The phosphorylation status of Ascl1 is a key determinant of neuronal differentiation and maturation in vivo and in vitro

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ABSTRACT
Generation of neurons from patient fibroblasts using a combination of developmentally defined transcription factors has great potential in disease modelling, as well as ultimately for use in regeneration and repair. However, generation of physiologically mature neurons in vitro remains problematic. Here we demonstrate the cell-cycle-dependent phosphorylation of a key reprogramming transcription factor, Ascl1, on multiple serine-proline sites. This multisite phosphorylation is a crucial regulator of the ability of Ascl1 to drive neuronal differentiation and maturation in vivo in the developing embryo; a phosphomutant form of Ascl1 shows substantially enhanced neuronal induction activity in Xenopus embryos. Mechanistically, we see that this un(der)phosphorylated Ascl1 is resistant to inhibition by both cyclin-dependent kinase activity and Notch signalling, both of which normally limit its neurogenic potential. Ascl1 is a central component of reprogramming transcription factor cocktails to generate neurons from human fibroblasts; the use of phosphomutant Ascl1 in place of the wild-type protein significantly promotes neuronal maturity after human fibroblast reprogramming in vitro. These results demonstrate that cell-cycle-dependent post-translational modification of proneural proteins directly regulates neuronal differentiation in vivo during development, and that this regulatory mechanism can be harnessed to promote maturation of neurons obtained by transdifferentiation of human cells in vitro.

KEY WORDS: Ascl1, Neurogenesis, Phosphorylation, Transdifferentiation

INTRODUCTION
Direct transdifferentiation of human fibroblasts into neurons using defined combinations of transcription factors has recently emerged as a powerful new approach for both disease modelling and repair (Vierbuchen et al., 2010; Caiazzo et al., 2011; Pang et al., 2011; Pfisterer et al., 2011; Yang et al., 2011). However, this is generally an inefficient process and neurons generated typically display phenotypic immaturity. Transdifferentiation using transcription factors has been further enhanced by adding small molecule inhibitors of growth factor pathways (Ladewig et al., 2012). Nevertheless, the mechanistic basis of the links between the cellular signalling environment and the ability of defined factors to drive neuronal differentiation is poorly characterized in both normal development and under transdifferentiation conditions.

During development, the proneural transcription factor Ascl1 (also known as Mash1) is a crucial regulator of multiple aspects of neurogenesis, including progenitor cell maintenance, neuronal differentiation and neurite outgrowth in the central and peripheral nervous systems (Bertrand et al., 2002; Castro and Guillemot, 2011). Moreover, Ascl1 overexpression alone converts ectodermal cells to ectopic neurons in Xenopus embryos (Talikka et al., 2002). Ascl1 is also a central and common component of a variety of methods developed for reprogramming of mouse and human fibroblasts into neurons in vitro (Vierbuchen et al., 2010; Caiazzo et al., 2011; Pang et al., 2011; Pfisterer et al., 2011). For instance, introduction of the ‘BAM’ factors Ascl1, Brn2 (Pou3f2 – Mouse Genome Informatics) and Myt1l, together with NeuroD, drives transdifferentiation of human fibroblasts to functional neurons (Yang et al., 2011). However, to enhance the efficacy of this approach, we must fully characterize mechanisms that regulate the activity of factors used for reprogramming. Here, we demonstrate that post-translational modification of Ascl1 by multisite phosphorylation regulates its ability to drive neuronal differentiation and transdifferentiation in vivo and in vitro.

RESULTS
Ascl1 is regulated by multisite phosphorylation
Ascl1 is a core component of a variety of transcription factor protocols that have been used to drive transdifferentiation of mammalian fibroblasts directly into neurons (Vierbuchen et al., 2010; Caiazzo et al., 2011; Pang et al., 2011; Pfisterer et al., 2011; Yang et al., 2011; Torper et al., 2013), but the post-translational control of this protein is largely unknown. Phosphorylation of a number of conserved serine-proline (SP) sites regulates the activity of Ngn2 (Neurog2 – Mouse Genome Informatics) and Olig2 basic helix-loop-helix (bHLH) proneural proteins (Ma et al., 2008; Ali et al., 2011; Gaber and Novitch, 2011). Ascl1 also contains multiple SP sites (supplementary material Fig. S1) that we hypothesized could be functionally modified through phosphorylation by proline-directed kinases. We see that mouse Ascl1 is subject to multisite phosphorylation in the complex and biologically relevant environment of interphase Xenopus egg extracts, resulting in slowed migration on SDS-PAGE that is reversed by phosphatase treatment (Fig. 1A, arrows). Moreover, incubation in mitotic extract leads to a greater reduction in mobility, indicating more phosphorylation in an environment with more Cdk kinase activity (Fig. 1A). Multisite phosphorylation occurs on serines of SP pairs; mutation of all six SP sites in Ascl1 to generate S-A Ascl1 prevented interphase extract-mediated modification of Ascl1 protein, and substantially reduced modification in mitotic extract, indicating that phosphorylation occurs on serines in SP pairs. Further mutational analysis where SP sites are either individually or additively mutated (supplementary material Fig. S1) revealed that multiple SP sites at both the N- and C-terminal either side of the bHLH domain are
phosphorylated (supplementary material Fig. S2); mutants with either N-terminal or C-terminal sites intact both show reduced mobility compared with wild-type protein in I and M extracts, although mutations of all SP sites to alanine-proline is required to restore SDS-PAGE mobility of extract-incubated to that of unincubated protein (Fig. 1A; supplementary material Figs S1 and S2).

Serine-proline or threonine-proline is minimally required for Cdk-dependent phosphorylation (Errico, 2010), and we see that Ascl1 undergoes cell cycle-dependent phosphorylation in Xenopus egg extracts on these sites (Fig. 1; supplementary material Fig. S2); a mutant in which SP sites have been mutated to alanine-proline shows a dramatic reduction in phosphorylation.

To determine whether Ascl1 can indeed act as a target for Cdks, we incubated Ascl1 protein with active recombinant cyclin/Cdk proteins. When wild-type Ascl1 was incubated with CyclinA/Cdk2, its migration on SDS-PAGE was significantly retarded, and a smear of slower-migrating Ascl1 protein indicates phosphorylation on more than one site (supplementary material Fig. S3). S-A Ascl1 in this assay shows markedly reduced retardation compared with wild-type protein in I and M extracts, although the physiological relevance of this observation is not clear; in vitro kinase assays can show target promiscuity not seen in a more physiological setting. Alternatively, it is possible that Cyclin/Cdks can phosphorylate and activate a kinase in the reticulocyte lysate used for in vitro translation that can go on to phosphorylate Ascl1 on non-SP sites.

We did not observe significant SDS-PAGE retardation of Ascl1 as a result of incubation with CyclinD/Cdk4 or CyclinB/Cdk1 (supplementary material Fig. S3). This could indicate an inability of these Cdks to target Ascl1 in this assay, but could still be compatible with phosphorylation on sites that does not result in a shift on SDS-PAGE, a phenomenon that likely to be amino acid context dependent. To confirm that Cdk2 is able to phosphorylate Ascl1 in Xenopus cytoplasm, we added recombinant Cdk inhibitor p27Xic1 (Vernon et al., 2003) to interphase egg extract and noted a marked increase in mobility of Ascl1 migration commensurate with inhibition of phosphorylation (supplementary material Fig. S4). As Cdk2 complexes with CyclinE and not CyclinA at this embryonic stage, and D-type cyclins are not expressed prominently in eggs (Richard-Parpaillon et al., 2004; Philpott and Yew, 2008), this indicates that Ascl1 can also be phosphorylated by CyclinE/Cdk2 (supplementary material Fig. S4).

Fig. 1. Ascl1 function is inhibited by SP-directed phosphorylation. (A) Wild-type and S-A Ascl1 translated in vitro in the presence of 35S-methionine and incubated in buffer (XB), Xenopus interphase or mitotic egg extracts with or without phosphatase, separated by SDS-PAGE. (B) Xenopus embryos injected unilaterally with 100 pg GFP, wild-type Ascl1 or S-A Ascl1, detecting β-III tubulin at stage 19 by in situ hybridisation. (C) The percentage of embryos with no difference (0), moderate increase (1) or substantial increase (2) of β-III tubulin expression comparing injected and un.injected sides (n≥57); ***P≤0.005. (D) qPCR analysis of Delta, Myt1 and β-III tubulin expression in stage 19 Xenopus embryos overexpressing 50 pg Ascl1 or S-A Ascl1 (mean ±s.e.m.; *P≤0.05). I, interphase; M, mitotic; WT, wild type.

To assess how SP site phosphorylation affects the ability of Ascl1 to drive ectopic neurogenesis in vivo, we overexpressed wild-type Ascl1 and phosphomutant Ascl1 (S-A Ascl1) in Xenopus embryos by mRNA microinjection into one cell of a two-cell embryo (Fig. 1B,C; supplementary material Fig. S1). Mutation of SP sites substantially enhanced the ability of S-A Ascl1 to induce ectopic neurogenesis compared with wild type. One possibility is that enhanced activity results from greater protein stability of S-A Ascl1. When protein levels were compared in Xenopus embryos, S-A Ascl1 was expressed at a modestly higher level than the wild-type protein (supplementary material Fig. S5A).

We then investigated whether increased S-A Ascl1 activity could be explained by enhanced activation of downstream targets that promote differentiation by the phosphomutant protein. To this end, we compared the effect of wild-type and S-A Ascl1 overexpression on activation of different direct downstream targets with functions in progenitor maintenance or differentiation. Quantitative PCR analysis of injected Xenopus embryos showed that S-A Ascl1 overexpression resulted in at least a sevenfold enhancement of
expression of Myt1 and Tubb (β-III tubulin), both markers of neuronal differentiation, compared with induction by wild-type Ascl1 (Fig. 1D). By contrast, expression of Delta, the Notch ligand involved in non-cell-autonomous progenitor maintenance pathways, is only upregulated twofold by S-A Ascl1 compared with wild type (Fig. 1D). This indicates that preventing phosphorylation of Ascl1 on SP sites may preferentially potentiate neuronal differentiation over progenitor maintenance.

Mutation of some SP sites, particularly the two sites in the N-terminus of Ascl1, led to a greater shift in SDS-PAGE mobility than mutation of other sites (supplementary material Figs S1 and S2), although it is not clear if this is because of differences in extent of phosphorylation or due to differences in the effect of phosphorylation of different sites on electrophoretic mobility. To see whether phosphorylation on both the N- and C-terminals contributes to the regulation of Ascl1 transcriptional activity, we overexpressed mutants of N- and C-terminal SP sites on Ascl1 mutants (supplementary material Fig. S1) and compared their activity to both wild type and S-A Ascl1 in Xenopus embryos (supplementary material Fig. S6). Neither mutation of the N-terminal or C-terminal SP sites alone was sufficient to significantly enhance neural beta tubulin expression, although an approximately 15-fold enhancement was seen when both N- and C-terminal sites were mutated together. This demonstrates that specific sites whose loss of SP phosphorylation may not result in a significant change in migration on SDSPAGE, nevertheless still contribute substantially to regulation of Ascl1 activity. We also compared relative activation of Delta and Myt1 by N- and C-terminal mutants. We saw that mutation of both the N- and C-terminal sites is needed for maximal activation of expression of these targets, but that Myt1 expression was much more sensitive to the phospho-status of Ascl1 than Delta expression (supplementary material Fig. S6).

Shortening the cell cycle by upregulation of cyclin-dependent kinase activity promotes progenitor maintenance (Richard-Parpaillon et al., 2004; Hindley, et al., 2012). We saw that overexpression of CyclinA2/Cdk2 inhibited endogenous primary neurogenesis in Xenopus embryos. Both endogenous neurogenesis and ectopic neurogenesis driven by ectopic wild-type Ascl1 were inhibited by increased Cdk activity, whereas S-A Ascl1 was resistant to this inhibition (Fig. 2A,B). Thus, mutation of SP sites on Ascl1 renders the protein insensitive to Cdk-dependent cues that would otherwise limit neuronal differentiation in vivo. Conversely, cell cycle shortening promotes neuronal differentiation (Lange and Calegari, 2010). We next investigated whether S-A Ascl1 may also potentiate neuronal differentiation by enhancing cell cycle lengthening/exit compared to wild-type Ascl1 by monitoring phospho-histone H3 (pH3) expression after Ascl1 overexpression. Both wild-type and S-A Ascl1 inhibited pH3 expression by similar amounts, indicating that phosphomutant Ascl1 does not enhance neurogenesis by potentiating cell cycle lengthening (Fig. 3A).

There are other mechanisms by which cell cycle regulators can influence proneural protein function beyond regulation by Cdk-dependent phosphorylation (Hindley and Philpott, 2012). For instance, we have previously shown that the Xenopus Cdk inhibitor, p27Xic1, is absolutely required for Ngn2-dependent differentiation of primary neurons in Xenopus beyond its ability to lengthen the cell cycle (Vernon et al., 2003). Cell cycle lengthening/exit upon Ascl1 expression, as evidenced by reduction in pH3 expression, is likely to be accompanied by upregulation of Cdkis (Farah et al., 2000). However, a requirement for Cdkis in Ascl1-mediated neurogenesis has not been investigated, nor has whether any such requirement is dependent on the ability of Ascl1 to be phosphorylated on Cdk sites. To investigate whether p27Xic1 is required for Ascl1-induced neurogenesis, Xenopus embryos were injected in one cell at the two-cell stage with Ascl1 or S-A Ascl1, with either a control antisense morpholino (Con Mo) or a morpholino directed against Xic1 (Xic1 Mo), which has previously been shown to efficiently block Xic1 protein expression (Vernon et al., 2003). The control morpholino had no discernible impact on neurogenesis, whereas Xic1 Mo injection inhibited both endogenous primary neurogenesis and ectopic neurogenesis induced by Ascl1 expression (Fig. 3B,C), demonstrating that wild-type Ascl1 requires Cdk inhibitor activity for the efficient induction of differentiated neurons. By contrast, S-A Ascl1 retained its ability to induce neuronal differentiation even in the absence of p27Xic1 protein. We then investigated whether enhancing p27Xic1 levels can promote Ascl1-driven neurogenesis as it can for Ngn2 (Vernon et al., 2003). Coinjection of low levels of p27Xic1 that are sufficient to substantially slow the cell cycle (Vernon et al., 2003) resulted in enhancement of Ascl1-mediated ectopic neurogenesis. However, significant synergy between p27Xic1 and S-A Ascl1 was not observed (Fig. 3B,C).

Proneural proteins including Ascl1 transcriptionally upregulate the Notch ligand Delta (Casanova et al., 1999) that inhibits neuronal differentiation in adjacent cells via Notch signalling in a process known as lateral inhibition (Chitnis et al., 1995). In Xenopus, overexpression of the constitutively active Notch intracellular domain (NICD) can inhibit both endogenous neurogenesis and proneural protein-driven ectopic neurogenesis. Post-translational inhibition of proneural proteins by Notch is relieved by expression

Fig. 2. Phosphomutant Ascl1 confers resistance to cell cycle inhibition of neurogenesis. (A) Xenopus embryos were injected unilaterally in one of two cells, injected side to right, with either 100 pg of GFP or wild-type/S-A Ascl1 mRNA, and 500 pg of CyclinA/Cdk2 (A2) mRNA, as indicated, and subject to in situ hybridisation for β-III tubulin at stage 19. (B) Graphical representation of percentage of injected embryos (as indicated) displaying no difference (0), substantial increase (1), substantial increase (2), moderate decrease (−1) and complete loss (−2) of neurons on the injected site compared with the uninjected side (n≥44), ***P≤0.005.
Phosphomutant proneural proteins enhance neuronal maturation in transdifferentiation protocols

Next, we sought to examine whether phosphoregulation of Ascl1 that we observe in *Xenopus* embryos is active in mammalian systems, and especially during transdifferentiation of adult somatic cells to neurons. Numerous studies have recently demonstrated that Ascl1 is a key factor required for direct conversion of mammalian fibroblasts into neurons *in vitro* (Vierbuchen et al., 2010; Caiazzo et al., 2011; Pang et al., 2011; Pfisterer et al., 2011; Yang et al., 2011) and more recently *in vivo* (Torper et al., 2013). Transdifferentiation of mouse fibroblasts can be achieved by overexpression of Brn2, Ascl1 and Myt1 (BAM) by lentiviral delivery, but addition of NeuroD to these ‘BAM factors’ (BAMN) is required for transdifferentiation of human fibroblasts (Pang et al., 2011). Although capable of firing action potentials, these neurons are morphologically immature with relatively short neurites (Yang et al., 2011). On the basis of its enhanced ability to drive neuronal differentiation *in vivo* in the presence of cues that would normally limit neurogenesis (Figs 1-4), we hypothesized that phosphomutant Ascl1 may also enhance neuronal transdifferentiation *in vitro*. To test this, and switching to human Ascl1 (ASCL1) protein for use in these human cells (supplementary material Fig. S1), we substituted S-A ASCL1 (supplementary material Fig. S1) for wild-type ASCL1 protein to compare neuron generation and maturation from human fibroblasts (Fig. 5). We saw that ASCL1 and S-A ASCL1 are expressed at equal mRNA and protein levels after lentiviral transduction into HFL1 cells (supplementary material Fig. S5B,C).

S-A BAM (S-A ASCL1, BRN2 and MYT1L) doubled the conversion efficiency of human fibroblasts to neurons compared with wild-type BAM (wild-type ASCL1, BRN2 and MYT1L), whereas the addition of NEUROD to S-A BAM (S-A BAMN) did not further enhance this efficiency (Fig. 5B). We next measured the morphological complexity and functional maturity of the neurons generated using BAM and BAMN protocols, using wild-type or S-A ASCL1. After 3 weeks of neuronal conversion *in vitro*, we measured several parameters of morphological maturity, including neurite length, neurite extension, branching and axonal complexity. Neurons generated using S-A ASCL1 had substantially enhanced morphological maturity compared with those generated using wild-type ASCL1 (Fig. 5C-G). In particular, we saw a significant enhancement in the number of neuronal branch points and the number of neurons displaying tertiary branching events. This enhanced branching is further evidenced by a 50% increase in the axonal complexity index, a numerical value that reflects the overall complexity of axonal projections from a population of neurons (Marshak et al., 2007).

To investigate electrophysiological maturity, patch-clamp recordings were performed on transdifferentiated neurons 6 weeks after infection.
Neurons were treated with stepwise current injections to determine their capacity to fire action potentials. Neurons generated using S-A ASCL1 showed a significantly enhanced ability to trigger successive action potentials in response to current injections when compared to wild-type ASCL1 (Fig. 5H). For both S-A BAM and S-A BAMN-derived neurons, we observed fast inward and outward currents in response to stepwise voltage depolarizations, corresponding to opening of voltage-gated Na+ and K+ channels, respectively (Fig. 5I). Larger currents were elicited in S-A BAMN-derived neurons, again signifying greater functional maturity.

Small molecule (SM) inhibitors of SMAD pathways and GSK3β have been included along with the proneural proteins ASCL1 and Ngn2 in human fibroblast reprogramming protocols to enhance neuronal maturity (Ladewig et al., 2012). We wished to investigate whether SMs work by dephosphorylating proneural proteins to increase their activity, which would result in no synergy between S-A BAM and SMs, or alternatively whether maturity could be further enhanced by combining SMs with phosphomutant proneural proteins, indicating parallel pathways. Consequently, we undertook fibroblast reprogramming in the presence of SMs using S-A ASCL1 alone with S-A mouse Ngn2, which we previously showed to have enhanced neuronal differentiation activity compared to wild-type Ngn2 (Ali et al., 2011). Again, neurons generated with these phosphomutant proneural proteins show significantly enhanced maturity (Fig. 6A), as evidenced by neurite length and innervation area (Fig. 6B) compared with wild type. In addition, branching was fourfold greater, of higher order and the axonal complexity index was doubled (Fig. 6B).

Whole cell electrophysiological recordings were performed on neurons generated using wild-type ASCL1+ wild-type Ngn2 and S-A ASCL1+ S-A Ngn2 6 weeks post-infection. Neurons generated by both wild-type and phosphomutant proneural factors had excitable membrane properties (Fig. 6C), with inward sodium and outward potassium currents (Fig. 6D). However, mean peak sodium current amplitudes in S-A ASCL1+ S-A Ngn2-induced neurons were notably larger than in wild-type-induced neurons (Fig. 6D), and resting membranes potentials in phosphomutant-derived neurons were significantly more negative compared with wild-type controls. In contrast to the weak action potentials elicited by wild-type factors, neurons induced with phosphomutant factors triggered sustained mature action potential firing (Fig. 6C). Altogether, these data demonstrate that neurons induced using S-A ASCL1 and S-A Ngn2 had neuronal membrane and action potential firing properties that were markedly more mature than neurons generated using a standard wild-type ASCL1 and wild-type Ngn2 protocol.

DISCUSSION

In this study, we demonstrate that the ability of the Ascl1 protein to drive neuronal differentiation is regulated by multisite phosphorylation on serine-proline sites. Ascl1 is phosphorylated on multiple sites on both its N- and C-terminals. Preventing phosphorylation results in upregulation of key differentiation targets such as Myt1 and neural beta-tubulin. Interestingly, the upregulation of these differentiation targets is greater than the Ascl1 target Delta, which mediates Notch-dependent non-cell autonomous progenitor maintenance, accounting for the enhanced neurogenesis seen with the phosphomutant protein.

We have previously shown that multisite phosphorylation of the proneural protein Ngn2 by Cdks inhibits both its ability to bind stably to DNA, and its ability to drive neuronal differentiation (Ali et al., 2011; Hindley et al., 2012) in a manner analogous to the phoshoregulation of Ascl1 we present here. We propose that, similar to Ngn2, differential sensitivity of downstream targets to Ascl1 phosphorylation probably results from differences in the requirement for epigenetic remodelling by Ascl1 for activation; epigenetically available targets tolerate a potentially low promoter dwell time of phosphorylated Ascl1 whereas those requiring substantial modification for activation, often genes associated with differentiation such as Myt1 and neural beta tubulin, require more stable association to bring about the epigenetic changes needed for activation (Ali et al., 2011). The similarities between the regulation of Ngn2 and Ascl1 raises the possibility that multisite S-P directed phosphorylation by Cdks may be a general mechanism to control differentiation activity of other proneural proteins in the nervous system in response to cell cycle and signalling cues.

In the case of Ngn2, we saw that it was the number of phospho-sites available that controlled the ability of the protein to drive neuronal differentiation, not their precise location (Ali et al., 2011). The same seems to be true for Ascl1. Ascl1 is phosphorylated on both N- and C-terminal sites, and preventing phosphorylation of both terminals of the molecule is required for maximal activity of the protein. Although mouse Ascl1 contains an additional N-terminal SP site not found in the human protein, both human and mouse Ascl1 S-A mutants show significantly enhanced activity driving neuronal differentiation, indicating that this SP site does not play an extra key regulatory role in Ascl1. We see that multiple sites can be phosphorylated by Cdks and its ability to drive neuronal differentiation (Ali et al., 2011; Hindley et al., 2012) in a manner analogous to the phosphoregulation of Ascl1 we present here. We propose that, similar to Ngn2, differential sensitivity of downstream targets to Ascl1 phosphorylation probably results from differences in the requirement for epigenetic remodelling by Ascl1 for activation; epigenetically available targets tolerate a potentially low promoter dwell time of phosphorylated Ascl1 whereas those requiring substantial modification for activation, often genes associated with differentiation such as Myt1 and neural beta tubulin, require more stable association to bring about the epigenetic changes needed for activation (Ali et al., 2011). The similarities between the regulation of Ngn2 and Ascl1 raises the possibility that multisite S-P directed phosphorylation by Cdks may be a general mechanism to control differentiation activity of other proneural proteins in the nervous system in response to cell cycle and signalling cues.
Notch signalling has been shown to both inhibit neurogenesis and suppress maturation of neurons already born (Giniger, 2012). The precise molecular mechanism of post-translational inhibition by Notch signalling is not clear, although inhibition of Ngn2 protein can be relieved by overexpression of *Xenopus* Myt1. As S-A Ascl1 upregulates Myt1 sevenfold more effectively than wild-type Ascl1, this is likely to be the way it escapes Notch-mediated inhibition. The ability of phosphomutant Ascl1 to resist Notch-mediated as well as Cdk-mediated inhibition may be key to its ability to overcome cellular signals limiting neuronal transdifferentiation in human fibroblasts, and its use in reprogramming cocktails results in enhanced neuronal differentiation *in vitro* compared with the wild-type protein (Figs 5 and 6).

Recently, Ascl1 has been identified as an ‘on-target pioneer factor’ defined as a transcription factor that can bind its targets when either accessible or nucleosome-bound (Wapinski et al., 2013). Nucleosome-bound targets of Ascl1 in fibroblasts are characterized by a trivalent chromatin signature consisting of enriched H3K4Me1, H3K27ac and H3K9me3, and cell types such as keratinocytes that do not show this trivalent signature at Ascl1 targets are resistant to BAM factor reprogramming. S-A ASCL1 is modestly better than the wild-type protein at converting fibroblasts to neurons (Figs 5 and 6) but is significantly better at inducing maturation of the neurons so generated. This may result from enhanced chromatin binding of the phosphomutant protein on promoters of key targets driving maturation (Hindley et al., 2012), although it is not yet clear whether enhanced maturity results from a higher expression of the same targets as are activated by wild-type Ascl1 (Fig. 1D), or whether S-A Ascl1 can activate additional targets with less favourable chromatin configurations. As genome-wide targets of Ascl1 in fibroblasts have now been identified, this question can be assessed, along with testing the ability of S-A Ascl1 to reprogram cell types that do not have these permissive trivalent marks at target promoters. As well as acting as a pioneer factor, Ascl1 recruits Brn2 to responsive promoters (Wapinski et al., 2013); enhanced co-factor recruitment is another possible mechanism to potentiate S-A Ascl1 ability to drive neuronal differentiation and transdifferentiation.
Our developmental findings pointed to a potentially enhanced ability of phosphomutant Ascl1 to enhance neuronal maturation after reprogramming of human fibroblasts, and this was indeed the case (Figs 5 and 6). A more in-depth study of mechanisms of developmental post-translational regulation of factors used in reprogramming protocols offers a potentially powerful platform to identify signalling pathways and regulatory mechanisms that facilitate and suppress differentiation and transdifferentiation in vivo and in vitro.

MATERIALS AND METHODS

Xenopus laevis extracts and embryos
Acquisition of Xenopus laevis eggs and embryos, preparation and injection of synthetic mRNA, staging of embryos, in situ hybridisation, pH3 analysis egg extract preparation and preparation and western blotting of embryo extracts were performed as described previously (Vernon et al., 2003; Vosper et al., 2007, 2009; Philpott and Friend, 1994; Richard-Parpaillon et al., 2004).

Xenopus embryos scoring scheme
Xenopus embryos scoring scheme was as described previously (Ali et al., 2011). Embryos were assigned scores from −2 to +2 based on the neuronal staining by in situ hybridisation on the injected side (identified by the co-injected lineage-tracer beta-gal, on the right in all figures) compared to the uninjected side: 0, no difference; −1, a small decrease in staining; −2, a large decrease or no staining; +1, a small increase in staining; +2, a large increase in staining, often with staining outside of the neural plate.

In vitro phosphorylation using recombinant Cdks
Ascl135S-labelled IVT protein was incubated with 250 ng of the indicated recombinant cyclin/Cdk complex (Cell Signaling Technology) with 87.5 μM ATP. The reaction was incubated at 30°C for 1 h, then denatured, resolved by SDS-PAGE and analysed by autoradiography.
Cell culture and lentiviral preparations
HFL1 (ATCC-CCL-145) cells were maintained and expanded in DMEM +GlutaMAX (Gibco) supplemented with 10% FBS (HyClone) and 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma). P19 cells were cultured in α-MEM with 7.5% newborn calf serum and 2.5% fetal bovine serum, 1% Glutamax, and 100 units/ml penicillin, 100 μg/ml streptomycin. Human open reading frame for Ascl1, S-A Ascl1, Bm2, Myt1l, NeuroD, Ngn2 and S-A Ngn2 were cloned into a doxycycline-regulated lentiviral vectors (pLVX-TRE3G). Viruses were generated in HEK293T cells and titres were determined using the Lenti-X Tet-On 3G Inducible Expression System (Clontech) according to the manufacturer’s instructions.

Quantitative PCR
For qPCR analysis, embryos were injected at the one-cell stage with mRNA as indicated. RNA extraction and qPCR analysis were performed as described previously (Ali et al., 2011). The sequences of the primers used were: Delta forward: GCCCCAGAGATGAGTCTTCC, Delta reverse: GCCTTGCCAACCCACTCTACATT, Myt1l forward: AAGGTTTGATGCTCAGGGT, β-III tubulin forward: ACACGGCATTGTACCTACAG, β-III tubulin reverse: AGCTCC-TTCGTTGTAATGAC.

Generation of transdifferentiated neurons and immunostaining
Neuronal conversion was performed as described previously (Pang et al., 2011, Cells). Were co-transduced with a Tet-On transactivator (LVX-Tet3G) and LVX-TRE3G- encoding genes of interest at ratio of 1:1 and multiplicity of infection of 5. Cells were left to proliferate for 5 days before induction with 1 μg/ml doxycycline. During neuronal conversion, cells were maintained in neuronal media (NDiff 227) (StemCells). Where indicated, small molecule inhibitors of the SMAD signalling pathway [SB431542 (Sigma), Noggin (R&D Systems) and LDN-193189 (Miltiemy Biotech) together with GSK-3β inhibitor CHIR99021 (Miltiemy Biotech)] were added for 2 weeks in neuronal media at the concentrations previously described (Ladewig et al., 2012). Neurotrophic factors BDNF, GDNF, NT3 (R&D Systems) and dACMP (Sigma) were supplemented in the neuronal media at the concentrations previously described (Ladewig et al., 2012) to promote prolonged neuronal survival. Six weeks post-transduction, cells were stained for β-III tubulin; 1:1000 (Covance) and chicken anti-MAP2 1:10,000 (Abcam) as previously described.

Electrophysiology
Whole-cell recordings were performed on cells with neuronal morphology at room temperature in artificial spinal fluid: 125 mM NaCl, 25 mM NaHCO3, 1.25 mM NaH2PO4, 3 mM KCl, 2 mM CaCl2, 25 mM glucose and 3 mM pyruvic acid, bubbled with 95% O2 and 5% CO2. Borosilicate glass electrodes (resistance 6-10 MΩ) were filled with an intracellular solution containing 135 mM potassium gluconate, 7 mM NaCl, 10 mM HEPES, 2 mM Na2ATP, 0.3 mM Na3GTP and 2 mM MgCl2. To detect sodium and potassium currents, step depolarizations were made from a holding potential of ~80 to ~40 mV in voltage clamp mode. Sodium currents were blocked with 1 μM tetrodotoxin (Tocris). To detect action potential firing, stepwise current injections were performed in current clamp mode. Recordings were made with a Multiclamp 700 A amplifier (Molecular Devices) and a Digidata 1440 (Molecular Devices). Signals were filtered at 6 kHz, sampled at 20 kHz with 16-bit resolution. Data acquisition and analysis was performed using pCLAMP (Molecular Devices).

Statistical analysis
Statistical analysis for qPCR and neuronal profiling were performed using a one-tailed Student’s t-test (**P<0.05; ***P<0.005); standard error of the mean (s.e.m.) calculated from at least two independent experiments. The Fisher exact statistical test was performed on the Xenopus embryos scoring scheme (**P<0.01; ***P<0.005).

References
Gaber, Z. B. and Novitch, B. G. (2011). All the embryo’s a stage, and Olig2 in its time plays many parts. Neuron 69, 833-835.
mediates axon arbor growth and synapse maturation during the establishment of retinotectal synaptic connectivity. J. Neurosci. 27, 2444-2456.


Figure S1: Schematic representation showing the distribution of SP sites and S-A mutations (AP) within mouse and human Ascl1 proteins. (A) Schematic of Ascl1 serine-proline sites, showing the positions of the serine-proline (SP) sites relative to the bHLH region in mouse and human Ascl1 (mAscl1 and hAscl1). Also shown are phosphomutant versions where SP sites have been mutated to alanine-proline (AP) to generate 6S-A mAscl1 (S-A mAscl1) and 5S-A hAscl1 (S-A hAscl1). (B) Schematic showing the positions of SP and mutated AP sites in a sequential S-A mutant mouse Ascl1 series used to investigate multi-site phosphorylation. (C) Schematic showing mutant mouse Ascl1 where SP sites were mutated to AP in the N-terminus (NT S-A), C-terminus (CT S-A), or both (6S-A).
Figure S2: Ascl1 is phosphorylated on multiple SP sites in Xenopus egg extract. mAscl1 constructs were translated \textit{in vitro} in rabbit reticulocyte lysate in the presence of $^{35}$S-methionine and incubated in Xenopus interphase (I) and mitotic (M) egg extract before separation by SDS-PAGE. (A) Numbers refer to the positions of sites of single serine to alanine (S-A) mutations. (B) Migration of the sequential phospho-mutant mAscl1 constructs described in Figure S1 B.
Figure S3: Phosphorylation of Ascl1 by recombinant cdks. WT and S-A mAcl 35S-labeled IVT proteins were incubated with the indicated recombinant cyclin/cdks in kinase buffer supplemented with 87.5 µM ATP for 1 hour prior to separation on SDS-PAGE, followed by autoradiography.

Figure S4: Ascl1 SP-site phosphorylation is inhibited by the cdk inhibitor Xic1. WT and S-A mAcl 35S-labeled IVT proteins were incubated in either interphase or mitotic Xenopus egg extract with or without addition of recombinant p27Xic1 (a cdk2 inhibitor), followed by separation by SDS-PAGE. P27Xic1 inhibits phosphorylation of WT mAcl1 but not S-A mAcl1 in I extract, but has no effect on migration in M extract, indicating Ascl1 is phosphorylated by cdk2.
Figure S5: Ascl1 and S-A Ascl1 are expressed at equal mRNA and protein levels.  

(A) Western blot analysis of mAscl1 and S-A mAscl1 proteins expressed at stage 19 in *Xenopus* embryos after injection of 50 pg of mRNA, as indicated, representative blot shown, β-Tubulin provides a loading control. 

(B)qPCR analysis of Ascl1 expression in induced neuronal cells 6 weeks following deoxycyclin induction. 

(C) HFL1 were infected with either GFP, hAscl1 or S-A hAscl1 lentiviruses at an MOI of 10. Cells were induced with 1 µg/mL doxycyclin to allow for transgene expression. Cell lysate were prepared 5 days following induction for western blot analysis, representative blot shown, β-Tubulin provides a loading control.
Figure S6: Mutations of SP sites in both the N- and C-terminal domains of Ascl1 is required to fully upregulate downstream target expression in Xenopus embryos. Transcriptional activitation of downstream targets of mAscl1 in embryos injected with 50 pg of the indicated mRNA. For each type of injection, five embryos were snap-frozen at stage 19 and RNA extracted for reverse transcription and qPCR analysis. The data presented as mean normalised to GFP-injected embryos +/- s.e.m from three independent experiments; *≤0.05 relative to WT mAscl1.