found that *Chx10*
167 expression was blocked by *sh-Sox14* (Fig. 4c-d'; none downregulated in control (n=5), 100%
168 downregulated in *sh-Sox14* (n=6)), suggesting that Sox14 is required for *Chx10* expression.

169 In contrast, the motor neuron domain, characterised by *Islet1*, was not affected by *sh-Sox14*
170 (Fig. 4e-f'; n=5 for control, n=6 for *sh-Sox14*), and the Sox2-expressing neural progenitor domain was
171 laterally expanded (Fig. 4g-h'; no ectopic expression in control (n=5), 83% displayed ectopic expression
172 in *sh-Sox14* (n=6)), suggesting that *Sox14* knockdown inhibited neuronal differentiation.

173 Therefore, Sox14 is required for the progression of the neuronal differentiation, particularly for
174 the V2a identity.

175

176 **Sox14 acts as a transcriptional activator**

177 Based on the amino acid sequences, SoxB transcription factors have been categorised into two
178 subgroups, SoxB1 and SoxB2, where SoxB1 includes Sox1-3, while SoxB2 is composed of Sox14 and
179 Sox21 [13]. While Sox1-3 are transcriptional activators [26], Sox14 and Sox21 are thought to act as
180 transcriptional repressors, as judged from their amino acid sequences [13]. Therefore, we attempted to
181 reveal the mode of action of Sox14 and generated chimeric constructs of the DNA-binding region of
182 Sox14 fused either with the transactivating domain of VP16 of the herpes simplex virus (Sox14_{DBD}-
183 VP16) or the *Drosophila Engrailed* repressor domain (Sox14_{DBD}-EnR) so that each construct mimics
184 transcriptional activation or repression (Fig. 5a). By the electroporation of *SOX14_{DBD}-VP16*, the
185 electroporated cells abolished the expression of the neural progenitor Sox2 (Fig. 5b,b'; n=10), and
186 *p57^{KIP2}* was found to be expanded (Fig. 5c,c'; n=10). Moreover, injection of IdU into the *SOX14_{DBD}-*
187 *VP16*-electroporated embryos revealed that the number of S-phase cells was reduced in the
188 electroporated cells (Fig. 5d,d'; n=7), suggesting that Sox14_{DBD}-VP16 mimics the activity of the wild-
189 type Sox14 (Fig. 3b,c).

190 In contrast, the electroporation of *SOX14_{DBD}-EnR*, a mimetic construct for transcriptional
191 repressor activity, induced the ectopic expression of Sox2 (Fig. 5e,e'; n=12) at the expense of *p57^{KIP2}*
192 (Fig. 5f,f'; n=12), suggesting that Sox14_{DBD}-EnR exerts opposing functions compared to wild-type
193 Sox14. In addition, the IdU injection did not change the rate of incorporation as in the control (Fig.
194 5g,g'; n=8), which differs from the phenotype observed in wild-type Sox14 (Fig. 3b,c).

195 Taken together, these results suggest that (i) Sox14 acts as a transcriptional activator and the
196 EnR-fused construct acts in an antimorphic manner, and (ii) Sox14 is essential for promoting the
197 differentiation of neural progenitor cells.

198 **Discussion**

199 **Sox14 expression begins earlier than initiation of neuronal differentiation.**

200 In this study, we analysed the function of one of the Sox transcription factors, Sox14, and
201 demonstrated that Sox14 is required for the progression of neuronal differentiation (Fig. 4). In addition,
202 we demonstrated that Sox14 acts as a transcriptional activator, as the VP16-chimeric construct mimics
203 wild-type Sox14 (Fig. 5).

204 Sox14 was initially identified as a SoxB subclass gene, which encompasses Sox1-3, Sox14,
205 and Sox21 [13]. A subsequent study demonstrated that Sox14 expression was found in the V2a neuronal
206 region, and its expression was overlapped, at least in part, with that of Chx10 and Lhx3/Lim3 [20].
207 Moreover, a recent study showed that electroporation of Chx10 induces ectopic expression of Sox14,
208 indicating that Chx10 is a sufficient upstream factor for *Sox14* [11]. In contrast, our analysis revealed
209 that Sox14 expression onset occurred earlier than that of *Chx10* (Fig. 1), suggesting that Chx10 is not
210 a single upstream regulator for the initiation of *Sox14* expression. One possible upstream factor is
211 retinoic acid (RA), as Sox14 expression is upregulated by RA during neural differentiation in embryonic
212 carcinoma cells [27]. In addition, RA has been shown to play an important role in the intermediate
213 region of neural progenitor cells [28], and Pax6 expression was upregulated by RA, as indicated by RT-
214 qPCR analysis of neural explants. However, Sox14 expression remained unchanged after RA treatment
215 (Supplementary Fig. S1), suggesting that Pax6 and Sox14 expression is initiated by different
216 mechanisms.

217 In addition, it is notable that there are multiple binding sites for Nkx2.2 and Olig2 in the
218 regulatory region of *Sox14* [29]. Considering that Nkx2.2 and Olig2 are induced by Shh [30] and are
219 transcriptional repressors [31], Sox14 may be expressed in neural progenitor cells as a default, and its
220 expression is restricted by Nkx2.2 and Olig2.

221

222 **Sox14 is required for the progression of neuronal differentiation of intermediate neurons.**

223 Sox14 and Chx10 are expressed in the same region of the V2a intermediate neuronal area.
224 *Chx10* is induced by Lhx3 and consolidates V2a identity by repressing the genes that characterise the
225 adjacent neuronal regions of non-V2a interneurons or MN [11]. Attenuation of the *Chx10* gene causes
226 aberrant upregulation of the *MN* gene, suggesting that Chx10 determines the direction of neuronal
227 identity [11].

228 In contrast, our analyses revealed that Sox14 is required for the promotion of neuronal
229 differentiation. Knockdown of the *Sox14* gene caused the abolishment of neuronal differentiation, rather
230 than producing different types of neurons (Fig. 4). Therefore, it can be said that Chx10 and Sox14 play
231 different functions during neuronal differentiation, Sox14 promotes differentiation, and Chx10
232 determines the direction.

233 Previous studies have shown that Sox14 plays multiple roles in neural and neuronal
234 development. For instance, Sox14 is required for terminal differentiation of dorsal midbrain
235 GABAergic neurons [32]. In addition, Sox14 activates the p53 pathway to induce cell death in
236 carcinoma cells [17]. While the context in which we analysed the Sox14 function is different from these
237 previous reports, the activities of Sox14 revealed from our analyses are consistent with these previous
238 results; Sox14 is required for the promotion of neuronal differentiation.

239 In our analyses, Sox14 acted as a transcriptional activator (Fig. 5). We recognise that Sox14
240 has been suggested as a transcriptional repressor, which is controversial for its mode of action. The
241 repressor assumption was based on its amino acid sequence [13], and we predicted that the mode of
242 action would change in a context-dependent manner.

243 In this study we demonstrated that Sox14 induces the *p57^{KIP2}* expression; however, whether this
244 induction is direct is still elusive. Thus future studies will focus on searching for the direct target genes
245 of Sox14 using chromatin immunoprecipitation. Moreover, it is highly possible that the target genes of
246 Sox14 in neural progenitor cells and those in V2a neurons are distinct. Therefore, in addition to
247 searching for different target genes depending on the neural differentiation steps, it would be useful to
248 identify cofactors that bind to Sox14. These cofactors would include the general proneural genes, such
249 as Neurogenin1/2 or NeuroD2/4/6, and would modulate the functions of Sox14.

250 Understanding the function of each transcription factor is useful for generating specific
251 functional neurons from stem cells [33]. We envision that the findings of this study will partly contribute
252 to regenerative medicine in the future.

253 **Methods**

254 **Animal experiments**

255 All animal experiments were performed under the approval of national and internal legislation; approval
256 number 1636 was obtained from the review panel of the animal experiments of the Nara Institute of
257 Science and Technology.

258

259 **Manipulations and histological analyses on chick embryos**

260 Chicken eggs were purchased from Yamagishi Farm (Wakayama prefecture, Japan). The
261 development of chick embryos was evaluated using the Hamburger and Hamilton criteria [34]. Embryos
262 were electroporated at HH stage 12 with the electroporator BTX830 and were incubated at 38 °C for
263 the indicated periods.

264 For the overexpression analyses, the coding regions of the genes of interest were subcloned into
265 the *pCIG* expression vector [35], which contains the chicken β -actin promoter and the *GFP* gene
266 downstream of the internal ribosomal entry site. *Drosophila* EnR [36] and herpes-simplex virus-derived
267 VP16 transactivation domain [37] chimeric constructs were generated by fusing the amino-acids 1-90
268 of chicken Sox14.

269 To construct *sh-Sox14*, the sequence AAGCCTCCGGTTGCCTACATATTAT was subcloned
270 into the vector *pRFPRNAi* [25], in which the electroporated cells were identified by RFP expression.

271 For tissue analyses, the harvested embryos were fixed with 4% paraformaldehyde for 90 min,
272 and were replaced with 15% (w/v) sucrose overnight with rotating incubation. These embryos were
273 embedded in OCT compound (Sakura) and cryosectioned to a thickness of 12 μ m using a Polar cryostat
274 (Sakura Finetek). The following antibodies were used in this study: Sox2 (rabbit; Millipore, #5603),
275 p27 (mouse; BD Transduction Laboratories, #610241), Olig2 (rabbit; Millipore; #9610), Nkx2.2
276 (mouse; DSHB; #74.5A5), Islet1 (mouse; DSHB; #39.4D5), Pax7 (mouse; DSHB), and GFP (sheep;
277 AbD Serotec; #4745-1051). The secondary antibodies used were rabbit (Jackson Laboratories; #711-
278 166-152 for Cy3; #711-606-152 for Cy5), mouse (#715-166-151 for Cy3; #715-606-150 for Cy5), and
279 sheep (#713-096-147 for FITC).

280 *In situ* hybridisation and TUNEL assays were performed as previously described [6, 38, 39].
281 For *in situ* hybridisation analyses, the expression of genes or *si-RNAs* was validated by the tracer
282 (GFP/RFP) expression on the adjacent sections. As a positive control for the TUNEL assay, *pCIG-*
283 *Ptch Δ* , encoding an insensitive mutant to the Hedgehog signal [23], was electroporated, as the blockade
284 of the Hedgehog signal has been shown to induce programmed cell death [24].

285 For IdU incorporation assays, 100 mM IdU (Wako, Japan) was injected into the neural tube
286 cavity at 22 hpt and incubated for 2 h. To identify the incorporated cells, the sectioned tissues were pre-
287 treated with 1M hydrochloric acid, and the IdU antibody (mouse; Thermo; MA5-24879) was used.

288

289 **Explants and RT-qPCR**

290 The intermediate neural explants were prepared as described previously [38, 39]. Briefly, HH
291 stage 9 embryos were placed in L-15 medium and the caudal stem zone was excised [40]. After
292 treatment with 10 µg/mL dispase II (Sigma), the intermediate region of the neural plate was further cut
293 out and embedded in the collagen gel (Sigma). The embedded cells were cultured with DMEM/F-12
294 supplemented with Mitoserum extender (BD Biosciences) and 1× penicillin/streptomycin/glutamine
295 mixture (Wako, Japan). A high concentration of Shh (Shh^H) was defined as the concentration at which
296 approximately 80 % of Nkx2.2-positive cells were found, whereas a low concentration of Shh (Shh^L)
297 resulted in approximately 60% of Olig2-positive cells at 24 h [41]. For RT-qPCR, RNA was extracted
298 using the Picopure RNA extraction kit (Thermo; #KIT0204) and analysed using a CFX qPCR machine
299 (Bio-Rad). Expression levels were normalised with that of *Glyceraldehyde-3-Phosphate*
300 *Dehydrogenase (GAPDH)*. The primer sequences used are shown in Supplementary Tab. S1.

301

302 **Image processing and statistics**

303 Images were captured using the LSM710 confocal microscope or AxioVision (Carl Zeiss) and were
304 processed using the software Photoshop (Adobe). Figures were formed using an Illustrator (Adobe).
305 Quantitative data are presented as means ± SEM, and differences were evaluated using the two-tailed
306 Student's *t* test. Statistical significance was set at $p < 0.05$, and p -values (*<0.05; **; $p < 0.01$, ***;
307 $p < 0.001$) are indicated in each graph.

308 **Declarations**

309 **Ethics approval and consent to participate**

310 This study does not include human participants, tissues or data.

311

312 **Consent for publication**

313 Not applicable.

314

315 **Competing interests**

316 The authors declare that they have no competing interests.

317

318 **Availability of data and material**

319 All relevant data are presented in the text and figures. The plasmids used in this study are available
320 from the corresponding author upon request.

321

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326

327 **Authors' contributions**

328 NS conceived the project; TK, NS, MS performed experiments and analysed the data; MK provided
329 essential materials; all authors joined the discussion; NS wrote the manuscript.

330

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333 guidance, and all other laboratory members for their support and discussions.

334 **Figure legends**

335 **Fig. 1 Sox14 expression begins before initiation of neuronal differentiation.** (a-f) Cross-sections of
336 the trunk level of the neural tube at HH stages 14 (a,d), 18 (b,e), and 24 (c,f) of chick embryos were
337 analysed either using *in situ* hybridisation with probes against *Sox14* (a-c) and *Chx10* (d-f). Expression
338 is indicated with a bracket (b) or with arrowheads (c,e,f). Scale bar in (a) = 100 μm for (a-f). (g,h)
339 Expression analysis of *Sox14* (g) and *Chx10* (h) using RT-qPCR on the explants cultured in the control
340 medium, or with Shh^{L} or Shh^{H} for various time periods.

341

342 **Fig. 2 Sox14 induces early neuronal differentiation during the neural tube development.** *pCIG*
343 (a,a',c,c',e,e') or *pCIG-Sox14* (b,b',d,d',f,f') were electroporated at HH stage 12 and embryos were
344 harvested at 48 hpt. Expression levels of *Sox2* (red; a-b') and $p27^{\text{KIP1}}$ (red; c-d') were analysed using
345 immunofluorescence, and the $p57^{\text{KIP2}}$ (e-f') was analysed using *in situ* hybridisation. Scale bar in (a) =
346 50 μm for (a-f'). (g) The effect of the electroporation of *pCIG* or *pCIG-Sox14* was analysed using RT-
347 qPCR at 36 hpt on the explants electroporated with *pCIG* (blue bars) or *pCIG-Sox14* (red bars). Note
348 that, in (e',f'), the GFP expression was taken from the adjacent sections.

349

350 **Fig. 3 Sox14 inhibits cell proliferation without inducing programmed cell death.** Either *pCIG*
351 (control; a,a',c,d,d',f,g,g',j) or *pCIG-Sox14* (b,b',c,e,e',f,h,h',j) were electroporated into HH stage 12
352 and embryos were subjected to the following assays. (a-c) IdU incorporation assay to detect the S-phase
353 cells. IdU was injected into the cavity of the neural tube at 22 hpt for 2 hours, and embryos were
354 harvested for immunofluorescence with the IdU antibody. (c) Quantification of the IdU-positive cells
355 in the total GFP-positive cells. (d-f) Immunofluorescence with the M-phase marker phospho-Histone 3
356 (pHH3). Embryos were harvested at 24 hpt and were subject to immunofluorescence with pHH3
357 antibody. (f) Quantification of the pHH3-positive cells over the GFP-positive cells on the apical side.
358 (g-j) TUNEL assay to detect programmed cell death. Embryos were harvested at 24 hpt as in (d-f) and
359 were analysed using a TUNEL assay. (i,i') For the positive control of the TUNEL assay, *pCIG-PtchA*
360 was electroporated and the same assay as in (g-h') was performed. (j) Quantification of the TUNEL-
361 positive cells in all GFP-positive cells. Scale bar in (a) = 50 μm for (a-b',d-e',g-i').

362

363 **Fig. 4 Sox14 is required for neuronal differentiation.** Embryos were electroporated either with
364 *pRNAi* (a,a',c,c',e,e',g,g') or *pRNAi-Sox14* (b,b',d,d',f,f',h,h') and were analysed at 48 hpt. *Sox14* (a-
365 b') and *Chx10* (c-d') expression levels were analysed using *in situ* hybridisation, and *Islet1* (e-f') and
366 *Sox2* (g-h') expression was analysed using immunofluorescence. Normal (a,b,c,d) or ectopic (h)
367 expression is indicated by filled arrowheads; reduced expression (b,d) by outlined arrowheads. Scale
368 bars in (a) for (a-d') and in (e) for (e-h') = 50 μm . Note that the RFP expression in (a',b',c',d') was
369 taken from the adjacent sections.

370

371 **Fig. 5 Sox14 acts as a transcriptional activator.** (a) Schematic of the Sox14_{DBD}-VP16 and Sox14_{DBD}-
372 EnR. DBD; DNA-binding domain of Sox14 (amino acid numbers 1-90 of chicken Sox14). (b-h) Either
373 *pCIG-Sox14_{DBD}-VP16* (b-d') or *pCIG-Sox14_{DBD}-EnR* (e-g') was electroporated into the neural tube and
374 the embryos were analysed using immunofluorescence with antibodies against Sox2 (b,b',e,e') and
375 p57^{KIP2} (c,c',f,f') at 48 hpt, or were subjected to an IdU incorporation assay at 24 hpt, as in Fig. 3a-c.
376 Ectopic (e,f) and reduced (b,c) expression is indicated by filled and outlined arrowheads, respectively.
377 Note that the GFP expression in (c',f') was taken from the adjacent sections. Scale bars in (b) for (b-
378 c',e-f') and in (d) for (d,d',g,g') = 50 μ m. (h) Quantification of the IdU-positive cells in all GFP-positive
379 cells. The ratio of the control condition is taken from Fig. 3c.

380

381 **Supplementary Figures**

382 **Supplementary Fig. S1 Sox14 expression is not upregulated by retinoic acid (RA).** Intermediate
383 neural explants were cultured either in the control medium or the medium with RA (100 nM) for 24
384 hours to be subjected for an RT-qPCR.

385

386 **Supplementary Table**

387 **Supplementary Tab. S1** The RT-qPCR primers used in this study.

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