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Lewis, Philip Alexander, Bradley, Isobel Claire, Pizzey, Alastair Robert et al. (2 more authors) (2017) N1-Src kinase is required for primary neurogenesis in *Xenopus tropicalis*. *Journal of neuroscience*. pp. 8477-8584. ISSN 1529-2401

<https://doi.org/10.1523/JNEUROSCI.3881-16.2017>

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N1-Src kinase is required for primary neurogenesis in *Xenopus tropicalis*

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Abbreviated title: N1-Src is essential for primary neurogenesis

No. of pages: 25

No. of words:

Abstract: 225 words

Introduction: 516 words

Discussion: 1276 words

Acknowledgements

PAL and ARP were funded by BBSRC PhD studentships (BB/F016751/1; BB/M011151/1). The authors declare no competing financial interests.

1 **Abstract**

2 The presence of the neuronal-specific N1-Src splice variant of the C-Src tyrosine
3 kinase is conserved through vertebrate evolution, suggesting an important role in
4 complex nervous systems. Alternative splicing involving a *N1-Src* specific microexon
5 leads to a five or six amino acid insertion into the SH3 domain of Src. A prevailing
6 model suggests that N1-Src regulates neuronal differentiation via cytoskeletal
7 dynamics in the growth cone. Here we have investigated the role of n1-src in the
8 early development of the amphibian *Xenopus tropicalis*, and find that *n1-src*
9 expression is regulated during embryogenesis, with highest levels detected during
10 the phases of primary and secondary neurogenesis. *In situ* hybridisation analysis,
11 using locked nucleic acid (LNA) oligo probes complementary to the *n1-src* microexon
12 indicate that *n1-src* expression is highly enriched in the open neural plate during
13 neurula stages and in the neural tissue of adult frogs. Given the *n1-src* expression
14 pattern, we investigated a possible role for n1-src in neurogenesis. Using splice site-
15 specific antisense morpholino oligos, we are able to inhibit *n1-src* splicing, whilst
16 preserving *c-src* expression. Differentiation of neurons in the primary nervous system
17 is reduced in *n1-src* knockdown embryos, accompanied by a severely impaired touch
18 response in later development. These data reveal an essential role for n1-src in
19 amphibian neural development and suggest that alternative splicing of C-Src in the
20 developing vertebrate nervous system evolved to regulate neurogenesis.

21

22 **Significance statement**

23 The Src family of non-receptor tyrosine kinases act in signalling pathways that
24 regulate cell migration, cell adhesion and proliferation. Srcs are also enriched in the
25 brain where they play key roles in neuronal development and neurotransmission.

26 Vertebrates have evolved a neuron-specific splice variant of C-Src, N1-Src, which
27 differs from C-Src by just five or six amino acids. N1-Src is poorly understood and its
28 high similarity to C-Src has made it difficult to delineate its function. Using antisense
29 knockdown of the *n1-src* microexon, we have studied neuronal development in the
30 *Xenopus* embryo in the absence of *n1-src*, whilst preserving *c-src*. Loss of *n1-src*
31 causes a striking absence of primary neurogenesis, implicating *n1-src* in the
32 specification of neurons early in neural development.

33

34 **Introduction**

35 The Src family of eleven non-receptor tyrosine kinases evolved to regulate key
36 signalling pathways involved in cell adhesion, migration and cell fate in multicellular
37 organisms (Thomas and Brugge, 1997). Several Src family members, including C-
38 Src, Fyn and Yes, are enriched in the vertebrate nervous system with roles in the
39 developing and mature brain and have been implicated in the pathology of
40 neurological disorders (Grant et al., 1992; Maness, 1992; Zhao et al., 2000; Ohnishi
41 et al., 2001; Kalia et al., 2004; Nygaard et al., 2014). Further complexity and
42 specificity of C-Src signalling in the brain, is conferred by neuronal-specific splicing to
43 yield N1- or N2-Src (Brugge et al., 1985; Pyper and Bolen, 1990). The N-Src splice
44 variants contain an additional six or seventeen amino acids respectively in the SH3
45 domain, and are encoded by microexons situated between exons three and four of
46 C-Src (Martinez et al., 1987). We and others have shown that N-Srcs have a higher
47 constitutive kinase activity and an altered SH3 domain substrate specificity compared
48 to C-Src (Dergai et al., 2010; Keenan et al., 2015), however, their *in vivo* substrates
49 are unknown.

50 C-Src expression has been identified in a wide range of animal groups,
51 including basal metazoans, such as sea sponges (Ottillie et al., 1992), but its
52 neuronal splicing to yield N1-Src only appears in the vertebrate lineage (Fig. 1A; Levy
53 et al., 1987; Martinez et al., 1987; Raulf et al., 1989) and N2-Src is restricted to
54 mammals (Pyper and Bolen, 1990). Within the N1-Src microexon, there are minor
55 differences in the length and sequence between vertebrate species. For example, a
56 six amino acid N1-Src insert has been detected in brain tissue from the teleost fish
57 *Xiphophorus* (Raulf et al., 1989), whereas the *c-src* locus of the diploid amphibian
58 *Xenopus tropicalis* and the two pseudoallelic loci of allotetraploid *Xenopus laevis*
59 contain five amino acid inserts (Collett and Steele, 1992). Identical six amino acid
60 neuronal Src inserts are observed in N1-Src of chicks, rodents and humans (Levy et
61 al., 1987; Martinez et al., 1987). The appearance and conservation of a neural-
62 restricted src isoform in the vertebrate lineage raises the intriguing possibility that n1-
63 src function is related to the evolution and development of the complex vertebrate
64 nervous system.

65 Previous studies in which N1-Src was overexpressed suggest N1-Src
66 regulates neuronal morphology through cytoskeletal modifications affecting neurite
67 outgrowth and axonogenesis (Worley et al., 1997; Kotani et al., 2007). However, no
68 studies have thus far observed the development of the nervous system in the
69 absence of N1-Src splicing. Here, we investigated n1-src function in the amphibian
70 *Xenopus tropicalis*. We found that *n1-src* expression is localised to the dorsal
71 ectoderm of the neural plate, which gives rise to the central nervous system during
72 development. Using antisense morpholino oligos, we have for the first time achieved
73 specific inhibition of *n1-src* splicing in a vertebrate nervous system, without affecting
74 *c-src* expression. The knockdown of *n1-src* caused abnormal touch responses in

75 larval stage embryos, with a concomitant reduction in neuronal-specific tubulin
76 (*tubb2a*) positive neurons during primary neurogenesis. We propose that neuronal
77 splicing of C-Src has evolved to be essential for vertebrate neurogenesis.

78

79 **Materials and Methods**

80 ***Sub-cloning of Xenopus n1-src***

81 A plasmid encoding C-terminal FLAG-tagged *Xenopus* n1-src (pFLAG-Xn1-Src) was
82 generated by amplifying the *Xenopus laevis* n1-src b variant open reading frame from
83 IMAGE clone: 5572523 with the following PCR primers incorporating 5' BglII and 3'
84 KpnI restriction sites. This codes for an n1 insertion identical to that of *Xenopus*
85 *tropicalis* n1-src, as determined by examination of the *Xenopus tropicalis* genome
86 and sequencing of relevant rt-PCR products.

87 **forward** 5'-AGATCTCTCTAGAACCATGGGTGCCACTAAAAGCAAGCCA-3'

88 **reverse** 5'-GGTACCGTAGATCCAAGGTGTTCCCCAGGCTGGTACTG-3'.

89 Digested product was ligated into pEGFP-N1 (Clontech, Mountain View, CA) in which
90 the GFP tag was replaced with a FLAG tag (pFLAG). The pCS2+-Xn1-src-FLAG
91 plasmid was generated by excising FLAG-tagged Xn1-src from pFLAG-Xn1-src with
92 XbaI and ligating into XbaI digested pCS2+. The preparation of pFLAG-C-Src and -
93 N1-Src was previously described (Keenan et al., 2015).

94

95 ***Fibroblast cell morphology assay***

96 Ten thousand COS7 fibroblast cells were plated onto 13 mm coverslips. Twenty four
97 hours after plating, cells were transfected with 1 µg plasmid DNA using Ecotransfect
98 (Oz Biosciences) according to the manufacturer's instructions. Cells were fixed 48 h
99 after transfection in 4 % paraformaldehyde, 4 % sucrose for 20 min and then

100 permeabilised in 0.1 % Triton, 1 % BSA and stained with primary antibodies (mouse
101 anti-FLAG (M2), 1:1000; rabbit anti-GFP, 1:500) in 1 % BSA in PBS for 2 h at room
102 temperature. After 3 washes in PBS, secondary antibodies (anti-mouse Alexa Fluor-
103 564 and anti-rabbit Alexa Fluor-488; Invitrogen, Paisley, UK) were applied at 1:500 in
104 1 % BSA in PBS for 1 h in the dark. Coverslips were mounted on slides using Mowial
105 mountant (10 % Mowial, 25 % glycerol in 0.1 M Tris pH 8.5) containing 1 µg/ml DAPI.
106 Images were acquired using a 40x objective on a Nikon TE200 epifluorescence
107 inverted microscope using a RoleraXR CCD (QImaging) camera controlled by
108 SimplePCI Software (Hamamatsu). The percentage of COS7 cells bearing neurite-
109 like processes, defined as being longer than the cell soma diameter and having a
110 width of less than 2 µm, was calculated and statistical analysis of the data was
111 performed with SigmaPlot software using a Kruskal-Wallis two tailed analysis of
112 variance. The experimenter was blind to the plasmid transfected in each condition.

113

114 ***Embryological methods***

115 *Xenopus tropicalis* embryos were produced as previously described (Khokha et al.,
116 2005; Winterbottom et al., 2010). Embryos were microinjected at the 2- or 4-cell
117 stage and cultured at 22°C in MRS/9+3% Ficoll, before transferring to MRS/20 for
118 long term culture. The sequences for the splice blocking antisense morpholino oligos
119 (GeneTools, LLC) are shown below.

120 **AMO a (splice acceptor)** 5'-GTCAGGTCTCCTATGGCACAGCATG-3'

121 **AMO d (splice donor)** 5'-GCCGCCGGATGGTCACATACCTCAT-3'

122 Videos of the locomotive phenotypes of Stage 28 or 41 embryos in response to touch
123 stimuli were acquired using a JVC TK-C1381 camera and processed with ArcSoft
124 ShowBiz software.

125 **RNA extraction and semi-quantitative rt-PCR**

126 Demembrated embryos or tissues dissected from male adult *Xenopus tropicalis*
127 were flash frozen on dry ice. Total RNA was extracted from tissues using Tri-Reagent
128 and precipitated with 7.5 M LiCl/50 mM EDTA (Warrander et al., 2016). First strand
129 cDNA was synthesised from 1-3 µg total RNA using random hexamer primers and
130 Invitrogen SuperScript II Reverse Transcriptase according to the manufacturer's
131 instructions (Warrander et al., 2016). Promega PCR Master Mix was used to amplify
132 cDNA from the embryos at the different developmental stages, with *rpl8* used as a
133 loading control. Primers used to detect gene expression were as follows.

134 ***rpl8* forward** 5'-GGGCTGTCGACTTCGCTGAA-3'

135 ***rpl8* reverse** 5'-ATACGACCACCACCAGCAAC-3'

136 ***c-src* forward** 5'-ATCTCGCACCGAGACAGACT-3'

137 ***c-src* reverse** 5'-CAGTCGCCTTCCGTGTTATT-3'

138 ***n1-src* forward** 5'-ACTGTGACCTGACGCCTTTT-3'

139 ***n1-src* reverse** 5'-CCTCATGTCAGGTCTCGTGT-3'.

140

141 ***In situ* hybridisation and β-galactosidase staining**

142 *Tubb2a* (*n-tubulin*) probe synthesis and *in situ* hybridisation were carried as
143 previously described (Winterbottom et al., 2010). A 19-mer digoxigenin end-labelled
144 locked nucleic acid (LNA) probe was designed against the 15 base *n1-src* microexon
145 sequence, with the addition of two bases from the flanking *c-src* exons. The probe
146 with the following sequence was synthesised by Exiqon (Vedbaek, Denmark; (Darnell
147 et al., 2010). Locked nucleotides are indicated in bold.

148 ***n1-src* microexon probe** 5'-TCCCTCATGTCAGGTCTCG-3'

149 It was confirmed that no off-target sequence identities of more than 12 nucleotides
150 were present in the *Xenopus tropicalis* genome. The LNA *in situ* hybridisation was
151 undertaken as previously described (Sweetman, 2011; Warrander et al., 2016).
152 Briefly, de-membrated embryos were fixed in 0.1 M MOPS, 2 mM EDTA, 1 mM
153 MgSO₄, 3.7 % formaldehyde. The hybridisation and washes were carried out at
154 57°C. Embryos were hybridised for 36 h with 20 nM LNA probe preabsorbed again
155 tailbud stage embryos. Colour was developed with BM Purple (Roche) substrate until
156 diffuse purple staining was visible, at which point embryos were washed for 12 h. The
157 staining and washing cycle was then repeated until strong specific staining was
158 present. For lineage tracing, *β-galactosidase* mRNA synthesis, embryo injection and
159 enzyme staining was undertaken as previously described (Pownall et al., 1996). Both
160 *in situs* and fixed phenotypes were imaged using a Leica MZ FLIII microscope
161 (Leica), a SPOT 14.2 Colour Mosaic camera and SPOT Advanced software
162 (Diagnostic Instruments Inc.).

163

164 **Results**

165 ***The Xenopus n1-src splice variant promotes neurite outgrowth***

166 We first investigated whether the activity of N1-src isoforms has been conserved
167 during vertebrate evolution. There are minor differences in the length and sequence
168 of the *n1-src* microexon between mammals, amphibians and fish, however, the
169 distribution of charged and hydrophobic residues is conserved (Fig 1A).
170 Overexpression of mammalian N1-Src (but not C-Src) was previously shown to elicit
171 morphological changes in *Xenopus* kidney epithelial cells (Worley et al., 1997) and
172 we therefore performed a similar assay to compare the biological activity of *Xenopus*
173 *n1-src* and mammalian N1-Src (Fig. 1B,C). COS7 fibroblasts were co-transfected

174 with soluble CFP (to aid the visualisation of cell morphology) and a C-terminal FLAG-
175 tagged Src construct (C-, N1- or n1-src) or a vector control. We and others have
176 previously shown that C-terminal fusion tags do not affect Src activity in cells
177 (Sandilands et al., 2004; Keenan et al., 2015). We assayed cell morphology by
178 quantifying the percentage of cells bearing neurite-like processes. In agreement with
179 previous findings, C-Src did not elicit process outgrowth compared to the vector
180 control, while approximately one third of N1-Src and n1-src transfected cells bore
181 processes (Fig. 1B,C), suggesting that, despite sequence differences their N1-Src
182 insertions, activities of the amphibian and mammalian N1-Src enzymes have been
183 highly conserved during evolution.

184

185 ***N1-src is expressed during neurogenesis***

186 We next examined the temporal expression of *Xenopus n1-src* during development.
187 Using splice variant specific PCR primer sets, we undertook RT-PCR analysis of *c-*
188 *src* and *n1-src* expression from cleavage to early larval stages (Fig. 2A). Expression
189 of *c-src* is relatively constant throughout early development. In contrast, *n1-src*
190 expression is highly regulated over the same period. Prior to the onset of
191 transcription from the zygotic genome at blastula stage 8, only very low levels of
192 maternally deposited *n1-src* mRNA are detected. Zygotic *n1-src* expression begins to
193 rise at gastrula stage 11, reaching its highest level at neurula stage 18, and this is
194 maintained through early tailbud stage 25. However, by early larval stage 35
195 expression has fallen dramatically. Fig 2B indicates that *n1-src* expression increases
196 again at stage 46, correlating with secondary neurogenesis of motor, inter- and
197 sensory neurons in the closed neural tube (Schlosser et al., 2002). Therefore, *n1-src*
198 expression is maximal during phases of neurogenesis in the primary nervous system.

199 We also examined the expression of *c-src* and *n1-src* in adult tissues and
200 found that the highest level of *n1-src* expression is within the adult brain, with heart
201 muscle the only other tissue, where we were able to detect low levels of *n1-src*
202 expression (Fig. 2C).

203

204 ***N1-src expression is enriched in the neural plate***

205 To visualise the spatial expression pattern of *n1-src* in the developing embryo we
206 used a 19-mer locked nucleic acid (LNA) probe specific for the *n1-src* microexon
207 sequence. Traditional antisense mRNA *in situ* probes are typically greater than ~150
208 bases in length and are unable to distinguish between the small sequence
209 differences exhibited by the *c-src* and *n1-src* splice variants. Early and late stage
210 neurula embryos were probed with a digoxigenin labelled *n1-src* specific LNA probe.
211 In keeping with our RT-analysis we find that *n1-src* expression is highly enriched in
212 the neural plate of neurula stage embryos (Fig. 3). Our analysis indicates general
213 expression of *n1-src* in cells of the neural plate at stage 14 (Fig 3A and B).
214 Expression is fairly constant along the anteroposterior axis of the neural plate, with
215 expression being detected in cells of the presumptive fore, mid and hindbrain
216 regions, as well as the spinal cord (Fig 3A, C and D). *n1-src* expression continues to
217 be enriched in the neural plate as it narrows and rolls up to form the neural tube in
218 late stage 19 neurula embryos (Fig. 3E and F).

219

220 ***Morpholino mediated knockdown of Xenopus n1-src disrupts the touch*** 221 ***response of embryos***

222 Morpholino oligos (MOs) are nucleic acid analogs with a modified backbone
223 chemistry which are able to hybridize to target RNA in a highly specific, sequence

224 dependent fashion. Antisense MOs (AMOs) are able to knockdown gene function in a
225 number of systems (Eisen and Smith, 2008). Typical knockdown strategies use
226 AMOs to block translation or nuclear pre-mRNA processing. AMOs targeted to splice
227 acceptor and donor sites in pre-mRNAs have been used to block normal splicing
228 events leading to the formation of aberrant mRNAs containing intron sequences, thus
229 disrupting the protein coding information normally found in the mature mRNA. AMOs
230 have also been successfully used to induce exon skipping (Goyenvalle et al., 2010;
231 Kang et al., 2011). Here we use this approach to block splicing involving the *n1-src*
232 specific microexon. Non-overlapping AMOs targeted to the splice acceptor (AMO a)
233 and donor (AMO d) sites of the *n1-src* microexon were designed (Fig.4A). *n1-src*
234 AMOs were delivered to the cells of the embryo by microinjection. In contrast to
235 uninjected control embryos, we are unable to detect *n1-src* expression in embryos
236 injected with a combination of AMO a+d. Furthermore, injection of AMO a or d alone
237 also effectively blocked *n1-src* expression (Fig. 4B). Consistent with an effect on
238 exon skipping, the expression of *c-src* was unaffected by the AMOs. We conclude
239 that AMOs represent highly specific tools for investigating the function of the
240 *Xenopus* *n1-src* isoform in early development.

241 Injection of a standard control MO has little effect on the phenotype of larval
242 stage 41 embryos, whereas injection of the AMO a+d mixture leads to a mild, but
243 highly penetrant phenotype, which is characterised by shortening and/ or kinking of
244 the tail, and variable disruption to the pigmented retina of the eye (Fig. 4C). To
245 assess the function of the primary nervous system in *n1-src* ablated embryos, we
246 applied a touch stimulus to the side of larval stage embryos, which elicits a dart
247 response. The neuronal circuitry for the touch reflex (Fig. 4D; Movie 1) is well
248 characterised and comprises Rohon-Beard sensory neurons, which activate

249 commissural interneurons that in turn synapse onto contralateral motor neurons to
250 stimulate muscle contraction, propelling the embryo away from the stimulus (Boothby
251 and Roberts, 1995; Li et al., 2003; Roberts et al., 2010). (Fig 4D; Movie 1, 3). We
252 tested the same embryos at developmental stages 28 and 41 (prior to and during the
253 onset of myelination) and the dart response was commonly abnormal or absent in *n1-*
254 *src* AMO a+d injected embryos (Fig. 4E). These embryos instead frequently
255 displayed an uncoordinated twitch or spasm response, indicating abnormal
256 development of the neural circuitry necessary for the dart response (Fig 4D; Movie 2,
257 4).

258

259 ***N1-src knockdown disrupts primary neurogenesis***

260 To ascertain which neurons in the touch reflex are affected by *n1-src* knockdown, we
261 next investigated the early development of the primary nervous system. During
262 primary neurogenesis, the motor, inter and Rohon-Beard sensory neurons
263 differentiate to form medial, intermediate and lateral columns respectively, either side
264 of the neural plate midline. These columns of differentiating neurons are separated
265 by non-differentiating, proliferative progenitors and can be identified by expression of
266 the neuronal specific *tubb2b* gene (Chitnis et al., 1995). Fig. 4F shows differentiating
267 primary neurons visualised by *in situ* hybridization for *tubb2b* mRNA. Unilateral
268 injection of AMO a+d resulted in penetrant reduction of *tubb2b* expression in all three
269 columns relative to the uninjected contralateral side. (90%, n=55 from four
270 independent fertilisations) or embryos unilaterally injected with a control MO (24%,
271 n=39 from four independent fertilisations). Thus we concluded that rather than
272 regulating the development of specific subsets of neurons, *n1-src* is required for

273 neurogenesis of all *tubb2b*-positive neurons in *Xenopus* primary nervous system
274 development.

275

276 **Discussion**

277 ***The activity of amphibian and mammalian n1-src is conserved***

278 The alternative splicing of neuronal src isoforms alters the ligand binding specificity of
279 the C-Src SH3 domain and the catalytic activity of its kinase domain (Brugge et al.,
280 1985; Keenan et al., 2015). These differences are believed to underpin the reported
281 differential activity of neuronal Srcs. The position of the N1-specific insertion into the
282 SH3 domain of C-Src is conserved between amphibians and amniotes, and we
283 investigated whether the differential biological activity of N1-Src isoforms has been
284 conserved in amphibians. We find that, unlike C-Src, both *Xenopus* n1-src, and
285 mammalian N1-Src, despite different SH3 inserts (5 versus 6 amino acid in
286 amphibians and mammals respectively), are able to induce neurite-like processes
287 when transfected into COS-7 cells. In keeping with this, it has been shown previously
288 that N1-Src overexpression in *Xenopus* A6 epithelial cells, induced neurite-like
289 processes, in contrast to the rounded phenotype of C-Src transfected cells (Worley et
290 al., 1997).

291

292 ***n1-src expression correlates with primary neurogenesis***

293 A previous study indicated that the expression of the *n1-src* isoforms of the tetraploid
294 amphibian *Xenopus laevis*, are initiated by mid-neurula stage 15 (Collett and Steele,
295 1992). We find that in the diploid amphibian *Xenopus tropicalis*, there is low level
296 maternal *n1-src* expression from the start of development, and, in contrast to the
297 previous study, activation of zygotic *n1-src* expression is initiated as early as mid to

298 late gastrula stages, and by early neurula stages expression is restricted to the open
299 neural plate.

300 The period from late gastrula to early neurula is a key phase in the
301 development of the primary nervous system; a simple functional nervous system,
302 characteristic of anamniotic aquatic vertebrates, including fish and amphibians
303 (Hartenstein, 1989). Differentiation of primary neurons enables the early development
304 of motility, and helps embryos avoid predation in an aquatic environment. Primary
305 neurons begin to differentiate at open neural plate stages in *Xenopus* embryos and
306 primary neurogenesis continues through neurula and early tailbud stages (Schlosser
307 et al., 2002). In keeping with our findings that *n1-src* expression is initiated during the
308 gastrula stage, a subsequent study by Collet and Steele (1993) showed that *n1-src*
309 expression is rapidly activated in competent gastrula dorsal ectoderm by endogenous
310 neural inducing signals and the neural inducing activity of the phorbol ester TPA in
311 the absence of protein synthesis.

312

313 ***Abnormal neural development in n1-src knockdown embryos***

314 We present the first analysis of the consequences of blocking the splicing events
315 required for the expression of *n1-src* during vertebrate development. An advantage of
316 our approach is that the morpholinos ablated *n1-src* expression whilst *c-src*
317 expression was unaffected. *N1-src* knockdown caused striking behavioural,
318 morphological and neuronal phenotypes in the *Xenopus* embryo. At the larval stage
319 we found that AMO injected embryos exhibited a severely abnormal locomotor
320 response to touch stimuli, with many observed to twitch or spasm following the touch.
321 The *Xenopus* touch reflex and subsequent swimming circuits involve the co-
322 ordination of sensory, inter and motor neurons (Roberts et al., 2010). In stage 14

323 embryos, and in keeping with the widespread expression of *n1-src* in the neural plate,
324 we found the columns of differentiating *tubb2a*-positive neurons that subsequently
325 form the touch and swimming circuits are reduced or absent. Due to the labile nature
326 of the morpholinos, we predict that *n1-src* expression will slowly return, leading to a
327 delay and perturbation in primary neurogenesis that generates the aberrant circuits
328 observed in the larval embryo. The reduced touch response could also result from a
329 defect in myelination, a process that begins at approximately stage 42 (Yoshida,
330 1997). Furthermore, oligodendrocytes arise from the same precursors as motor
331 neurons (Park et al., 2002). However, our observation that both stage 28 and stage
332 41 embryos exhibit the same defects rules out myelination as the sole cause of the
333 phenotype.

334 Morphologically, *n1-src* knockdown embryos exhibited a loss of retinal
335 pigmentation and a kinked tail. The optic stalk, retina and retinal pigmented
336 epithelium develop from an outpocketing of the diencephalon (Fuhrmann et al.,
337 2014). Therefore, the loss of eye pigmentation in *n1-src* knockdown embryos may
338 indicate a common role for *n1-src* in regulating the differentiation of cells derived from
339 the neuroepithelium. The morphogenesis of the vertebrate main body axis involves
340 coordinated cell movements in the axial mesoderm and the neuroepithelium
341 (Nikolopoulou et al., 2017). Posterior axial defects have been observed in embryos in
342 which normal convergent extension within the neuroepithelium has been inhibited
343 through interference with components of the planar cell polarity signalling pathway
344 (Goto and Keller, 2002). We speculate that the kinking of the posterior axis observed
345 in *n1-src* knockdown embryos arises through deregulation of the process of
346 convergent extension, which drives elongation and narrowing of the neuroepithelium

347 and reflects abnormal signalling within the neuroepithelium in the absence of n1-src
348 activity.

349 At present we are unable to say with any confidence where n1-src functions in
350 the pathway leading to neuronal differentiation. However, n1-src's general neural
351 expression is similar to a group of neural stabilization genes, including members of
352 the Sox, Zic and Iroquois families (reviewed by Moody and Je, 2002). These code for
353 transcription factors and, as is the case with n1-src, several of these genes are
354 expressed in response to neural induction. Neural stabilization genes have multiple
355 overlapping functions, providing a link between the signals that induce the neural
356 plate and the hierarchy of proneural and neurogenic genes that are required for
357 neuronal specification and differentiation. Thus, early expressed neural stabilization
358 genes have roles in regulating the competence of ectodermal cells to respond to
359 neural inducing signals, and later expressed ones regulate the progression from
360 neuronal progenitor to differentiated neuron (Moody and Je, 2002). Current evidence
361 is suggestive of a role for n1-src in the process of neural stabilization. However,
362 future studies will be required to investigate the regulatory interactions between the
363 n1-src kinase and known components of the vertebrate neurogenic pathway.

364

365 ***N1-Src function and neurogenesis in higher vertebrates***

366 Our data show that following primary neurogenesis *n1-src* expression falls, but is
367 again elevated during a second phase of neurogenesis in late larval stages. A
368 primary nervous system is absent in amniotes, and it is the later phase of secondary
369 neurogenesis in the closed neural tube that is more akin to the neurogenesis of
370 amniotes, including mice and humans (Wullimann et al. 2005). A connection between
371 neuronal differentiation and N1-Src function in amniotes is supported by an analysis

372 of N1-Src (also termed pp60⁺) activity in the developing mouse brain, which showed
373 a rapid increase in N1-Src activity at E12 and which peaks at E18, when increasing
374 numbers of neuroblasts are exiting the cell cycle and differentiating (Wiestler and
375 Walter, 1988). Furthermore, cultured neurons of the rat striatum contain little
376 detectable N1-Src activity, however, neuronal differentiation induced by serum
377 starvation leads to a seven-fold increase in N1-Src activity relative to C-Src
378 (Cartwright et al., 1987). Similarly, embryonic carcinoma cells treated with retinoic
379 acid to induce neuronal differentiation express increased levels of N1-Src (Lynch et
380 al., 1986), and there is an increase in both N1-Src and N2-Src expression during
381 differentiation of the neuroblastoma cell line LAN-5 (Matsunaga et al., 1993a).

382 We present evidence for an early role for n1-src in neural development
383 regulating the transition from neural progenitors to differentiated neuron. However,
384 there is also evidence that N1-Src has roles regulating the cellular architecture and
385 morphogenesis of neurons. Transgenic mice expressing N1-Src in Purkinje neurons
386 of the cerebellum display defects in migration and dendrite morphology, which might
387 be linked to defects in microtubule structure (Kotani et al., 2007). Conversely, in
388 *Xenopus laevis*, overexpression of mammalian N1-Src in the optic tectum enhanced
389 axonogenesis of retinal progenitors. Thus n1-src is likely to have multiple roles in
390 neural development regulating neuronal specification and morphogenesis.

391

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498 **Figure legends**

499 **Figure 1.** *Xenopus* n1-src elicits neurite-like processes in fibroblasts.

500 **A**, Amino acid alignment of the N1-microexon in mammalian, *Xenopus*, and fish
501 species. +=basic; -=acidic and Φ =hydrophobic amino acid sidechains. **B**,
502 Representative COS7 cells co-transfected for four days with Src-FLAG and CFP
503 constructs. Cells were stained for Src (anti-FLAG) and CFP. **C**, Quantification of
504 process outgrowth in COS7 cells. Each process was defined as an extension longer
505 than one cell soma diameter and less than 2 μ m in diameter. Data are plotted as
506 mean \pm SEM, n=3 independent experiments. Kruskal-Wallis two-tailed analysis of
507 variance. ***, P<0.001. Scale bar = 10 μ m.

508

509 **Figure 2.** *n1-src* mRNA expression levels during *Xenopus tropicalis* development
510 and in adult tissues.

511 **A**, rt-PCR analysis of *c-src* and *n1-src* mRNA expression levels from early cleavage
512 stage 4 to tailbud stage 35. *rpl8* is used as a ubiquitously expressed loading control. -
513 rt= no reverse transcriptase control and water= no template control. **B**, rt-PCR
514 analysis of *c-src* and *n1-src* expression levels during (stage 25) and after (stage 35)
515 primary neurogenesis, and during secondary neurogenesis (stage 46). **C**, rt-PCR
516 analysis of *c-src* and *n1-src* expression in a range of adult tissues.

517

518 **Figure 3.** Expression pattern of *n1-src* during *Xenopus tropicalis* primary
519 neurogenesis.

520 *In situ* hybridisation analysis of *n1-src* mRNA expression using a 19-mer digoxigenin
521 end-labelled antisense probe directed against *n1-src* specific sequence. **A**, **B**, **C** and
522 **D** are early neurula stage 14 embryos. **E** and **F** are late neurula stage 19 embryos. **A**,

523 dorsal view, anterior to left. **B**, lateral view anterior to the left. **C**, anterior view, dorsal
524 to the top. **D**, posterior view, dorsal to the top. **E**, dorsal view anterior to the left. **F**,
525 anterior view dorsal to the top. *F+mb* = presumptive forebrain and midbrain, *sc* =
526 presumptive spinal cord, *bp* = blastopore.

527

528 **Figure 4.** Abnormal touch response and primary neurogenesis in *n1-src* knockdown
529 embryos

530 **A**, diagram showing the sequences and corresponding RNA target sequences of the
531 splice acceptor (AMO a) and donor (AMO d) splice blocking antisense morpholinos.

532 **B**, rt-PCR analysis of *c-src* and *n1-src* mRNA expression at stage 16 in control
533 uninjected and injected with a total of 20 ng of AMO a, AMO d or AMO a+d. *rpl8* is
534 used as a ubiquitously expressed loading control. -rt = no reverse transcriptase

535 control and water = no template control. **C**, representative phenotypes of embryos at
536 larval stage 41 bilaterally injected at the 2- or 4-cell stage with 10 ng total of a

537 standard control MO or the AMO a+d combination. Embryos were co-injected with
538 100 pg nuclear β -galactosidase and subsequently stained with X-gal (light blue

539 colour) to demonstrate successful injection targeting. **D**, cartoon of embryo touch
540 reflex. Touching the skin stimulates Rohon-Beard sensory neurons (s), which

541 synapse onto commissural interneurons (i) that activate contralateral motor neurons
542 (m), leading to muscle contraction. **E**, Quantitation of touch response phenotype of

543 the same embryos at larval stage 28 and 41 bilaterally injected at the 2- or 4-cell
544 stage with 10 ng total of a standard control MO or the AMO a+d combination. Data

545 are plotted as mean \pm SEM, n=4 independent experiments. **F**, *in situ* hybridisation
546 analysis of *tubb2b* expression in differentiating primary neurons of open neural plate

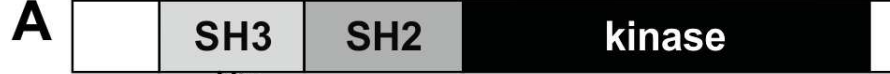
547 stage 14 embryos unilaterally injected with 5 ng total of a standard control MO or the

548 AMO a+d combination. The injected side shows faint blue nuclear staining with the β -
549 *galactosidase* lineage tracer, and is indicated with a black asterisk, anterior is to the
550 left. m=motor neurons; i=interneurons; s=sensory neurons.

551

552 **Movies 1-4.** Normal and abnormal touch responses in stage 28 and stage 41
553 *Xenopus tropicalis* embryos.

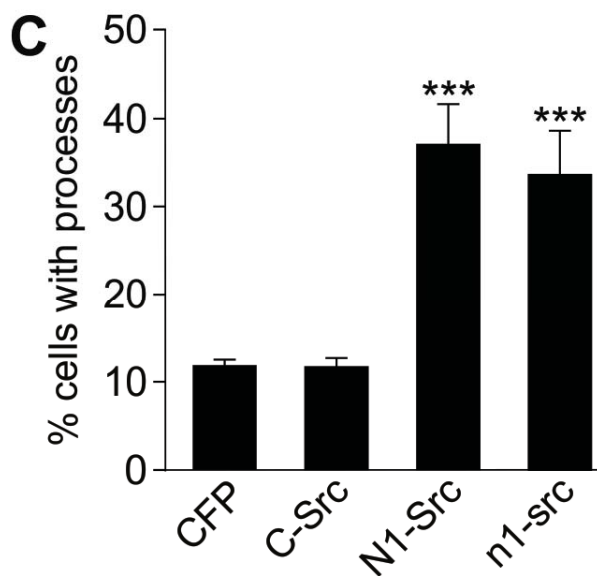
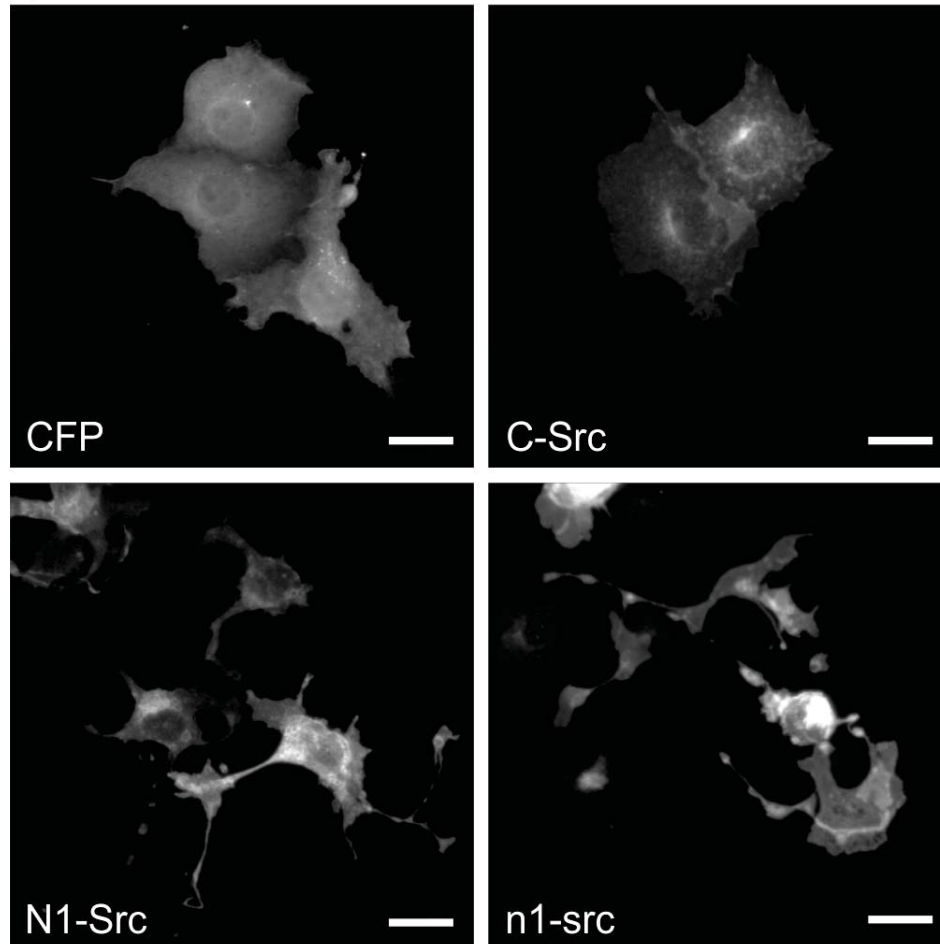
554 **Movies 1 & 3,** real time videos showing the normal touch response of stage 28
555 (Movie 1) or stage 41 (Movie 3) embryos injected with 10 ng of a standard control
556 MO. Embryos right themselves and swiftly swam a short distance from the point of
557 contact. **Movies 2 & 4,** real time videos of the abnormal touch response in stage 28
558 (Movie 2) or stage 41 (Movie 4) embryos injected with 10 ng *n1-src* AMO a+d. Non-
559 responding phenotypes remain horizontal and moved slowly from the point of contact
560 with uncoordinated twitching movements.

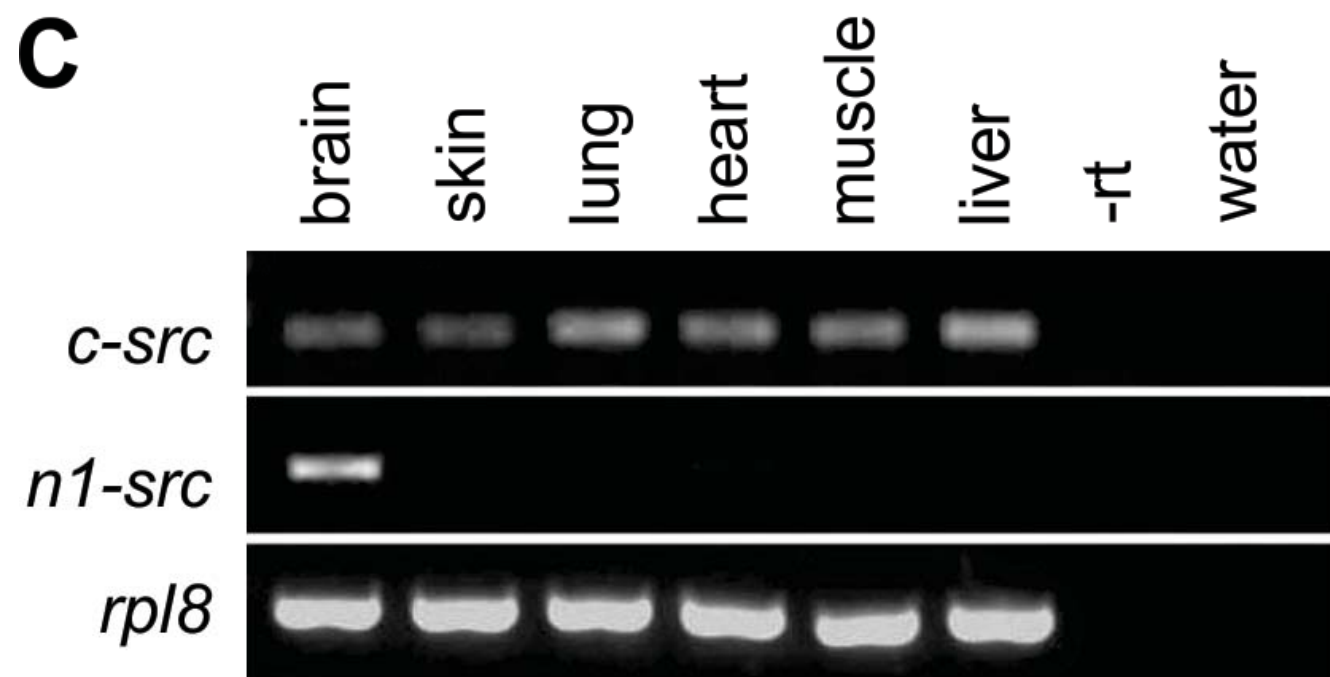
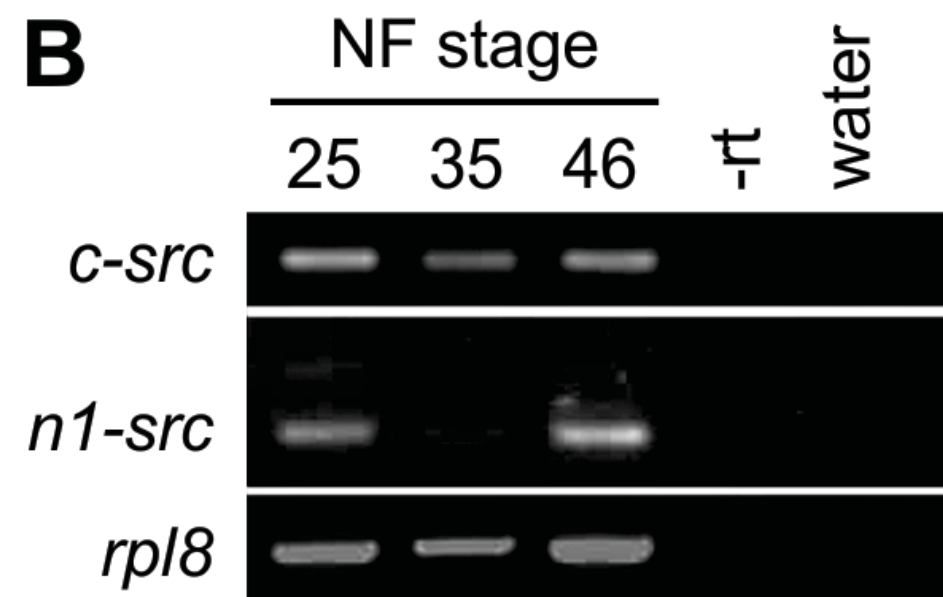
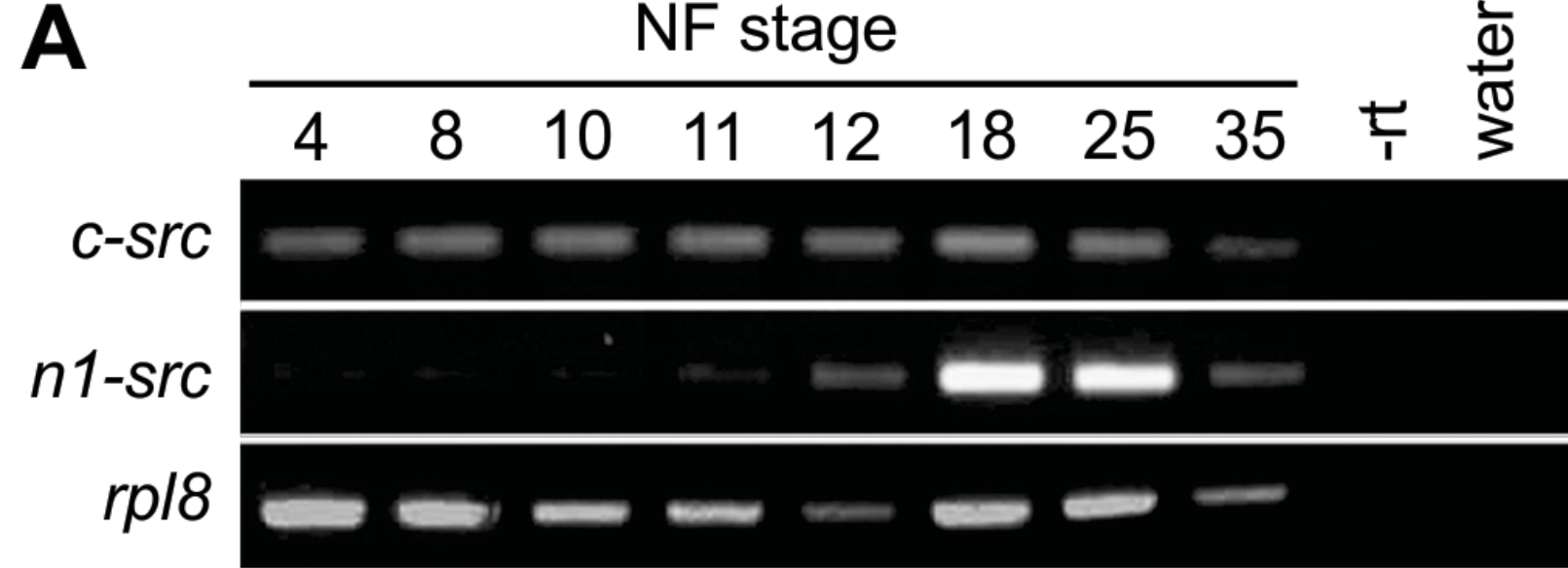


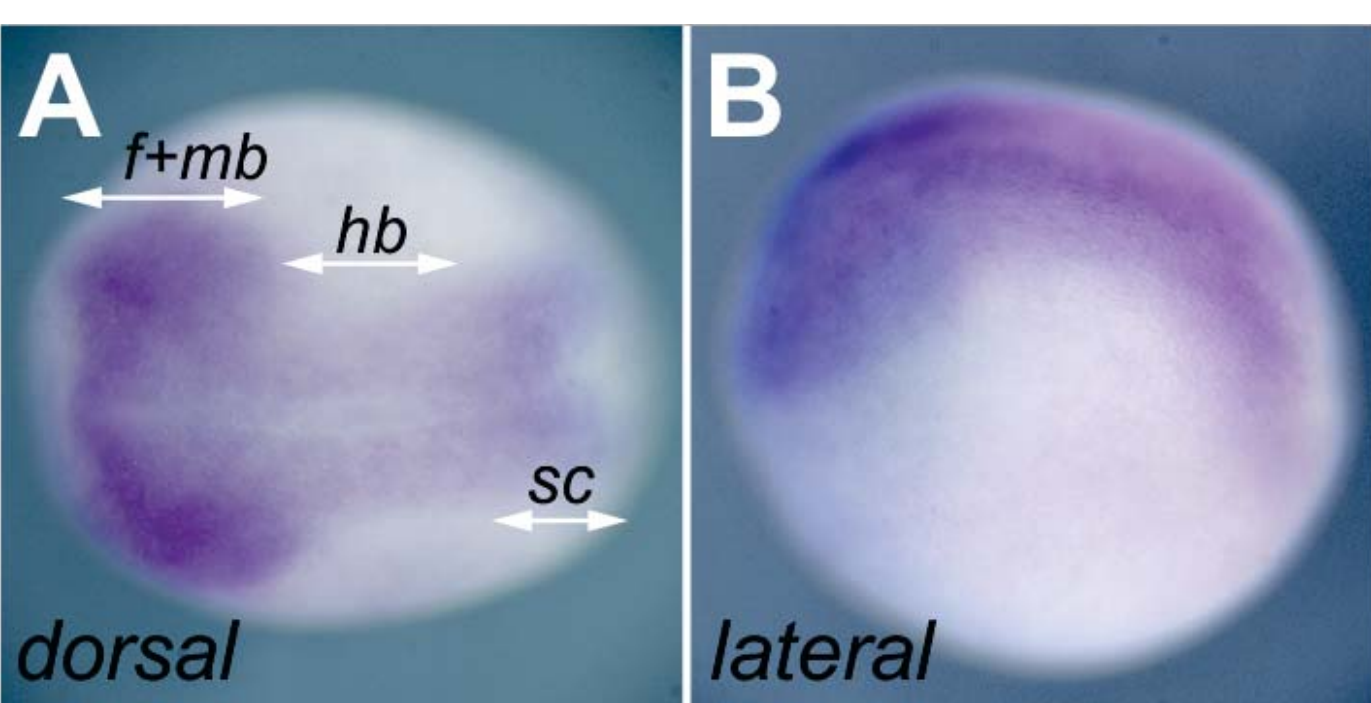
<i>H sapiens, M musculus</i>	VNNT	+ ϕ - ϕ +	EGDW
<i>R rattus, G gallus</i>	VNNT	R	EGDW
<i>X laevis a</i>	VNNT	R	EGDW
<i>X laevis b</i>	VNNT	R	EGDW
<i>X tropicalis</i>	VNNT	R	EGDW
<i>D rerio</i>	VNNT	R	EGDW
<i>Scyliorhinidae (catshark)</i>	VNNT	R	EGDW

...exon 3 N1 exon 4...
microexon

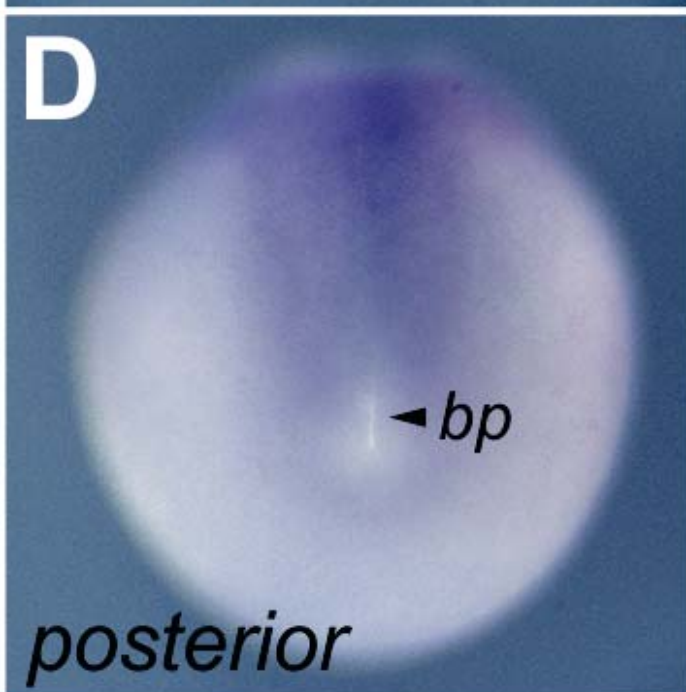
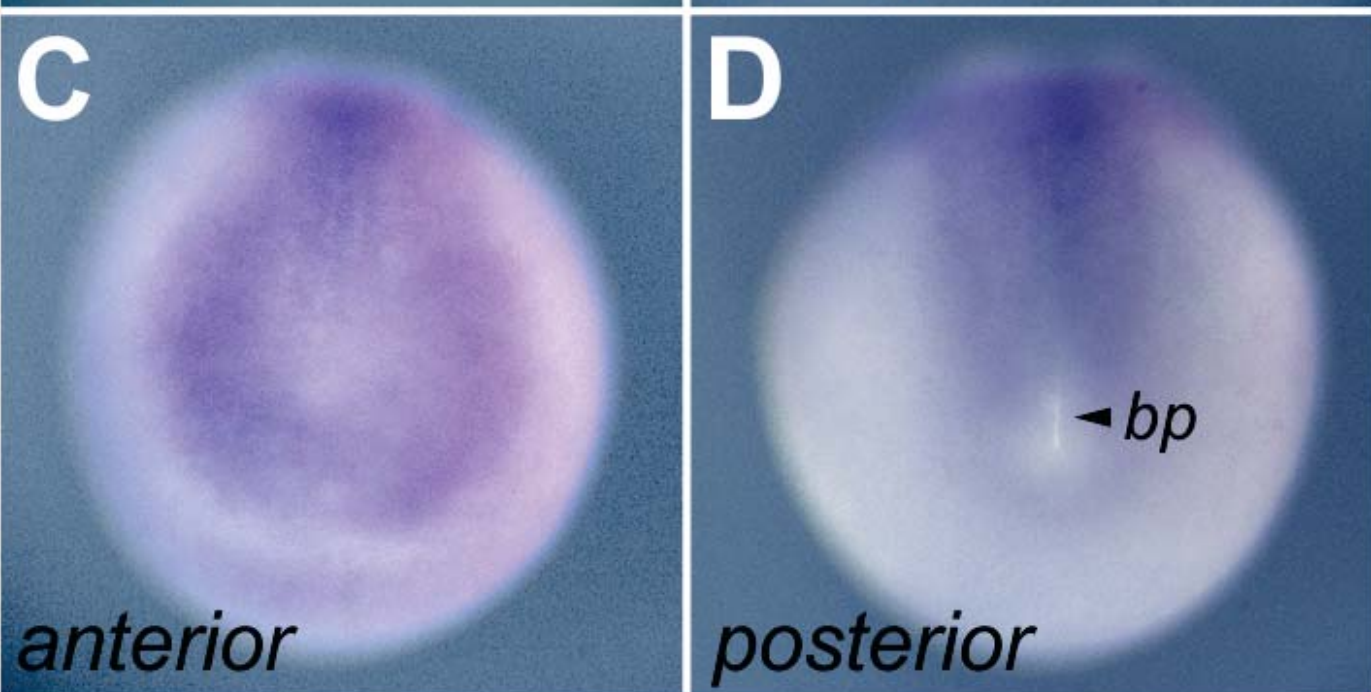
B







early neurula
(stage 14)



late neurula
(stage 19)

