



RESEARCH NOTE

The N terminus of Ascl1 underlies differing proneural activity of mouse and *Xenopus* Ascl1 proteins [version 1; referees: 2 approved]

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Abstract

The proneural basic-helix-loop-helix (bHLH) transcription factor Ascl1 is a master regulator of neurogenesis in both central and peripheral nervous systems *in vivo*, and is a central driver of neuronal reprogramming *in vitro*. Over the last three decades, assaying primary neuron formation in *Xenopus* embryos in response to transcription factor overexpression has contributed to our understanding of the roles and regulation of proneural proteins like Ascl1, with homologues from different species usually exhibiting similar functional effects. Here we demonstrate that the mouse Ascl1 protein is twice as active as the *Xenopus* protein in inducing neural- β -tubulin expression in *Xenopus* embryos, despite there being little difference in protein accumulation or ability to undergo phosphorylation, two properties known to influence Ascl1 function. This superior activity of the mouse compared to the *Xenopus* protein is dependent on the presence of the non-conserved N terminal region of the protein, and indicates species-specific regulation that may necessitate care when interpreting results in cross-species experiments.

Keywords

Ascl1, neurogenesis, proneural, *Xenopus*, bHLH.

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	1	2
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Introduction

Proneural basic-helix-loop-helix (bHLH) transcription factors have conserved roles from *Drosophila* to vertebrates, acting in cascades to drive and coordinate the multiple stages of neurogenesis¹. Vertebrate homologues of the *Drosophila achaete-scute complex* include mammalian Ascl1 (mash1/mAscl1)², and *Xenopus* Ascl1 (Xash1/xAscl1)³. In mammals, Ascl1 is involved in development of GABAergic neurons in the ventral telencephalon and dorsal spine⁴, mesencephalic dopaminergic neurons⁵, rhombencephalic serotonergic neurons⁶ and central and peripheral noradrenergic neurons⁷. In parallel, *Xenopus* Ascl1 is expressed pre and post-metamorphically in the ventricular zone of the pros-, mes-, and rhombencephalon³, with a distinct role in GABAergic fate in the retina⁸, and in noradrenergic identity in antero-ventral neural precursors⁹.

Development of *Xenopus* primary neurons expressing neural- β -tubulin has provided a simple assay for investigating proneural protein activity and regulation *in vivo*, for example¹⁰⁻¹². Proneural gene homologues from different species are often used interchangeably due to the striking conservation of expression and function as outlined above. Ascl1 is also a key cellular reprogramming factor in the regenerative medicine field¹³ and its molecular mechanisms of action are a current area of study where species-specific phenomena may be critical for selection of appropriate model systems. We present a short study to compare the activity of mouse and *Xenopus* Ascl1 proteins in driving neurogenesis in *Xenopus* embryos. Surprisingly, we observe a marked difference in potency between the mouse and *Xenopus* Ascl1 homologues, and show that the differences in potency map to the non-conserved residues in the N terminus of the protein.

Methods

Animal care

All work has been carried out under UK Home Office Licence and has passed an Institutional ethical review committee assessment at the University of Cambridge.

Plasmids and constructs

The coding regions of mouse *Ascl1* (Genbank accession number [NM008553](#)) and *Xenopus Ascl1* (Genbank accession number [NM001085778](#)) were cloned into pCS2+ vectors between EcoRI and XhoI or BamHI and XhoI sites respectively, adding a single HA tag at 3' end. The chimeric N-m/bHC-xAscl1 construct was generated by double digest of both plasmids with BamHI and BglI. The N terminal fragment of the mouse gene was purified as the insert and the bHLH and C terminus of the *Xenopus* gene in pCS2+ was used as the vector for ligation. The NTdelxAscl1 construct was generated by PCR amplification of the conserved N terminal region, bHLH and C terminus of the *Xenopus* gene, (therefore removing the non-conserved 5' region of the N terminus) using primers

[5' GATCGGATCCACCATGAAGAGGCAACGCTCGG] and

[3' GATCCTCGAGTCAGAACCAAGTGGTGAAGTC] with cloning into pCS2+ between BamHI and XhoI. Nucleotide and

protein sequence alignments were conducted with ClustalW software¹⁴.

Xenopus laevis embryo manipulation

All efforts are made to ameliorate suffering to any animal. For example, the colony of approximately 80 *X. laevis* females are housed and cared for by a dedicated team of animal technicians operating under Home Office Licence. Each experiment requires eggs from 2 or 3 females (depending on N = 2 or 3) and females are used on rotation within the colony with at least a 3-month rest period after laying. A single male frog is sacrificed under humane conditions and Home Office Licence to provide testes for at least 16 experiments. Embryos obtained from fertilised eggs are used for the experiments and development is stopped 48 hours post fertilisation when embryos reach late neurula stage and prior to formation of tadpoles.

Thus, *X. laevis* eggs were obtained by standard hormone methods of induction, and subsequently fertilised *in vitro*. pCS2+ constructs were linearised and capped mRNA was transcribed *in vitro* using the SP6 mMessage mMachine[®] kit (Ambion). Embryos were injected unilaterally at the two cell stage with mRNA as indicated in the text, with GFP (for qPCR) or β -gal (ISH) as lineage tracers. Embryos were cultured at 18°C in Ficol solution and staged according to¹⁵. At stage 18, embryos were either snap-frozen for qPCR analysis or fixed in MEMFA for 90 minutes, washed twice in PBS, followed by PBS supplemented with 2mM MgCl₂ and embryos stained in 1 mg/ml X-gal in X-gal mixer. For recipes see¹². Embryos were again washed twice in PBS before dehydration and storage in methanol.

Whole mount *in situ* hybridisation (ISH)

Dig-oxigenin-labelled anti-sense probes were synthesised from the following plasmids: *X. laevis neural- β -tubulin*¹⁶ and *X. laevis xMyt1*¹⁷. Whole mount ISH was performed as described in [12](#) and embryos were scored for the extent and pattern of marker expression as described in data analysis.

Quantitative real-time PCR (qPCR)

GFP expression was used to confirm successful injection and samples of four embryos were snap frozen. Whole embryo RNA was extracted using the RNeasy[®] Mini kit (Qiagen) and template cDNAs synthesised with the QuantiTect[®] Reverse Transcription Kit (Qiagen). qPCR was performed using the Quantifast[®] SYBR Green PCR kit (Qiagen) in a LightCycler[®] 480 (Roche). Thermal cycling conditions: 95°C for 5 minutes, then 45 cycles of 95°C for 10s, 60°C for 10s and 72°C for 20s. EF1 α reference gene (Genbank accession [NM001087442](#)): Forward, CACCATGAAGCCCTTACTGAG; Reverse, TGATAACCTGT-GCGGTAAATG. N- β -Tubulin target gene (Genbank accession [NM001086064](#)): Forward, TGGATTGGGAACCAGGCA; Reverse, GCTCAGCTCCTTCGGTGTA.

Western blotting

For western blot analysis, 12 embryos were snap frozen at stage 12.5 and whole embryo protein was extracted as described in [12](#). For detection of phospho-status, samples were incubated

in the presence or absence of lambda protein phosphatase (NEB) for 1 hour at 30°C. 60µg total protein was loaded on to pre-cast BioRad Criterion™ TGX 18% gels in Tris-Glycine buffer. Primary antibodies were used at 1:2000 dilution for at least 1 hour at room temperature (tubulin) or at 4°C overnight (HA): Rat HRP-conjugated anti-HA clone 3F-10 antibody (Roche; 12013819001) and mouse anti-α-tubulin clone B-5-1-2 antibody (Sigma; T5618). Anti-tubulin antibody was detected with a sheep HRP-conjugated anti-mouse antibody at 1:10000 dilution (GE Healthcare; NA931V).

Data analysis

For ISH data, embryos were scored for the extent and pattern of gene expression on the injected side relative to uninjected side and to uninjected embryos. Scores were assigned as 0, no difference; +1, mild increase in expression within the neural tube with or without occasional ectopic expression on the injected side; +2, moderate increase with ectopic expression occurring in patches on the injected side and sometimes bilaterally; +3, substantial increase with extensive ectopic expression in a more homogenous pattern on the injected side and sometimes bilaterally. Experiments were conducted in independent duplicate in different batches of eggs and the N numbers refer to the range of total numbers of embryos in each injection category.

For qPCR data, mRNA expression was normalised to expression of reference gene EF1α and mRNA levels in the injected embryos were calculated relative to stage-matched uninjected controls. Mean values are plotted and error bars show the standard error of the mean from three independent experiments. Statistical significance was calculated by a paired two-tailed student T test; NS = not significant; * = $p < 0.05$; ** = $p < 0.025$; *** = $p < 0.0125$. Western blots show two independent experiments. For protein quantification, Image J software was used as described in¹².

Results

Mouse Ascl1 is more active than *Xenopus* Ascl1 in the *Xenopus* primary neurogenesis assay

Xenopus primary neurons endogenously develop in the trigeminal ganglia and three bilateral stripes on the neural plate¹⁸, but over-expression of proneural proteins by micro-injection of mRNA can lead to expansion of the endogenous domains and induction of neural-β-tubulin expression across the lateral and ventral epidermis¹². In order to directly compare the effects of over-expression of the mouse and *Xenopus* Ascl1 proteins, equivalent plasmids were constructed with the coding region of each wild-type gene placed directly after the SP6 promoter and with a single C terminal HA tag.

Two cell-stage embryos were unilaterally injected with 100pg mRNA encoding either mAscl1 or xAscl1 and with βgal as a lineage tracer. At stage 18, embryos were assayed by *in situ* hybridisation (ISH) for expression of N-β-tubulin as a marker of primary neurogenesis¹⁹, and for xMyt1 that is induced downstream of proneural proteins and mediates resistance to lateral inhibition¹⁷ (Figure 1A-C). xMyt1 expression mirrors N-β-tubulin expression, consistent with both of these genes being induced

by the Ascl1 proteins. In comparison, mouse Ascl1 produces a moderate increase in expression of N-β-tubulin and xMyt1 across the lateral epidermis, while the *Xenopus* protein results in less pronounced ectopic neurogenesis that is concentrated in the region of the developing neural tube. As these experiments involve protein translation *in vivo* from over-expressed coding sequence mRNA, the observed differences in Ascl1 activity must be mediated at a post-translational level, rather than differences in Ascl1 gene expression or mRNA processing.

Mouse and *Xenopus* Ascl1 proteins are both phosphorylated and show similar protein stability

Proneural transcription factors are known to be unstable proteins, which are turned over rapidly by the ubiquitin-proteasome system²⁰. To explore potential species differences in protein accumulation/stability following mRNA expression, Ascl1 levels in whole embryo extracts were compared by western blot, probing for the HA-tag at the C terminus of each protein (Figure 1D). Mouse Ascl1 migrates as a single band and at a higher molecular weight than xAscl1, in part expected from the extended N terminus in the mouse protein (see below). In contrast, *Xenopus* Ascl1 migrates as a broad or multiple bands that may represent various post-translationally modified isoforms. When the amount of mAscl1 and xAscl1 is measured relative to the tubulin loading controls in two independent experiments (shown), there are no significant differences in protein accumulation; 1.60 ± 0.11 units for xAscl1 and 1.54 ± 0.08 units for mAscl1, indicating that accumulation and stability are similar for the two proteins and cannot account for the differences in activity.

Multi-site phosphorylation has been shown to have a marked inhibitory effect on Ascl1 proneural activity²¹. To determine whether there are differences in phosphorylation between mAscl1 and xAscl1, extracts from embryos expressing the two proteins were treated with lambda protein phosphatase prior to separation and western blot (Figure 1E). The multiple bands of xAscl1 protein collapse to a single faster migrating band in the presence of phosphatase enzyme, suggesting that the multiple bands in the untreated samples represent xAscl1 phospho-forms. Similarly, the single band of the mouse protein also undergoes a prominent increase in migration after phosphatase treatment, indicating that mouse Ascl1 is also phosphorylated, and indeed may be more uniformly phosphorylated than the *Xenopus* protein. Thus both proteins accumulate to comparable levels following over-expression and both undergo phosphorylation, so these properties cannot account for the observed *in vivo* differences in activity.

Mouse and *Xenopus* proteins are highly conserved except for the N terminus

Protein sequence alignment of mAscl1 and xAscl1 reveals a high degree of species conservation in all but the N terminus (Figure 2A). The bHLH domains of mouse and *Xenopus* proteins (shown in blue and green) are identical, and the C terminus (purple) differs by just four amino acids that are broadly conserved alternative residues. Furthermore, the 35 residues in N terminal fragment adjacent to the bHLH domain

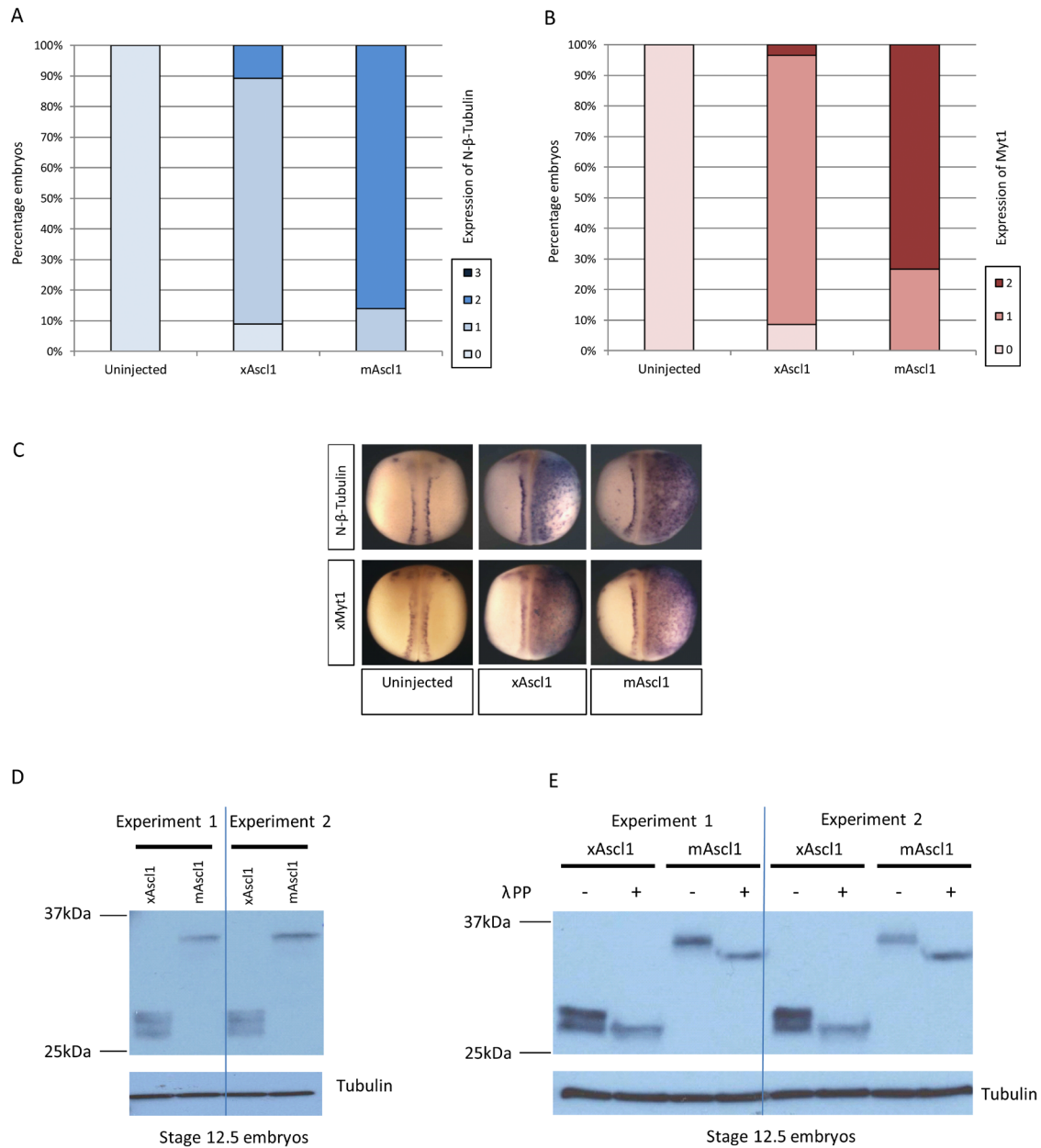


Figure 1. mAscl1 is more active than xAscl1 despite no difference in protein stability. (A–C) Two cell stage embryos were unilaterally injected with 100pg of mRNA encoding either xAscl1 or mAscl1. At stage 18, embryos were assayed by *in situ* hybridisation (ISH) and scored for gene expression relative to uninjected control embryos. (A) N- β -tubulin expression [N=50-78 embryos per category from two experiments]. (B) Myt1 expression [N=58-63 embryos from two experiments]. (C) Representative images of embryos with injected side to the right, stained with pale blue β -gal tracer. Injection of equal amounts of mRNA results in greater gene upregulation by mAscl1 than xAscl1. (D) Western blot analysis of stage 12.5 whole embryo extracts from two independent experiments, over-expressing 200pg of each HA-tagged construct and detected with anti-HA antibody; tubulin as a loading control. There are no significant differences in protein expression between the two constructs. (E) Whole embryo extracts from (C) were incubated with or without lambda protein phosphatase enzyme prior to western blot as before. Both mouse and *Xenopus* Ascl1 are phosphorylated.

are identical in all but two residues between species. However, outside of this, the most N terminal regions of the two proteins diverge in both sequence and length.

To investigate the potential role of the N terminus in conferring Ascl1 protein activity, a chimeric construct was made

(Figure 2B) by substituting the non-conserved N terminal region of the *Xenopus* protein with the mouse equivalent (N-m/bHC-xAscl1). Similarly, a deletion mutant was made to remove this non-conserved N terminus from the *Xenopus* protein, leaving only the conserved regions from KRQRS... (NTdelxAscl1).

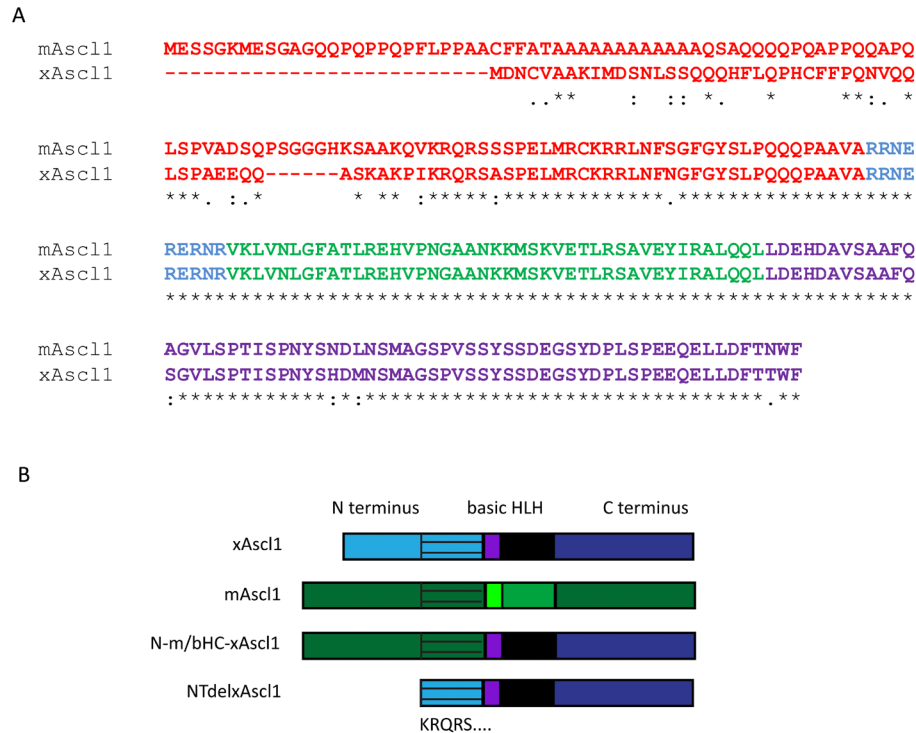


Figure 2. mAscl1 and xAscl1 proteins differ in their N terminal sequences. (A) Protein sequence alignment of mAscl1 and xAscl1 using ClustalW software. N terminus shown in red; basic domain in blue; HLH domain in green; C terminus in purple. A consensus line is shown below the alignment to indicate the degree of conservation of amino acids at each position: (*) denotes identical residues; (:) denotes highly conserved residues; (.) denotes weakly conserved residues. (B) Schematic representation of constructs made to investigate the importance of differences in the N terminus of the Ascl1 protein. The conserved region of the N terminus (from KRQRS) is shown with black stripes through the respective blue (xAscl1) or green (mAscl1) region, and this is retained in NTdelxAscl1 so that only the non-conserved region of the N terminus is deleted.

The non-conserved region of the N terminus mediates species differences in Ascl1 activity

Using the *Xenopus* neurogenesis assay as before, mRNA encoding chimeric and truncated forms of Ascl1 (Figure 2B) was injected unilaterally in two-cell stage embryos, and expression of N- β -Tubulin was detected in stage 18 embryos by qPCR and ISH (Figure 3). Exchange of the non-conserved N terminal region of mouse Ascl1 in place of the shorter N-terminal region of xAscl1 results in enhanced activity of the chimera to the level of native mAscl1; by qPCR, both mAscl1 and N-m/bHC-xAscl1 induce around two-fold higher level of N- β -Tubulin expression than wild-type xAscl1. This indicates that the non-conserved region of the N terminus of the mouse protein mediates its relatively greater potency. Interestingly, deletion of the corresponding non-conserved region of the *Xenopus* protein without its replacement by the respective region from mAscl1 also increases N- β -Tubulin expression relative to wild-type xAscl1, although this is not significant on qPCR analysis. This may suggest that while the N terminus of the mouse protein enhances Ascl1 activity, the corresponding region of the *Xenopus* protein may have an inhibitory influence.

Discussion/conclusions

In this study we have determined that mouse Ascl1 protein is approximately twice as active as the *Xenopus* protein at inducing ectopic primary neurons in *Xenopus* embryos. This requires the presence of the non-conserved N terminal residues in the mouse protein, but is not mediated through changes in protein accumulation or Ascl1 phosphorylation.

Different activity between mouse and *Xenopus* homologues in *Xenopus* embryos has previously been reported for another master regulatory bHLH protein in muscle, MyoD. The C terminus of xMyoD causes cytoplasmic retention of the protein until mesoderm induction, while mMyoD is constitutively nuclear²². The differing activity of mouse and *Xenopus* Ascl1 *in vivo* in *Xenopus* embryos may similarly be due to differences in nuclear localisation, although we have yet to explore this possibility. Species differences in activity could also be due to cofactor binding involving the N terminus of the protein, for instance, association of mouse Ascl1 with an activating partner or reduced association with an inhibitory factor. As such, if using *Xenopus* to dissect the molecular mechanisms of

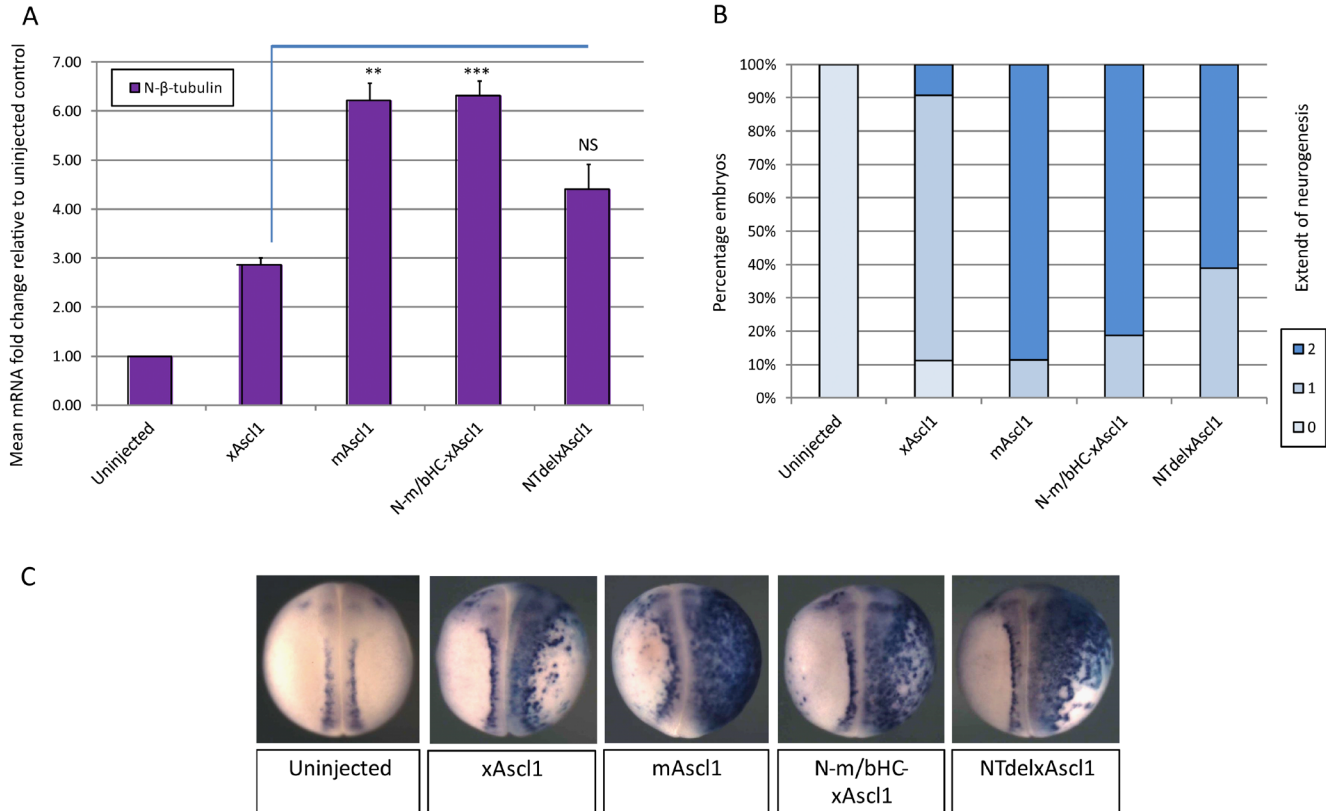


Figure 3. Differential activity between mAscl1 and xAscl1 maps to the N terminus of the protein. Two cell stage embryos were unilaterally injected with 100pg of mRNA encoding each construct, as labelled. At stage 18, embryos were assayed for expression of N- β -tubulin relative to uninjected control embryos. **(A)** qPCR data [N=3] with significance calculated by paired student T test; NS = not significant; * = $p < 0.05$; ** = $p < 0.025$; *** = $p < 0.0125$. **(B)** Semi-quantitative scoring of grade of neurogenesis after in situ hybridisation [N=49-75 embryos per category from two experiments]. **(C)** Representative images of embryos with injected side to the right. The non-conserved N terminal region of mAscl1 is able to confer the activity of the mouse protein on chimeric xAscl1. Deletion of the non-conserved N terminus in xAscl1 also enhances the activity of xAscl1 but this deletion mutant is still not as active as mAscl1.

proneural activity, consideration should be given to potential differences in activity between homologous proteins from different species.

Data availability

Raw data files are available in Open Science Framework: The N terminus of Ascl1 underlies differing proneural activity of mouse and *Xenopus* Ascl1 proteins: <https://doi.org/10.17605/OSF.IO/CN76S23>

See *Methods* section for description of data analysis and ISH scoring. Datasets presented are as follows:

- Fig1A_ISH and Fig1B_ISH: Semi-quantitative scoring of expression of N- β -Tubulin (Fig1A) or xMyt1 (Fig1B) for each injection category showing total number of embryos from two independent experiments.
- Fig1C_embryos: Representative images from 50–78 embryos in each category in two independent experiments.
- Fig1D_WesternBlot.
- Fig1E_WesternBlot.

- Fig3A_qPCR: Mean fold change in N- β -tubulin expression relative to uninjected controls; three independent experiments.
- Fig3B_ISH: Semi-quantitative scoring of grade of neurogenesis for each injection category showing total number of embryos from two independent experiments.
- Fig3C_embryos: Representative images from 49-75 embryos in each category in two independent experiments.

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](https://creativecommons.org/licenses/by/4.0/) (CC0 1.0 Public domain dedication).

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References

1. Bertrand N, Castro DS, Guillemot F: **Proneural genes and the specification of neural cell types.** *Nat Rev Neurosci.* 2002; **3**(7): 517–30.
[PubMed Abstract](#) | [Publisher Full Text](#)
2. Lo LC, Johnson JE, Wuenschell CW, *et al.*: **Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells.** *Genes Dev.* 1991; **5**(9): 1524–37.
[PubMed Abstract](#) | [Publisher Full Text](#)
3. Ferreiro B, Skoglund P, Bailey A, *et al.*: ***XASH1*, a *Xenopus* homolog of *achaete-scute*: a proneural gene in anterior regions of the vertebrate CNS.** *Mech Dev.* 1993; **40**(1–2): 25–36.
[PubMed Abstract](#) | [Publisher Full Text](#)
4. Parras CM, Schuurmans C, Scardigli R, *et al.*: **Divergent functions of the proneural genes *Mash1* and *Ngn2* in the specification of neuronal subtype identity.** *Genes Dev.* 2002; **16**(3): 324–38.
[PubMed Abstract](#) | [Publisher Full Text](#)
5. Park CH, Kang JS, Kim JS, *et al.*: **Differential actions of the proneural genes encoding *Mash1* and neurogenins in Nurr1-induced dopamine neuron differentiation.** *J Cell Sci.* 2006; **119**(Pt 11): 2310–20.
[PubMed Abstract](#) | [Publisher Full Text](#)
6. Pattyn A, Simplicio N, van Doorninck JH, *et al.*: ***Ascl1/Mash1* is required for the development of central serotonergic neurons.** *Nat Neurosci.* 2004; **7**(6): 589–95.
[PubMed Abstract](#) | [Publisher Full Text](#)
7. Hirsch MR, Tiveron MC, Guillemot F, *et al.*: **Control of noradrenergic differentiation and *Phox2a* expression by *MASH1* in the central and peripheral nervous system.** *Development.* 1998; **125**(4): 599–608.
[PubMed Abstract](#)
8. Mazurier N, Parain K, Parlier D, *et al.*: ***Ascl1* as a novel player in the *Ptf1a* transcriptional network for GABAergic cell specification in the retina.** *PLoS One.* 2014; **9**(3): e92113.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
9. Parlier D, Ariza A, Christulia F, *et al.*: ***Xenopus* zinc finger transcription factor *IA1 (Insm1)* expression marks anteroventral noradrenergic neuron progenitors in *Xenopus* embryos.** *Dev Dyn.* 2008; **237**(8): 2147–57.
[PubMed Abstract](#) | [Publisher Full Text](#)
10. Chitnis A, Kintner C: **Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in *Xenopus* embryos.** *Development.* 1996; **122**(7): 2295–301.
[PubMed Abstract](#)
11. Hindley C, Ali F, McDowell G, *et al.*: **Post-translational modification of *Ngn2* differentially affects transcription of distinct targets to regulate the balance between progenitor maintenance and differentiation.** *Development.* 2012; **139**(10): 1718–23.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
12. Hardwick LJ, Philpott A: **Multi-site phosphorylation regulates *NeuroD4* activity during primary neurogenesis: a conserved mechanism amongst proneural proteins.** *Neural Dev.* 2015; **10**: 15.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
13. Vierbuchen T, Ostermeier A, Pang ZP, *et al.*: **Direct conversion of fibroblasts to functional neurons by defined factors.** *Nature.* 2010; **463**(7284): 1035–41.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
14. Chenna R, Sugawara H, Koike T, *et al.*: **Multiple sequence alignment with the Clustal series of programs.** *Nucleic Acids Res.* 2003; **31**(13): 3497–500.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
15. Nieuwkoop PD, Faber J: **Normal table of *Xenopus laevis*.** New York: Garland Publishing; 1994.
[Reference Source](#)
16. Vernon AE, Devine C, Philpott A: **The cdk inhibitor p27^{Kic1} is required for differentiation of primary neurones in *Xenopus*.** *Development.* 2003; **130**(1): 85–92.
[PubMed Abstract](#) | [Publisher Full Text](#)
17. Bellefroid EJ, Bourguignon C, Hollemann T, *et al.*: **X-MyT1, a *Xenopus* C2HC-type zinc finger protein with a regulatory function in neuronal differentiation.** *Cell.* 1996; **87**(7): 1191–202.
[PubMed Abstract](#) | [Publisher Full Text](#)
18. Henningfeld KA, Locker M, Perron M: ***Xenopus* Primary Neurogenesis and Retinogenesis.** *Functional Development and Embryology.* 2007; **1**(1): 26–36.
[Reference Source](#)
19. Oschwald R, Richter K, Grunz H: **Localization of a nervous system-specific class II beta-tubulin gene in *Xenopus laevis* embryos by whole-mount in situ hybridization.** *Int J Dev Biol.* 1991; **35**(4): 399–405.
[PubMed Abstract](#)
20. McDowell GS, Hardwick LJ, Philpott A: **Complex domain interactions regulate stability and activity of closely related proneural transcription factors.** *Biochem Biophys Res Commun.* 2014; **450**(4): 1283–90.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
21. Ali FR, Cheng K, Kirwan P, *et al.*: **The phosphorylation status of *Ascl1* is a key determinant of neuronal differentiation and maturation *in vivo* and *in vitro*.** *Development.* 2014; **141**(11): 2216–24.
[PubMed Abstract](#) | [Publisher Full Text](#)
22. Rupp RA, Snider L, Weintraub H: ***Xenopus* embryos regulate the nuclear localization of *XMyoD*.** *Genes Dev.* 1994; **8**(11): 1311–23.
[PubMed Abstract](#) | [Publisher Full Text](#)
23. Hardwick L: **The N Terminus of *Ascl1* Underlies Differing Proneural Activity of Mouse and *Xenopus Ascl1* Proteins.** *OSF.* 2018.
<http://www.doi.org/10.17605/OSF.IO/CN76S>

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In this research note, the authors have compared the proneural activity of *Xenopus* and mouse transcription factor *Ascl1*, by mRNA injection in *Xenopus* embryos. They demonstrated that the mouse *Ascl1* protein is surprisingly more active in inducing ectopic neurons than the *Xenopus* one. Using chimeric and truncated forms of *Ascl1*, the authors determined that this species difference in *Ascl1* activity is mediated by the N terminus of *Ascl1*. This is interesting considering that this region was identified as poorly conserved between the two species. The molecular mechanisms underlying its distinct influence in *Xenopus* and mouse remain to be investigated.

Overall, the research note is clearly written, the methodology is sound and the data are convincing. The illustration in Fig. 1C could however be improved. It is explained that *Xenopus* *Ascl1* mRNA injection leads to ectopic neurogenesis that is concentrated in the region of the developing neural tube (compared to broader ectopic neurogenesis observed following injection of the mouse mRNA). The figure indeed shows an embryo injected with *Xenopus* *Ascl1* mRNA, displaying restricted ectopic N-tubulin expression. However, it also has a restricted β -gal staining to the same region. It would have been more convincing to show an embryo with β -gal staining on the whole side of the embryo and ectopic N-tubulin expression restricted to the neural tube region.

Although the data appear convincing in graphs of Fig. 1A,B and Fig. 3B, statistical tests would have been appropriate to draw the conclusions.

The authors wrote that *Ascl1* is a central driver of neuronal reprogramming *in vitro*. Specifying *in vitro* is not necessary since it has also been shown *in vivo* that forced *Ascl1* expression can trigger reprogramming, for instance in Müller cells in response to injury. The authors could cite Ueki *et al.*, PNAS 2015.

On western blots, it should be written alpha-tubulin since it could be confusing with the N-tubulin expression that is analyzed elsewhere in the manuscript.

The authors determined that mouse *Ascl1* protein is approximately twice as active as the *Xenopus* protein at inducing ectopic primary neurons in *Xenopus* embryos. It is not clear how this two-fold difference has been calculated.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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In this Research Note, Hardwick and Philpott use a simple assay to assess the ability of mouse and *Xenopus* Ascl1 to generate primary neurones in *Xenopus*. It is already known that *Xenopus* Ascl1 mRNA injected into an early stage *Xenopus* embryo will cause an increase in neurogenesis, the interesting result here is that the mouse version, injected into *Xenopus*, is even more active in this process. By using a chimeric mouse-*Xenopus* Ascl1 and a deleted version of the *Xenopus* protein they provide evidence that the poorly conserved N-terminal part of the protein is responsible for differences in its activity.

The report is concise and informative and the approach technically sound. The results clearly implicate the N-terminal region of the proteins. Other factors are excluded, since experiments on the broad phosphorylation status of the proteins indicate both are to some degree phosphorylated within the embryo and there are no significant differences in the accumulation of the Ascl1 proteins, suggesting that they have similar stabilities.

The inclusion of two additional constructs, a truncated version of the mouse protein and a chimeric form with the *Xenopus* N-terminus in front of the rest of the mouse protein, would have provided a more complete comparison. The results as they stand, however, give a clear indication of the significance of the N-terminal region, which is the main aim of this short report, even if the underlying molecular mechanism

remains uncertain.

As a minor point, a comment in the Methods section that the construction and cloning of the deleted version places a start codon directly adjacent to the remainder of the protein (KRQS...) might avoid some confusion.

One aspect that could be covered in a more detail concerns the lack of conservation in the N-terminal region of Ascl1. It raises several questions: How different is the sequence between the model organisms *Xenopus*, zebrafish, chicks and mice, for example and how conserved is the N-terminal region between two mammals such as mice and humans? Adding a brief comment in the text or a few more lines to the Clustal alignment to make the level of conservation more apparent would be helpful and would clarify whether *Xenopus* (or mouse) is an odd case, or whether this sequence just isn't well conserved across the vertebrates.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
