# Conserved roles for Oct4 homologues in maintaining multipotency during early vertebrate development

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# Conserved roles for Oct4 homologues in maintaining multipotency during early vertebrate development

**Gillian M. Morrison and Joshua M. Brickman***

All vertebrate embryos have multipotent cells until gastrulation but, to date, derivation of embryonic stem (ES) cell lines has been achieved only for mouse and primates. ES cells are derived from mammalian inner cell mass (ICM) tissue that express the Class V POU domain (PouV) protein Oct4. Loss of Oct4 in mice results in a failure to maintain ICM and consequently an inability to derive ES cells. Here, we show that Oct4 homologues also function in early amphibian development where they act as suppressors of commitment during germ layer specification. Antisense morpholino mediated PouV knockdown in *Xenopus* embryos resulted in severe posterior truncations and anterior neural defects. Gastrulation stage embryos showed reduced expression of genes associated with uncommitted marginal zone cells, while the expression of markers associated with more mature cell states was expanded. Importantly, we have tested PouV proteins from a number of vertebrate species for the ability to substitute Oct4 in mouse ES cells. PouV domain proteins from both *Xenopus* and axolotl could support murine ES cell self-renewal but the only identified zebrafish protein in this family could not. Moreover, we found that PouV proteins regulated similar genes in ES cells and *Xenopus* embryos, and that PouV proteins capable of supporting ES cell self-renewal could also rescue the *Xenopus* PouV knockdown phenotype. We conclude that the unique ability of Oct4 to maintain ES cell pluripotency is derived from an ancestral function of this class of proteins to maintain multipotency.

**KEY WORDS: Gastrulation, Self-renewal, Embryonic stem cell, Lineage commitment, Pou protein, Pou5f1, *Xenopus***

## INTRODUCTION

Germ layer specification involves an elaboration of intrinsic cell-autonomous factors and non-autonomous signalling molecules. Embryonic cells prior to gastrulation have the capacity to respond to various signalling pathways and are therefore uncommitted (Gardner and Beddington, 1988; Snape et al., 1987). This pluripotent feature of early embryonic cells is mirrored in vitro by embryonic stem (ES) cells (Smith, 2001). ES cells are self-renewing pluripotent cell lines derived from the ICM of the mammalian blastocyst that, in mouse, have been shown to be capable of populating all the lineages of the foetus and adult (Beddington and Robertson, 1989). Unlike ES cells, early embryonic cells cannot be considered stem cells as they do not self-renew indefinitely. However, mammalian embryos still need to maintain a population of non-committed cells through a significant amount of cell division to generate the number of cells necessary to complete gastrulation. Although lower vertebrates undergo reductive rather than proliferative cell division during early cleavage (Frederick and Andrews, 1994; Kane et al., 1992), they still require active mechanisms to ensure against premature cellular differentiation. Indeed, studies in *Xenopus* have shown that the future ectoderm cells and the ventral marginal zone cells are multipotent until late gastrulation (Domingo and Keller, 2000; Okabayashi and Asashima, 2003).

A number of factors have been identified that are necessary for the maintenance of ES cell pluripotency, including the cytokines LIF and BMP4, and transcription factors such as Nanog and Oct4 (encoded by *Pou5f1*, referred to here as *Oct4*) (Chambers, 2004). In addition to its role in ES cells, Oct4 is also required for the maintenance of the germ cell lineage (Kehler et al., 2004) and thus appears to have an in vivo role in maintaining multipotency. The ICM and then the epiblast express *Oct4* until gastrulation, when *Oct4* remains expressed only in the posterior epiblast and the primitive streak, but not in cells that have undergone mesendoderm induction (Yeom et al., 1996; Yoshimizu et al., 1999). This expression pattern is consistent with Oct4 having a role in maintaining cells as multipotent until it is the appropriate time for them to become committed to mesoderm, endoderm or ectoderm.

In the absence of Oct4, ES cells lose the capacity to self-renew and subsequently differentiate into extra-embryonic trophectoderm (Niwa et al., 2000). Similarly, *Oct4*$^{-/-}$ embryos die at peri-implantation stages because of the conversion of ICM into trophectoderm (Nichols et al., 1998). The involvement of Oct4 in embryonic versus extra-embryonic fate decisions and its role in the maintenance of primordial germ cells (PGCs) has led to the proposal that Oct4 arose in mammals to perform these specific functions (Pesce et al., 1998). Alternatively, Oct4 and the molecular programmes it regulates might represent an older evolutionary innovation designed to maintain a non-committed cell population in the early embryo until the appropriate time for induction of the germ layers.

Oct4 is a Class V POU domain protein, containing both a POU specific domain (POUs) and a POU homeodomain (POUh) (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990). Based on the degree of conservation within these domains and the linker region, the POU domain proteins have been divided into five classes. Class V POU domain proteins (referred to here as PouV proteins) are present in a number of other species and have been implicated in early embryonic development (Bachvarova et al., 2004; Frank and Harland, 1992; Hinkley et al., 1992; Takeda et al., 1994; Whitfield et al., 1993). To date, a single PouV protein has been identified in teleost fish (Burgess et al., 2002), zebrafish Pou2,

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referred to here as *DrPou2*. Interestingly, DrPou2 has recently been shown to have a role in endoderm induction (Lunde et al., 2004; Reim et al., 2004).

Although no self-renewing ES cell lines have been generated from lower vertebrates, we considered whether the mechanisms governing self-renewal were conserved. Here, we report that a PouV gene from *Xenopus laevis*, *Xlpou91*, has the capacity to maintain murine ES cells in the absence of Oct4. As *Xenopus* has neither induced PGCs nor extra-embryonic development, we believe that ES cell pluripotency may be based in part on conserved, rather than mammalian-specific, aspects of vertebrate development. Moreover, two other *Xenopus* genes, *Xlpou25* and *Xlpou60*, and *axolotl-Oct4* (AmOct4, also referred to as Axoct4) have some ability to rescue ES cell self-renewal, whereas the zebrafish protein Pou2 has none. Knockdown phenotypes of the three *Xenopus* XlpouV genes results in elevated expression of endodermal, organizer and neural markers, aspects of which can be rescued by axolotl and mouse Oct4. These data support our hypothesis that PouV proteins represent a class of transcription factor required for the maintenance of multipotent non-committed cell populations in both *Xenopus* and mouse.

## MATERIALS AND METHODS

### *Xenopus* embryo manipulations and biological assays

*Xenopus laevis* embryos were obtained by in vitro fertilization, staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) and cultured according to Slack et al. (Slack et al., 1984). Embryos were injected at the two-cell stage in both blastomeres with RNA and/or morpholino oligonucleotides. Dextran fluorescein (Molecular Probes) was used as a lineage label (Brickman et al., 2000).

Animal caps were dissected from stage 8 blastula and cultured in the presence or absence of partially purified activin (a gift from J. Smith). Activin units (U) are defined by Cooke et al. (Cooke et al., 1987). In situ hybridization was carried out as described in Brickman et al. (Brickman et al., 2000). Morpholino oligonucleotides were purchased from GeneTools. Sequences were as follows: Xlpou25 MO, 5′-ACATGGTGTCCAAGAGC-TTGCAGTC-3′; Xlpou60 MO, 5′-GTACAATATGGGCTGGTCCATC-TCC-3′; Xlpou91 MO, 5′-GTAGGTCTGTTGGTTATACATGATC-3′. The control morpholino was the standard provided by GeneTools.

The whole-mount TUNEL staining was carried out as previously described (Hensey and Gautier, 1997).

### In vitro translation

Wild-type XlPouV mRNA or non-complementary (NC) XlpouV mRNA were used as a template in a Red Nova Lysate Translation kit (Novagen) according to the manufacturer's instructions in the presence of $^{35}$S-methionine. Translation productions were fractionated by SDS-PAGE and visualized by autoradiography.

### Expression constructs and transcription

Wild-type Xlpou25, Xlpou60, mouse Oct4, DrPou2 and AmOct4, and non complementary (NC) Xlpou25, Xlpou60 and Xlpou91 expression constructs were generated by PCR in pCS2+. Plasmids for RNA injection were linearized with *Not*1 and mRNA generated according to Smith (Smith, 1993). cDNAs were inserted into pCAGIP (Niwa et al., 2002; Niwa et al., 1991) for ES cell rescue.

### Embryonic stem cell culture and transfection

ES cells were cultured according to Li et al. (Li et al., 1995). For the rescue experiments, $2\times10^7$ ZHBTc4 ES cells were electroporated with 100 μg of linearized plasmid DNA followed by culture with or without 2 μg/ml tetracycline (Tc) (Sigma) for 2 days. Cells were then cultured in 2 μg/ml puromycin (Sigma) with or without Tc for 7 days. The resulting colonies were staining for alkaline phosphatase (AP) activity (Sigma-Aldrich) or expanded as clonal lines.

### Luciferase reporter assays

ZHBTc4 ES cells ($1\times10^5$) were plated on a 24-well plate with 2 μg/ml Tc. Twenty-four hours later, 75 ng of reporter plasmids and 150 ng of the test plasmid was transfected according to Brickman et al. (Brickman et al., 2001). For *Xenopus* embryos, 50 pg of reporter plasmid with or without morpholino was injected into both blastomeres of a two-cell stage embryo.

### RNA isolation and real-time RT-PCR

Total RNA was prepared from pools of 15 embryos, 25 animal caps or $10^6$ ES cells using Absolutely RNA RT-PCR (Stratagene). RNA (1 μg) was used as a template for cDNA synthesis. Real-time RT-PCR was performed using a LightCycler (Roche) and the LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green 1 (Roche). Standard curves were generated either from diluted cDNA derived from control embryos or from plasmid. Samples were normalized to ornithine decarboxylase (*Odc*) for *Xenopus* embryos and β-actin for ES cells. PCR primers and conditions can be provided on request.

## RESULTS

### Protein identity, chromosomal synteny and expression profiles indicate conservation between XlpouV proteins and Oct4

To compare the protein sequences of mammalian, amphibian and zebrafish PouV proteins, we performed a clustal-based alignment. Identity is immediately apparent in the POUs and POUh domains, and outside these regions there exists only weak homology (Fig. 1A). As both the POU domain and either the N- or C-terminal activation domains of Oct4 have been shown to be required for the maintenance of ES cell self-renewal (Niwa et al., 2002), we looked at these regions specifically. Both global and regionally focused alignments of these proteins failed to reveal any conserved motifs within these regions indicative of shared functional properties that might be present within a subset of this family (Fig. 1A; Table 1).

The identification of three *Xenopus laevis* PouV proteins, all of which are conserved in *Xenopus tropicalis,* has been confirmed by extensive database analysis. The three genes are arranged in tandem and are within syntenic groups conserved between *Xenopus*, mouse and zebrafish (Burgess et al., 2002) (Fig. 1B).

Analysis of mRNA expression by in situ hybridization for *Xlpou60*, *Xlpou25* and *Xlpou91* was performed (Fig. 1C). *Xlpou60* mRNA is maternally expressed and restricted to the animal hemisphere in both unfertilized oocytes and early cleavage stage embryos. Expression remains in the animal and marginal zones of the embryo but is reduced by the late blastula stage and undetectable by the early gastrulation stages. *Xlpou25* and *Xlpou91* are first transcribed at the onset of zygotic transcription in the animal and

**Table 1. Sequence identity of PouV protein domains**

| Protein sequence | M/91 | M/60 | M/25 | M/D | 91/60 | 91/25 | 91/D | 60/25 | 60/D | 25/D |
|---|---|---|---|---|---|---|---|---|---|---|
| Complete | 33.6 | 31.2 | 32.3 | 36.2 | 35.5 | 49.0 | 31.7 | 40.8 | 29.8 | 31.9 |
| POUs+h | 66.0 | 57.7 | 65.3 | 68.0 | 63.8 | 76.0 | 62.0 | 67.8 | 59.7 | 65.3 |
| C-terminal | 8.3 | 8.3 | 7.3 | 11.6 | 36.5 | 54.2 | 40.0 | 37.5 | 35.8 | 36.8 |
| N-terminal | 25.5 | 28.4 | 20.7 | 15.9 | 33.9 | 39.1 | 13.3 | 34.7 | 12.9 | 13.3 |

The percentage of sequence identity of the various domains of the PouV proteins was calculated using ClustalW alignments (as in Fig. 1).
Abbreviations: POUs+h, POU specific domain and POU homeodomain; M, mouse Oct4; 91, Xlpou91; 60, Xlpou60; 25, Xlpou25; D, zebrafish Pou2.

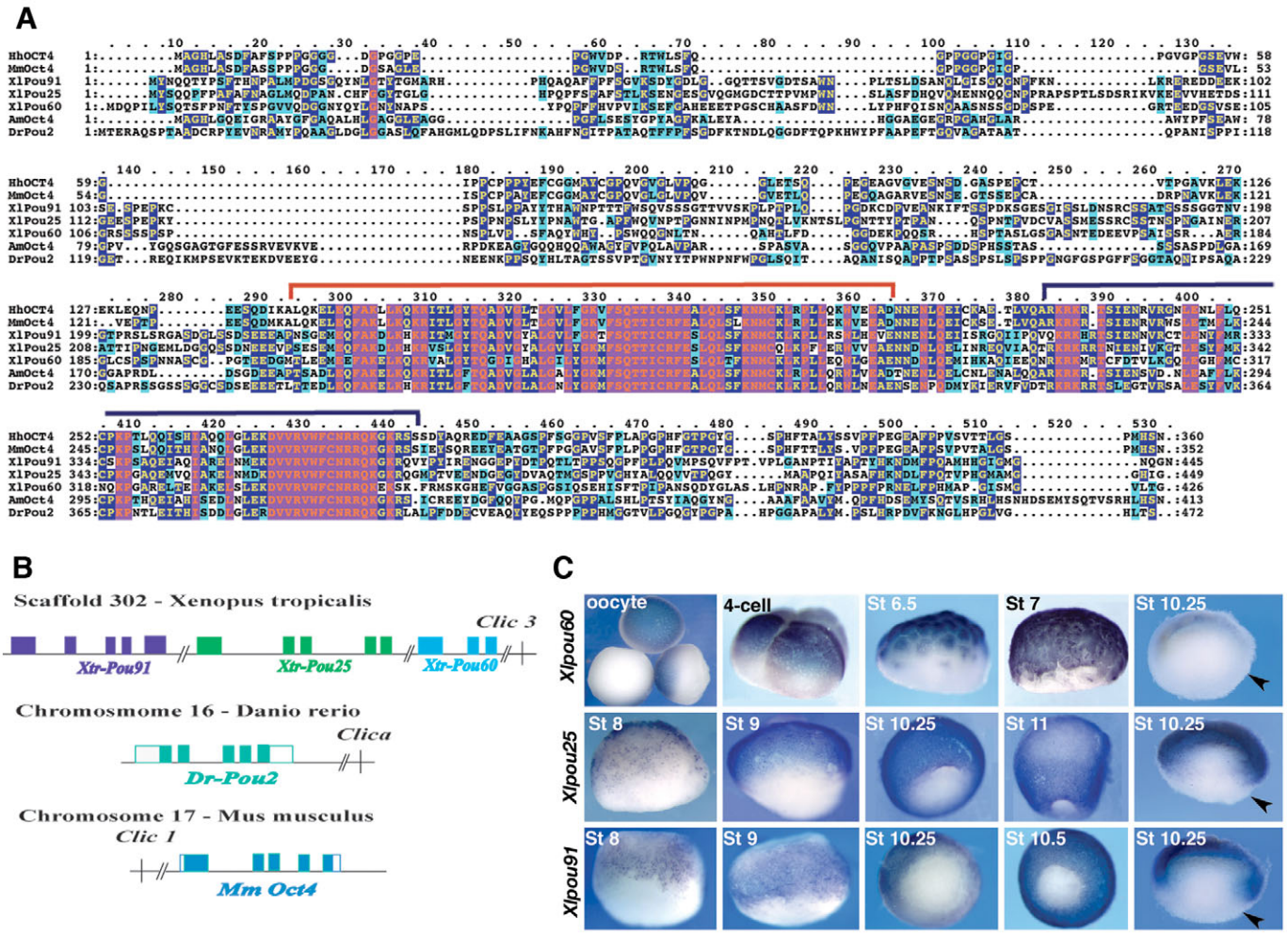


**Fig. 1. Protein sequence, chromosomal synteny and expression profile of *Xenopus* PouV genes.** (**A**) Alignment of human (HsOCT4), mouse (MmOct4), *Xenopus* (Xlpou91, Xlpou25, Xlpou60), axolotl (AmOct4) and zebrafish (DrPou2) PouV proteins. POUs and POUh domains are overlined in red and blue, respectively. Pink indicates identical residues, light blue indicates similar residues, dark blue indicates conservation within a subset of residues. (**B**) Syntenic relationship between the *Xenopus tropicalis* sequence scaffold 302, zebrafish chromosome 16 and mouse chromosome 17. (**C**) Expression analysis of the *XlpouV* genes. Embryos on the right were bisected prior to in situ hybridization. The arrowheads indicate the dorsal lip.

marginal zone, similar to *Xlpou60*. *Xlpou25* and *Xlpou91* continue to be expressed throughout the process of gastrulation but only in cells that have not undergone involution. *Xlpou25* and *Xlpou91* expression is then rapidly downregulated as cells begin the process of involution and commitment to their germ layer fate (Fig. 1C). Thus, the composite expression pattern of the *Xenopus* PouV genes is reminiscent of *Oct4*.

Later during development, we observe two additional domains of *Xlpou25* and *Xlpou91* expression in the developing anterior neural tissue and in the posterior neural tube (see Fig. 5A,D). We believe these expression domains to be de novo activation independent of the earlier gastrula expression as transcripts are undetectable at the intervening developmental stages.

## *Xenopus* PouV proteins can rescue self-renewal in Oct4 depleted ES cells

As Oct4 is an absolute requirement for ES cell self-renewal, we could assay the ability of the different PouV family members to substitute for Oct4 through the use of an inducible Oct4 knockout ES cell system (Niwa et al., 2000). Briefly, ZHBTc4 ES cells have both alleles of the endogenous *Oct4* gene inactivated, and expression of Oct4 is maintained within these cells by a tetracycline (Tc) regulatable Oct4 transgene.

Using this system, we could measure the extent of Oct4 rescue conveyed by the different PouV proteins in three ways: (1) the generation of ‘ES cell like’, alkaline phosphatase (AP) positive colonies (the rescue index); (2) long-term self-renewal (generation of clonal cell lines); and (3) expression of ES cell specific markers.

To ensure that differences in ES cell phenotypes were not due to expression level or a global defect in transcriptional regulatory activity, we first tested the ability of all PouV proteins to activate transcription of octamer binding reporter genes. Co-transfection of the PouV proteins with an Fgf4 enhancer reporter in ZHBTc4 ES cells all resulted in similar levels of induced transcription (Fig. 2A) with the exception of mouse Oct4 (V267P), a variant of Oct4 known to be unable to bind DNA when expressed in ES cells (Niwa et al., 2002). We also tested the activity of these proteins by co-transfection with a second reporter gene that contained six reiterated copies of the octamer binding motif (Niwa et al., 2002). Interestingly all three *Xenopus* proteins were potent activators in this context, whereas

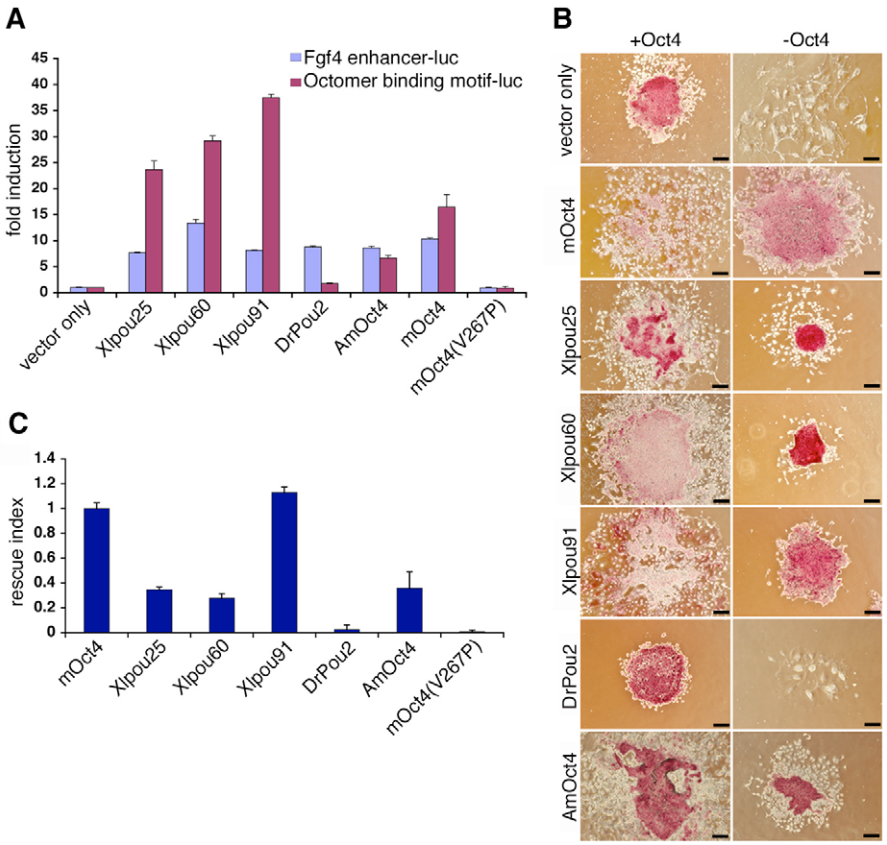


**Fig. 2. Amphibian PouV proteins can support mouse ES cell self-renewal.** (**A**) The indicated PouV cDNAs were co-transfected with the *Fgf4* enhancer or the octamer-binding motif luciferase reporters. Fold induction represents the increase in transcription compared with the vector-only control. Experiments were carried out in triplicate. (**B**) Morphology of day 9 puromycin resistant colonies transfected with the indicated cDNA in the presence or absence of Oct4 transgene expression. Colonies are stained for AP activity (red). Scale bars: 100 μm. (**C**) Rescue index for PouV proteins. The rescue index=the number of ES cell colonies in the absence of Oct4 /number of ES cell colonies in the presence of Oct4. Mouse Oct4 was assigned a value of 1 to which all other values were normalized. Data represents the mean values obtained from three independent experiments.

mouse Oct4, AmOct4 and DrPou2 were less active to varying degrees (Fig. 2A). The ability of all of these proteins to activate transcription from the Fgf4 enhancer reporter indicates that any differences in function they display in ES cells was not due to variability in protein stability.

ES cells normally give rise to AP-positive colonies with a characteristic undifferentiated morphology. In the absence of Oct4, ES cells differentiate into trophoblast. Typical examples of the morphology of cells resulting from transfection of the PouV genes into ZHBTc4 cells in the presence or absence of *Oct4* expression are shown in Fig. 2B. All PouV proteins tested, except mouse Oct4 (V267P) (data not shown) and DrPou2, showed some degree of rescue of the Oct4-null phenotype. Interestingly, only cells transfected with Xlpou91 produce colonies with a morphology and growth rate indistinguishable from ES cells. This result was confirmed quantitatively by culturing half of each transfection in the absence of Oct4 and dividing the number of undifferentiated (AP-positive) colonies growing in the absence of Oct4 by the number of undifferentiated colonies growing in the presence of Oct4. These values, rescue indices, are shown in Fig. 2C.

A rigorous test of whether these genes can support long-term self-renewal is to derive and expand ES cell lines in which the introduced genes are stably expressed in the absence of Oct4. The experimental strategy for expanding and analysing the resultant clonal cell lines is shown in Fig. 3A. For each transfection that produced colonies in the absence of Oct4, colonies were picked and expanded to generate clonal lines. All colonies from the mouse *Oct4* transfection survived for 10 passages (p10), as did those transfected with *Xlpou91*. However only 17% and 80% of colonies from the *Xlpou25* and *Xlpou60* transfection, respectively, survived 10 passages. In cell culture conditions that normally sustain ES cell self-renewal (+LIF) and in the absence of mouse *Oct4* expression (Fig. 3B, left panel), both Xlpou91 and mouse Oct4 lines appear undifferentiated, while Xlpou60 and AmOct4 lines contained a significant number of differentiated cells. Xlpou25 lines were almost all differentiated, with clumps of cells that appear to grow as colonies. DrPou2 cell lines, similar to the parental ZHBTc4 cell line, could not be maintained in the absence of mouse Oct4.

ES cell expansion is dependent on the cytokine LIF and, in its absence, ES cell lines rapidly differentiate (Smith et al., 1988; Williams et al., 1988). We observed that all of our cell lines also differentiated upon LIF withdrawal (Fig. 3B, centre panel) and thus the ability of these rescued cell lines to differentiate is not blocked by the presence of the amphibian PouV proteins. ES cells differentiate in response to increased Oct4 protein levels (Niwa et al., 2000), an effect also observed in our PouV cell lines when the Oct4 transgene was reactivated (Fig. 3B, right panel).

Fig. 3C shows the average expression levels calculated from the individual cell lines derived for each different PouV mRNA transfection. The very low standard deviations of these values illustrate that each clonal cell line, within the set, expressed similar levels of their particular PouV mRNA, indicating that ES cell self-renewal has a specific crucial threshold for each individual PouV protein. Interestingly, the expression level of Xlpou91 transcripts was similar to that of mouse Oct4, whereas the number of transcripts produced in the Xlpou25 and Xlpou60 rescued cell lines proved to be on average 15 fold and 116 fold higher, respectively (Fig. 3C).

Quantitative molecular marker analysis was performed on RNA from the rescued cell lines by real-time RT-PCR (Fig. 3D). We first confirmed that Oct4 expression had been suppressed in these lines. The values presented in Fig. 3D represent the mean level of gene expression calculated from at least two independent cell lines

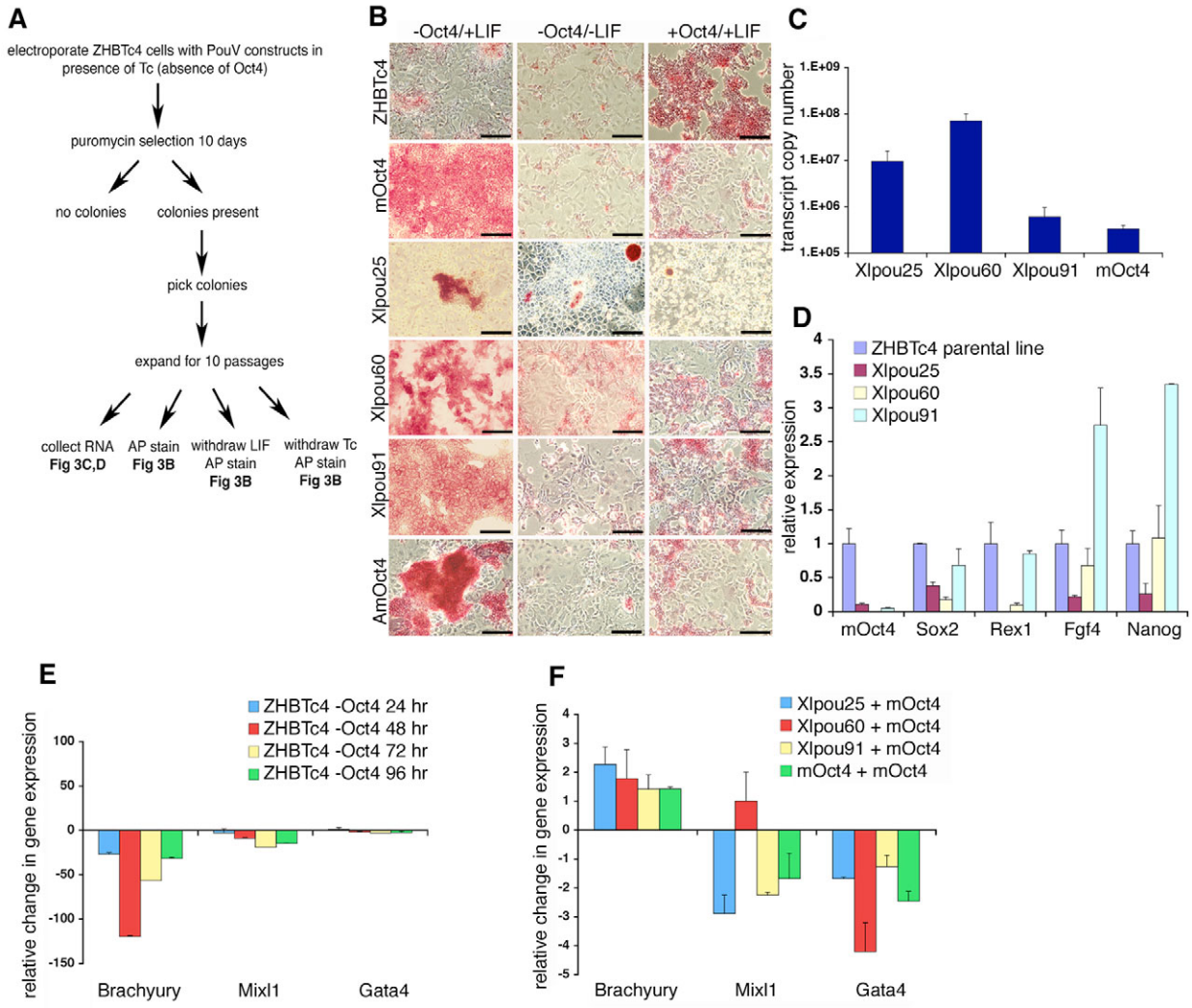


**Fig. 3. Derivation of PouV clonal cell lines.** (**A**) Experimental strategy to expand and analyse PouV clonal cell lines. (**B**) Cell lines stained for AP activity (red). The –Oct4/+LIF cells are at passage 10 (p10), apart from the ZHBTc4 line, shown 72 hours after Oct4 transgene repression. The –Oct4/-LIF cells are at p10, 5 days after LIF withdrawal. The +Oct4/+LIF cells are at p10, 5 days after Oct4 transgene re-expression. Scale bars: 100 μm. (**C**) Real-time RT-PCR analysis for PouV expression. RNA was collected at p10. Values represent the mean expression levels calculated from all available cell lines: Xlpou25 (*n*=2), Xlpou60 (*n*=8), Xlpou91 (*n*=10), mouse Oct4 (*n*=10). (**D**) Real-time RT-PCR analysis of ES cell markers genes from PouV ES cell lines. RNA was obtained at p10. Values represent the mean expression levels calculated from at least two independent cell lines. (**E**) Real-time RT-PCR analysis of ZHBTc4 ES cells following the shut-down of Oct4 transgene expression. RNA was collected at the indicated timepoints following shut down of Oct4 transgene expression. Values were normalized to β-actin and the relative change in gene expression for the marker genes analysed was calculated by dividing the –Oct4 values by the +Oct4 control value. Experiments were carried out in triplicate. (**F**) Real-time RT-PCR analysis of PouV protein overexpressing ES cells. RNA was collected from cell lines maintained in the absence of Oct4 and from cell lines 72 hours after Oct4 transgene re-expression. Values were normalized to β-actin and the relative change in gene expression of the marker genes analysed was calculated by dividing the +Oct4 values by the –Oct4 values. Experiments were carried out in triplicate.

following rescue using the different PouV proteins. Thus, although the real-time PCR technique we employed detected very low residual levels of Oct4 expression on average, we have cell lines supported by each PouV protein that do not express detectable levels of Oct4 (data not shown). Moreover, we have confirmed that this residual level of Oct4 mRNA does not produce detectable levels of Oct4 protein, as determined by immunohistochemistry (data not shown).

In agreement with the degree of rescue produced by the individual *Xenopus* proteins, Xlpou91 cell lines displayed the highest expression levels of the ES cell markers *Sox2*, *Rex1*, *Fgf4* and *Nanog*. The higher degree of differentiation displayed in Xlpou25 and Xlpou60 supported cell lines (Fig. 3B) is consistent with the reduced levels of ES cell gene expression observed in these cells.

As there is no trophoblast lineage in *Xenopus*, we considered whether Oct4 might have a broader function than ES cell self-renewal. RNAi-mediated knockdown of Oct4 in both mouse and human ES cells suggested that Oct4 suppressed the endodermal marker *Gata4* (Hay et al., 2004), while expression of both brachyury and *Gata4* appeared to be lost following Oct4 shut down in ZHBTc4 ES cells (Niwa et al., 2000). Owing to this discrepancy, we thought it important to examine the response of both mesodermal and endodermal markers to variations in Oct4 levels. We found that *Gata4* expression is maintained in the absence of Oct4, while brachyury expression was rapidly downregulated in the ZHBTc4 ES cell line following depletion of Oct4 (Fig. 4E). We also observe that re-expression of the Oct4 transgene in the mouse

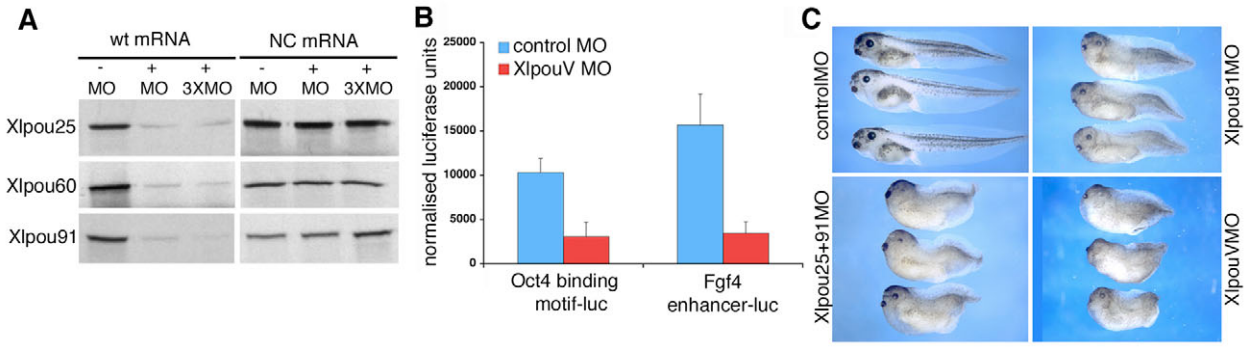


**Fig. 4. Depletion of XlPouV proteins causes posterior truncation and anterior defects.** (**A**) In vitro translation assay. The antisense MOs specifically prevent translation of their target mRNA but not the non-complementary (NC) mRNA. 3×MO indicates that a mix of all three XlpouV MOs was used. Capped mRNAs (1 μg) were used for translation and MOs were used at a concentration of 1 μM. (**B**) Two-cell stage embryos were injected in both blastomeres with control MO or XlpouV MO, and the octamer binding motif or the Fgf4 enhancer luciferase reporters. Embryos were assayed for luciferase activity at stage 9.5. The experiment was carried out in triplicate. (**C**) Two-cell stage embryos were injected in both blastomeres with 60 ng (control MO only) or 20 ng of each indicated MO and collected at stage 40. The term XlpouV MO was used to indicate that all three MO had been injected simultaneously.

Oct4, Xlpou25 and Xlpou91 ES cell lines results in reduced expression of *Gata4* and *Mixl1* and an increase in brachyury expression (Fig. 4F). It has previously been shown that both an increase or decrease in Oct4 levels leads to ES cell differentiation (Niwa et al., 2000). Marker analysis in our cell lines helps to explain this phenomenon by demonstrating that enhanced Oct4 drives ES cells towards a brachyury-positive state, whereas reduced levels of Oct4 leads to both endodermal and trophoblast differentiation.

## PouV protein depleted *Xenopus* have a truncated body axis and anterior defects

The ability of the *Xenopus* PouV proteins to rescue Oct4-deficient ES cells suggests a conserved function for the PouV proteins predating mammalian evolution. To gain insight into the embryonic function of PouV proteins in *Xenopus*, we depleted the levels of XlpouV proteins through the use of antisense morpholino oligonucleotides (MO). An in vitro translation assay demonstrated that the MOs block translation of the wild-type mRNAs but not the non-complementary (NC) Xlpou mRNAs containing non-coding changes to the sequence around the ATG that do not match the morpholino sequence (Fig. 4A). An in vivo luciferase assay confirmed that MO treatment significantly reduced the levels of functional PouV protein in *Xenopus* embryos (Fig. 4B). The residual luciferase activity is presumably caused by the presence of other octomer binding proteins such as XOct1 and XBrn3 during *Xenopus* embryogenesis (King and Moore, 1994).

Embryos injected with control MO, Xlpou25 MO or Xlpou60 MO alone developed with no observable phenotype. Protein knockdown with Xlpou91MO alone, Xlpou25 and Xlpou91 MO, or with a combination of all three MOs (XlPouV MO) resulted in a failure in axis elongation, of varying severity (Fig. 4C, Table 2). Although the most prominent phenotype was posterior truncation, we also observed an anterior defect consisting of reduced head and eye size. The depletion of all three XlPouV proteins resulted in the most severe phenotype (Fig. 4C, Table 2).

## *Xenopus* PouV proteins function in anterior and posterior neural development

*Xlpou25* and *Xlpou91* are expressed in the developing anterior neural tissue (Fig. 5A) and later in the posterior neural tube (Fig. 5C). The abnormal anterior appearance of the XlpouV MO-treated embryos suggested a possible role in anterior neural patterning. Fig. 5B demonstrates the dramatically reduced expression of *Fgf8* at the midbrain hindbrain boundary (MHB) of XlPouV-depleted embryos. The other anterior domains of *Fgf8* expression (the prospective hatching gland and an epidermal crescent outside the neural plate area) were not so severely affected (data not shown), while the posterior periblastoporal domain of *Fgf8* expression was also reduced. *En2*, a marker specific to the MHB region was initiated appropriately at the early neurula stage but then became dramatically reduced at later stages (Fig. 5B). This defect also extended to the anterior hindbrain as expression of *Krox20* in rhombomeres 3 and 5 was initiated but subsequently not maintained in rhombomere 3 (Fig. 5B).

**Table 2. Percentage phenotypes resulting from XlpouV protein depletion**

| Morpholino (ng/embryo) | | | Severe AP truncation (%) | Moderate AP truncation (%) | Anterior defect (%) | Normal (%) | *n* |
|---|---|---|---|---|---|---|---|
| Xlpou25 | Xlpou60 | Xlpou91 | | | | | |
| 40 | – | – | 0 | 8 | 0 | 89 | 109 |
| – | 40 | – | 0 | 8 | 5 | 89 | 109 |
| – | | 40 | 5 | 62 | 41 | 21 | 147 |
| 40 | – | 40 | 58 | 35 | 36 | 6 | 214 |
| 10 | 10 | 10 | 0 | 25 | 14 | 50 | 96 |
| 20 | 20 | 20 | 13 | 56 | 61 | 8 | 149 |
| 40 | 40 | 40 | 68 | 26 | 61 | 3 | 284 |
| Control (120 ng) | | | 0 | 0 | 3 | 97 | 164 |

Abbreviations: AP, anterior-posterior axis; *n*, number of embryos.

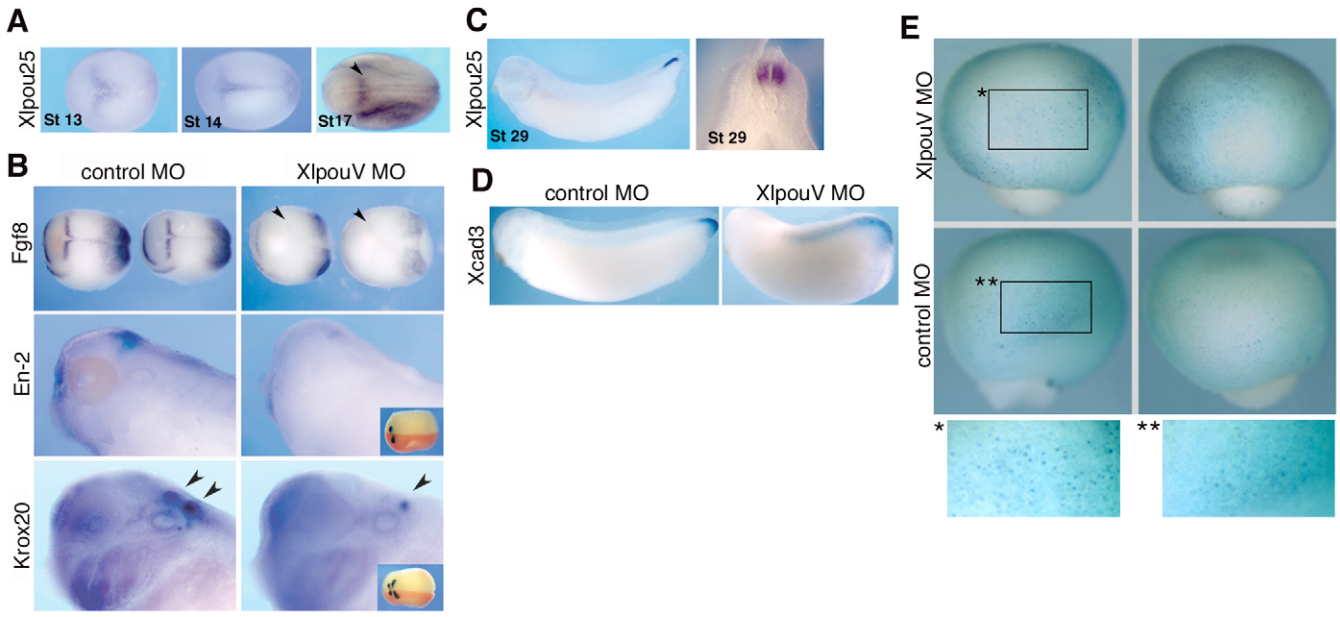


**Fig. 5. XlpouV depletion results in defective anterior and posterior neural patterning.** (**A**) In situ hybridization for expression of Xlpou25 at neurula stages. Xlpou25 expression is visible in the developing neural tube at stages 13 and 14 and is then present in the midbrain hindbrain boundary (MHB) at stage 17. The arrowhead indicates the MHB. Xlpou91 showed an identical expression pattern (data not shown). (**B**) Embryos were injected with control MO or XlpouV MO as in Fig. 4C, and in situ hybridization carried out for *Fgf8* expression at stage 16 and for *En-2* and *Krox20* expression at both stage 16 and stage 35. The insets show stage 16 embryos injected in one blastomere at the two-cell stage with MO and a lineage trace (orange). The arrowheads in the *Fgf8* panel indicate the MHB. The arrowheads in the *Krox20* panel indicate rhombomere 3 and 5 in the control MO-treated embryo and rhombomere 5 in the XlpouV MO-treated embryo. (**C**) In situ hybridization for expression of Xlpou25 at tailbud stages. The right panel is a transverse section through the posterior region of a stage 29 embryo. Xlpou91 showed an identical expression pattern (data not shown). (**D**) Depletion of XlpouV protein results in increased *Xcad3* expression. Embryos were injected with control MO or XlpouV MO as in Fig. 4C, and in situ hybridization for Xcad3 expression performed on stage 28 embryos. (**E**) Whole-mount TUNEL staining. Embryos were injected with control MO or XlpouV MO as in Fig. 4C and TUNEL staining carried out at stage 11.5. Embryos are positioned with the blastopore to the bottom and both sides of the embryo are shown. The boxed areas are shown at higher magnification below the main images.

The expression of both *Xlpou25* and *Xlpou91* in the posterior neural tube prompted us to investigate the effects of XlpouV depletion on the expression of Xcad3, a member of the caudal (Cdx) gene family. Xcad3 is also expressed in the posterior neural tube at this stage and was of particular interest as it is a homologue of mouse *Cdx2*, a gene whose expression was shown to be increased following mouse Oct4 depletion in ES cells (Niwa et al., 2000). Depletion of PouV protein in *Xenopus* embryos also resulted in an expansion of *Xcad3* expression (Fig. 5D). We also observed an increase in *Xcad3* expression during gastrulation, although at a lower level (data not shown).

## XlPouV protein depletion disrupts the expression of genes associated with early lineage commitment

To investigate whether the loss of posterior tissue was due to increased cell death of late gastrulating marginal zone cells, whole-mount TUNEL was performed on XlpouV-depleted embryos and control MO-treated embryos. We observed no difference between XlpouV depleted embryos and control MO-treated embryos during late gastrulation (Fig. 5E).

The loss of posterior tissues in the absence of cell death suggests that Xlpou depletion might have resulted in defects early during cell specification. Fig. 6A shows RNA in situ hybridization for a number of gastrulation stage markers in XlPouV-depleted embryos. Interestingly, we observed a dramatic loss of *Bmp4* and *Xom* (*Xvent2*) expression, both genes associated with the inhibition of differentiation (Cao et al., 2004; Constance Lane et al., 2004; Ying et al., 2003). We also observed a loss of *Fgf8* and brachyury (*Xbra*) expression, genes normally expressed in the marginal zone. Expression of marker genes associated with both endoderm (*Mixer*, *Sox17α* and endodermin) and the organizer region [goosecoid (*Gsc*), chordin and cerberus] were all expanded (Fig. 6A,B). These results were confirmed by real-time RT-PCR analysis of XlPouV-depleted embryos (Fig. 6B). Depletion of individual XlPouV proteins revealed some differences in function within the *Xenopus* gene family. For example, depletion of Xlpou25 protein alone did not result in the loss of *Xbra* and *Bmp4* expression or an increase in *Sox17α* expression (data not shown). This difference in the function of Xlpou25, compared with Xlpou60 and Xlpou91, may explain its reduced capacity to rescue ES cell self-renewal.

Fig. 6C shows the post-gastrulation consequences of XlPouV depletion. All three Xlpou proteins are expressed in the animal hemisphere and following their depletion we found that the neural plate marker *Sox2* and a pro-neural gene, *Ngnr1*, were both upregulated. Marginal zone cells from Xlpou-depleted embryos express high levels of organizer and anterior endoderm markers, consistent with the precocious conversion of uncommitted marginal zone into a committed cell fate. In agreement with the early conversion of an abnormally large region of this tissue to early endoderm, we found an expansion of later Hex expression, which normally marks the earliest endoderm to involute during gastrulation. In normal stage 35 tadpoles, Hex expression is

restricted to the descendants of this tissue in the liver, whereas in Xlpou-depleted embryos there is both an expanded liver domain and ectopic Hex expression (Fig. 6C). These embryos also exhibit a dramatic reduction in posterior myosin-light chain 1-3 (*MLC1-3*) expression. This result is consistent with a premature conversion of uncommitted marginal zone cells to organizer or anterior endoderm early in gastrulation, leaving an insufficient number of cells to form the posterior somites. Thus, the anterior somites appear normal but towards the posterior of the embryo the expression of *MLC1-3* is decreased to a point where no segmented somites are visible (Fig. 6C).

Overexpression of XlPouV proteins produced the opposite effects on gene expression to that of XlpouV depletion. At the gastrulation stage, overexpression of all three *Xenopus* PouV proteins enhanced the expression of *Xbra* and *Bmp4* and reduced the expression of *Mixer* and goosecoid (Fig. 6D). These results paralleled those obtained from the depletion or overexpression of PouV proteins in ES cells (Fig. 3E,F) and indicate that mammalian and *Xenopus* PouV proteins have similar downstream targets and may function through similar molecular pathways.

## Rescue of knockdown phenotype

To confirm that the changes in gene expression were specific to depletion of PouV proteins, we co-injected XlpouV MO with XlpouV RNAs non-complementary (NC) to the MO-targeted sequence. Luciferase assays demonstrated that the NC proteins activated transcription as efficiently as the wild-type proteins (data not shown). As expected, some specificity in the ability of the

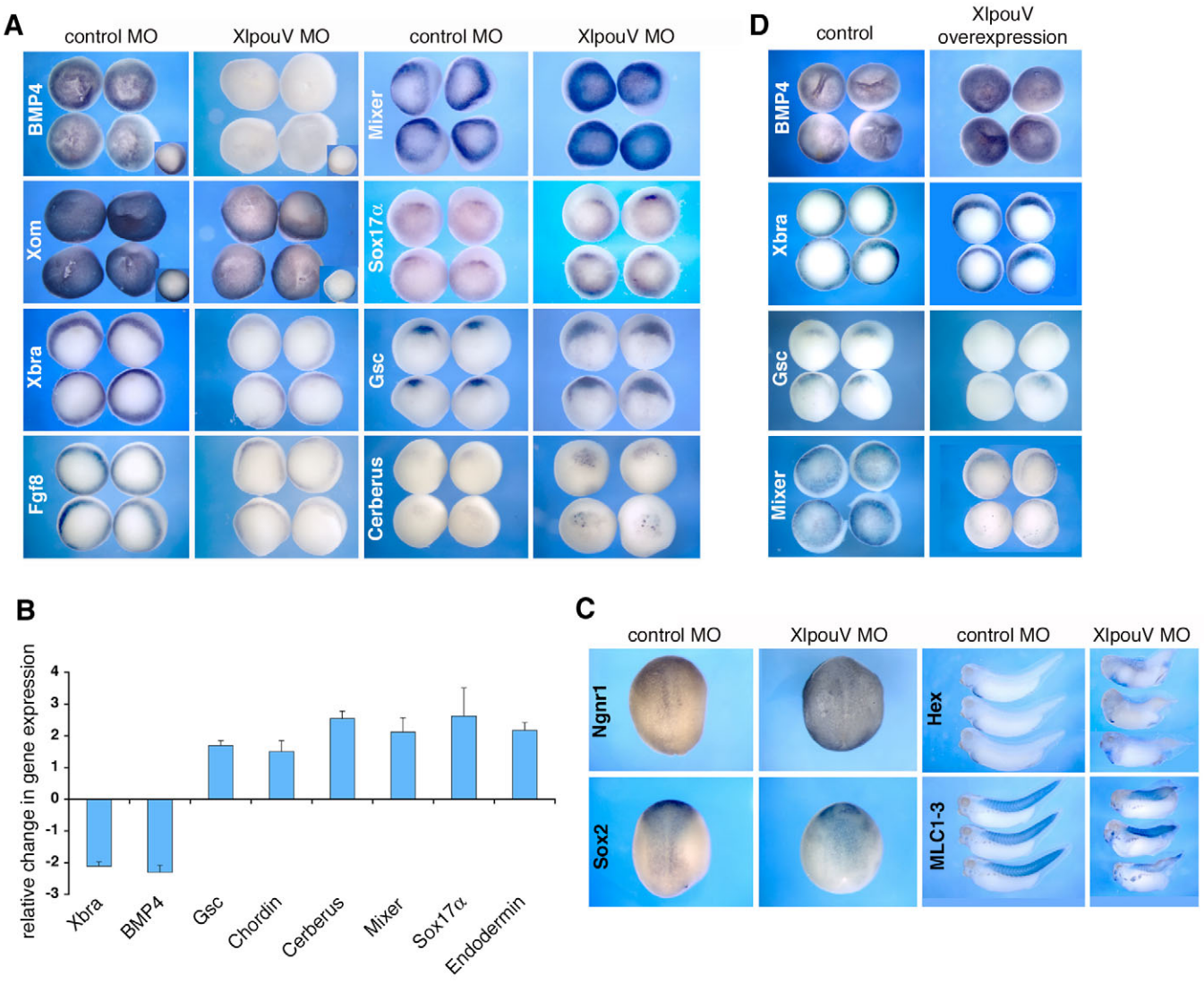


**Fig. 6. Depletion of XlPouV proteins affects germ layer induction.** (**A**) Two-cell stage embryos were injected in both blastomeres with 60 ng of control MO or 20 ng of each of the XlpouV MOs. In situs were performed on stage 10.25 embryos. Embryos are positioned with the vegetal hemisphere upwards and the dorsal lip towards the top apart from Bmp4 and Xom, which are positioned animal hemisphere upwards and insets with vegetal hemisphere upwards. (**B**) Real-time RT-PCR analysis of XlpouV protein-depleted embryos. Embryos were injected with control MO or XlpouV MO as in Fig. 6A and RNA extracted at stage 10.25. The relative change in gene expression was calculated by dividing the XlpouV MO values by the control MO values. Experiments were carried out in triplicate. (**C**) Embryos were injected with control MO or XlpouV MO as in Fig. 6A. *Sox2* and *Ngnr1* expression was analysed at stage 13 and *Hex* and *MLC1-3* expression at stage 35 embryos. (**D**) Two-cell stage embryos were injected in both blastomeres with 250 pg of each RNA and in situs performed on stage 10.25 embryos. All embryos are positioned with the vegetal hemisphere facing upwards and the dorsal lip towards the top, apart from Bmp4 embryos that are positioned animal hemisphere upwards.

**Table 3. Percentage rescue of the XlpouV protein depletion phenotype by PouV proteins**

| | Xbra expression | | Bmp4 expression | | Mixer expression | |
|---|---|---|---|---|---|---|
| RNA | Complete | Moderate | Complete | Moderate | Complete | Moderate |
| Xlpou25 | 10 | 20 | 0 | 50 | 40 | 40 |
| Xlpou60 | 20 | 30 | 0 | 50 | 50 | 40 |
| Xlpou91 | 30 | 40 | 20 | 60 | 40 | 30 |
| Xlpou25/60/91 | 50 | 50 | 70 | 20 | 70 | 10 |
| mOct4 | 40 | 50 | 30 | 20 | 10 | 60 |
| AmOct4 | 40 | 60 | 40 | 40 | 0 | 40 |
| DrPou2 | 0 | 10 | 0 | 20 | 10 | 10 |

mRNA (250 pg) was injected into each blastomere of a two-cell stage embryo. For Xlpou25/60/91, 100 pg of each mRNA was injected into each blastomere of a two-cell stage embryo.

different POU proteins to rescue the MO phenotype was detected, but addition of all three XlPouV NC proteins fully rescued the MO-induced phenotype (Table 3). Mouse Oct4 and AmOct4 also efficiently rescued the defect in *Xbra* expression and could partially rescue *Mixer* expression, indicating a degree of functional conservation between the amphibian and mammalian members of the PouV family. By contrast, DrPou2 showed very little rescue ability (Table 3).

## Depletion of PouV proteins alters germ layer induction by activin in animal cap explants

Our data suggested that the depletion of PouV proteins causes multipotent, non-committed marginal zone cells to differentiate much more readily when exposed to differentiation signals. To investigate this further we used animal cap explants, which can be used to reproduce the in vivo induction of both marginal zone and endoderm or organizer derivatives (Okabayashi and Asashima, 2003).

The molecular effects of XlpouV knockdown in animal caps was monitored by the expression of specific marker genes. *Bmp4* was the only marker analysed that is expressed in un-induced (no activin) animal caps, and we observed a significant decrease in *Bmp4* expression in uninduced animal caps following XlPouV protein depletion (Fig. 7A). Although differences could be observed between the control MO and XlpouV MO-treated explants that had not been induced with activin, we do not consider these to be significant because of their very low expression levels (Fig. 7A).

Treatment of animal caps with 8 U/ml of activin induces the expression of *Xbra* in control MO explants, but this induction was reduced in XlpouV-depleted explants (Fig. 7A). Using 16 U/ml of activin, we also observed elevated expression of the organizer and endodermal associated markers *Gsc*, chordin, cerberus, *Mixer*, *Sox17α* and endodermin in XlPouV-depleted explants. Moreover, *Gsc* and cerberus expression can only be induced at 16 U/ml activin in control MO-treated animal caps whereas the lower dose of 8 U/ml activin was sufficient to induce these organizer markers in the XlPouV depleted animal caps (Fig. 7B). Thus, depletion of XlPouV proteins sensitizes animal cap cells to activin treatment, while reducing expression of marginal zone markers such as *Xbra* and *Bmp4*. We also observed that overexpression of XlPouV proteins in activin-treated animal caps dramatically reduced the induction of *Gsc* (data not shown).

## DISCUSSION

Maintenance of cells in a non-committed state prior to gastrulation is a fundamental aspect of triploblastic organisms. Here, we have shown that *Xenopus* PouV proteins have the ability to maintain ES cells in an undifferentiated

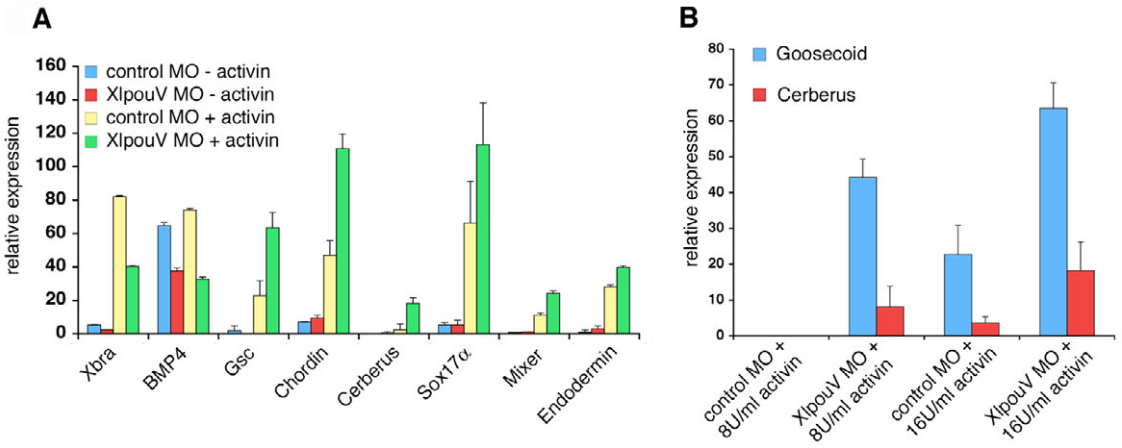


**Fig. 7. Depletion of PouV proteins produces a heightened activin response in animal cap explants.** (**A**) Real-time RT-PCR analysis of XlpouV protein-depleted animal cap explants. Embryos were injected with control MO or XlpouV MO as above. Animal cap explants were isolated at stage 8 and cultured with or without activin until sibling embryos reached stage 10.25. Relative expression represents values normalized to *Odc*. 16 U/ml activin was used for induction apart from the *Xbra* and *Bmp4* analysis that used 8 U/ml activin. Experiments were carried out in triplicate. (**B**) Embryos were injected with control MO or XlpouV MO as above and animal caps explants isolated and induced with either 8 U/ml or 16 U/ml activin. Relative expression represents the *Gsc* and cerberus values normalized to *Odc*. Experiments were carried out in triplicate.

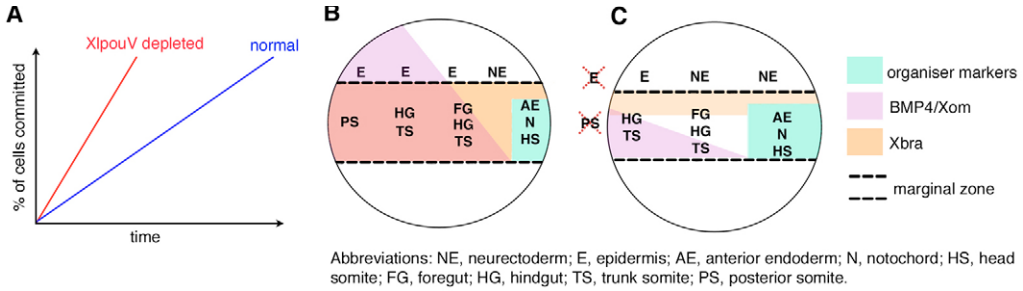


**Fig. 8. Effect of PouV depletion on the timing of cell fate commitment.** (**A**) Graph representing the premature commitment of cells in the XlpouV-depleted embryos. (**B**) Cartoon of a control embryo. (**C**) Cartoon of an XlpouV-depleted embryo depicting the shift in cell fate domains caused by premature commitment.

state and that their role during *Xenopus* embryogenesis is to maintain cells as multipotent and non-committed during gastrulation.

## Conservation and diversification of PouV proteins

Our data indicate a role for PouV proteins in the maintenance of non-committed cell populations in *Xenopus* (Fig. 8A). In mouse, PouV function is a known requirement for both ES cell self-renewal and primordial germ cell development. The similarities between these roles suggest PouV proteins have a conserved role in maintaining multipotent, non-committed cells. Thus, *Xenopus* PouV proteins can sustain ES cell self-renewal and mouse Oct4 can rescue PouV depletion phenotypes in *Xenopus*. By contrast, the zebrafish PouV protein, DrPou2, showed no ability to substitute for Oct4 in ES cells or rescue the *Xenopus* PouV depletion phenotype. Consistent with these observations, DrPou2 was shown to have a distinct function during gastrulation, as a mediator of endoderm induction and differentiation in zebrafish (Lunde et al., 2004; Reim and Brand, 2002; Reim et al., 2004).

Although the zebrafish protein DrPou2 was unable to rescue Oct4 null ES cells or the gastrulation stage XlpouV depletion phenotype, its role in neural patterning shows some conservation with *Xenopus*. Xlpou25 and Xlpou91 were found to be required for maintenance of gene expression in the MHB and the anterior hindbrain. A similar role has been reported for DrPou2 and this aspect of the XlPouV depletion phenotype is similar to the spiel-ohne-grenzen (spg) (DrPou2 mutant) phenotype (Belting et al., 2001; Hauptmann et al., 2002; Reim and Brand, 2002). Consistent with the conservation of function during neural development, overexpression of mouse Oct4 mRNA in spg mutant zebrafish can rescue a defect in *Pax2.1* expression in the MHB. However, although mouse Oct4 rescues this aspect of the zebrafish spg phenotype, there is currently no evidence for a requirement for Oct4 in neural development. In addition, despite the rescue of *Pax2.1* expression in neural patterning there is no evidence that mouse Oct4 can rescue the zygotic or maternal/zygotic defects in endoderm induction. Moreover, DrPou2 induces endoderm cooperatively with the SRY box protein, Sox32 (also known as Casanova) (Reim et al., 2004). As PouV proteins usually bind DNA coordinately with a member of the Sox family (Dailey and Basilico, 2001) and no Sox32-like protein has been identified in other vertebrates, the Sox32-Pou2 endoderm induction mechanism may be unique to teleosts. It is possible that the genes encoding Sox32 and Pou2 arose from a duplication event in which an ancient PouV-Sox gene set retained original function and the other acquired a novel endoderm inducing activity. However, despite the large number of POU genes present in the sequenced teleost genomes, we have been unable to find an obvious candidate. This is particularly surprising as sequences resembling both Pou2 and Oct4 have been identified in sturgeon, a species that occupies a more basal phylogenetic position than teleosts (Andrew Johnson, personal communication). The divergent function of DrPou2 in endoderm induction may explain why it was unable to rescue ES cell self-renewal or the gastrulation stage XlPouV depletion phenotype.

As *Xenopus* lacks trophectoderm and the XlpouV depletion does not affect germ cell development (data not shown), the conserved PouV function in *Xenopus* and ES cells argues against Oct4 function being a unique innovation of mammals for these specific lineages. In support of this, the relationship between Caudal (Cdx) and PouV proteins does not appear to be exclusive to trophoblast versus ICM cell fate decisions in mammals. *Xenopus* lacks trophectoderm tissue, yet an orthologue of *Cdx2*, *Xcad3*, exists (Pownall et al., 1996). Loss of PouV proteins in both ES cells and *Xenopus* embryos results in ectopic *Cdx2* (Nichols et al., 1998; Niwa et al., 2000) and *Xcad3* expression (Fig. 5D), respectively. Moreover we have found similar sets of genes to be regulated by PouV proteins in ES cells and during *Xenopus* gastrulation. Thus, we conclude that mouse Oct4 is not a mammalian-specific protein, but rather the function of PouV proteins in suppressing differentiation and commitment has been adopted by mammals to maintain pluripotency in ES cells, in part by blocking commitment to the trophoblast lineage.

## A function for PouV proteins during gastrulation

It is likely that the pivotal role of Oct4 in the maintenance of ES cell pluripotency depends on the ability of this protein to suppress cell fate commitment in multiple lineages and is therefore derived from an ancient role in gastrulation. In *Xenopus*, the future germ layer domains are specified early by the asymmetrical localization of maternal mRNAs such as Vg1, VegT, Wnt11 and ectodermin (Dupont et al., 2005; Joseph and Melton, 1998; Tao et al., 2005; Xanthos et al., 2001). Although this process regionalizes the embryo into broad domains, cells become committed to specific lineages only as they begin involution in response to these signals. Cells continue to be committed to their particular cell fate throughout gastrulation, but the embryo requires a pool of multipotent precursors to enable the formation of more posterior lineages later during this process. A similar pool of cells is required in mammalian development and the expression of Oct4, initially throughout the epiblast and later in the region of the primitive streak, may indicate the location of these populations (Snape et al., 1987; Wylie et al., 1987). ES cell derivation represents the capture of these epiblast, non-committed populations in vitro. However, in *Xenopus*, reductive cell division would make it difficult to capture and sustain these populations (Frederick and Andrews, 1994). Regardless of the feasibility of creating ES cell lines, all vertebrates must posses the

ability to prevent premature commitment. We propose here that PouV proteins posses this function, which we have found to be conserved in a number of vertebrate species.

A model illustrating how precocious differentiation in the absence of PouV affects cells fate is shown in Fig. 8. In *Xenopus*, the organizer tissue is the first to gastrulate and become committed, giving rise to dorsal anterior structures while cells from the remaining marginal zone gastrulate later and populate progressively posterior regions of the embryo (Constance Lane et al., 2004; Lane and Smith, 1999). In the PouV-depleted embryos we observe a decrease in the expression of markers associated with non-committed cell types (*Bmp4*, *Xom* and brachyury), whereas the expression domains of markers associated with early commitment (organizer and anterior endoderm) were expanded. The concurrent loss and gain of uncommitted and committed cells, respectively, in the absence of increased cell death leads us to conclude that there is a premature progression of uncommitted marginal zone cells to a more committed cell fate early during gastrulation.

Following PouV depletion in *Xenopus*, there is a striking downregulation in the expression of *Bmp4*. This extrinsic factor has been associated with maintaining cells in a non-committed state in both ES cell lines and in *Xenopus* embryos where expression of a constitutively active BMP receptor blocks the onset of germ layer commitment (Constance Lane et al., 2004; Ying et al., 2003). We found the expression of both *Bmp4* and its downstream target *Xom* was dependent on PouV in *Xenopus*. Interestingly, Xom belongs to the same class of transcription factors as the ES cell marker, Nanog.

We also observed a reduction in the expression of *Xbra* in the PouV-depleted embryos. There is an accumulating body of evidence in mouse that brachyury is a transient marker of both future mesoderm and endodermal cells (Kubo et al., 2004; Wilson et al., 1995). In addition *Xbra* interacts directly with components of the BMP signalling pathway to induce *Xom*, a direct antagonist of the organizer associated gene goosecoid (Messenger et al., 2005). Thus, PouV proteins may block commitment in bipotent precursors of the mesoderm and endoderm in part by maintaining brachyury expression.

Loss of XlpouV expression also leads to an expansion of endodermal markers not associated with the organizer. Interestingly, mouse Oct4 has been shown to have a role in inhibiting the expression of endodermal associated genes through an interaction with Foxd3 (Guo et al., 2002). An increase in endodermal gene expression was also observed in Oct4-depleted mouse and human ES cells (Hay et al., 2004).

As the depletion of PouV function in *Xenopus* results in a premature progression to a committed cell type and XlPouV proteins can sustain undifferentiated ES cells, we propose that there is a conserved role for these proteins in maintaining cell multipotentcy. This would strongly imply that the ability of mouse Oct4 to maintain ES cells as self-renewing and pluripotent is derived from this ancient PouV function.

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