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On the function of *Xenopus* Oct4 protein homologs:
Molecular construction of dominant interference variants and
functional analysis in early frog development



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1 Zusammenfassung/Summary

Die Embryonalentwicklung stellt einen hochkomplizierten, multifaktoriellen Prozess dar. Hierbei müssen Spezifizierung, Musterbildung und Differenzierung von Zellen und Geweben zeitlich und räumlich genauestens kontrolliert werden. Dies impliziert die Regulierung einer Vielzahl unterschiedlicher Gene. Nur eine kleine Anzahl von Transkriptionsfaktoren scheint jedoch für diese Regulierung verantwortlich zu sein.

Oct4, Sox2 und Nanog bilden ein Netzwerk, das eine wichtige Rolle bei der Pluripotenzhaltung und der zeitlichen Regulierung der Differenzierung spielt. Die zentrale Rolle von Oct4 in Säugetieren wurde durch jüngste Forschungsergebnisse unterstrichen, die zeigten, dass Oct4 entscheidend bei der Reprogrammierung somatischer Zellen beteiligt ist. Dennoch ist bislang wenig über die molekularen Regulationsmechanismen dieses Transkriptionsfaktors während der Normogenese bekannt.

Beim *Xenopus laevis* (Krallenfrosch) entwickeln sich die Embryonen extrauterin. Seine Embryonalentwicklung ist genauestens studiert und Techniken wie embryonale RNA- und DNA-Injektionen sind gut etabliert. Daher stellt *Xenopus laevis* einen idealen Modellorganismus für die Untersuchung von Oct4 homologen Proteinen in der frühen Embryonalentwicklung dar.

Im *Xenopus laevis* sind drei Oct4 Paraloge – Oct25, Oct60 und Oct91 – bekannt, welche eine ähnliche Größe besitzen und hohe Sequenzhomologien mit dem Säugetier Oct4 aufweisen. Es gibt starke Hinweise, dass *Xenopus* Oct Proteine ähnliche Funktionen wie Oct4 innehaben.

Um die Funktion der Oct Proteine genauer zu studieren, habe ich dominant aktivierende (VP16-Oct60), dominant reprimierende (EnR-Oct60) und hormoninduzierbare (GR-Oct60) Transkriptionsfaktor-Varianten für alle drei *Xenopus* Oct Proteine generiert. Die Proteinexpression wurde sowohl *in vitro*, als auch *in vivo* verifiziert.

Verglichen mit anderen *Xenopus* Oct Proteinen zeigt Oct60 ein einzigartiges Expressionsmuster: Oct60 wird maternal transkribiert und seine RNA ist in reifen Oocyten nachweisbar. Die Expression nimmt während dem Gastrulastadium, wenn die Expression der anderen *Xenopus* POU Proteine beginnt, wieder ab. Somit gehört Oct60 zu den ersten Genen die exprimiert werden. Daher haben wir uns entschlossen uns vorerst auf die Untersuchung von Oct60 zu konzentrieren.

Die transaktivierenden Eigenschaften der Oct60 Funktionsgewinn- („gain of function“) Proteine wurden *in vivo* durch einen Luciferase-Assay mit zwei unterschiedlichen Oct4 Reporterkonstrukten untersucht. Hierbei zeigte sich eine starke Aktivierung beider Reporter durch Oct60 und VP16-Oct60 sowie eine Repression durch EnR-Oct60.

Durch die Injektion von Oct60 und seinen „gain of function“ Varianten konnten wir mehrere, verschiedene Körperregionen betreffende, phänotypische Veränderungen beobachten. Neben einer schwerwiegenden Behinderung der Differenzierung im Bereich des Kopfes, hervorgerufen durch VP16-Oct60 und Oct60 Überexpression, konnten wir eine starke Hyperpigmentierung in EnR-Oct60 und Oct60 injizierten Embryonen beobachten. Weiterhin zeigten EnR-Oct60 injizierte Embryonen hyperpigmentierte Auswüchse im Bereich der Flanke. Alle injizierten Embryonen wiesen verkürzte Körperachsen mit einer spezifischen Krümmung abhängig von der injizierten RNA auf. Um die zugrunde liegenden molekularen Mechanismen dieser phänotypischen Veränderungen zu analysieren führten wir *in situ* Hybridisierungen durch. Diese zeigten, dass alle untersuchten Konstrukte die Bildung von neuroektodermalem Gewebe fördern und gleichzeitigen die Mesodermbildung hemmen. Diese Ergebnisse weisen darauf hin, dass Oct60 die Induktion und Spezifikation der Keimblattbildung beeinflusst. Durch die Klonierung und Charakterisierung verschiedener neomorpher Proteinvarianten ist es uns gelungen wichtige Werkzeuge für die weitere Untersuchung von Oct4 Homologen im *Xenopus laevis* zu entwickeln.

Summary

Embryonic development represents a sophisticated multistep process. Hereby, specification, patterning and differentiation of cells and tissue need to be extremely well regulated in a temporo-spatial manner. This is based on repression and activation of a vast number of cell-type specific genes, but only a small number of transcription factors seem to be responsible for their regulation.

The transcription factor network of Oct4, Sox2 and Nanog are thought to play an essential role in the maintenance of pluripotency and in timing the onset of differentiation. The importance of mouse Oct4 in the regulation of pluripotency is underscored by recent findings providing evidence that Oct4 is essential for reprogramming somatic cells. Nevertheless, little is known on the molecular function of this transcription factor during normogenesis. Given the extra-uterine development of the embryos, the well-studied early development and the established manipulation methods like injection of RNA or DNA, *Xenopus laevis* offers an ideal model organism to study the role of Oct4 homologs in early development.

In *Xenopus laevis* three Oct4 paralogs – Oct25, Oct60 and Oct91 – are known, which are similar in size and have a high sequence homology compared to mammalian Oct4. There are strong evidences that *Xenopus* Oct proteins and mammalian Oct4 share similar functions.

To gain further insights into the function of Oct proteins I generated dominant activating- (VP16-Oct60), dominant repressing- (EnR-Oct60) and hormone inducible (GR-Oct60)

transcription factor variants for all three *Xenopus* Oct proteins. Protein expression was verified *in vitro* as well as *in vivo*.

Oct60 shows a unique expression pattern among *Xenopus* Oct proteins: Oct60 is maternally transcribed and its RNA is detectable in mature oocytes. Expression is downregulated in the gastrula, when the expression of other *Xenopus* POU proteins begins. Therefore, it is one of the earliest genes to be expressed. I decided to concentrate first efforts on Oct60.

The transactivating functions of the Oct60 G.o.F. variants were tested in a luciferase assay on two different Oct4 reporter constructs *in vivo*. Oct60 and VP16-Oct60 acted as strong activators whereas EnR-Oct60 repressed both reporter constructs.

By overexpression of Oct60 and its G.o.F. variants, several phenotypes were observed that affected distinct parts of the body. Beside impaired head differentiation, observed by overexpression of VP16-Oct60 and Oct60, a strong hyperpigmentation was observed by injection of EnR-Oct60 and Oct60. Additionally, EnR-Oct60 injected embryos showed hyperpigmented outgrowths in the trunk region. All injected embryos possessed a shortened body axis that was specifically curved depending on the injected mRNA.

In situ hybridizations were performed to investigate the molecular mechanism of the observed phenotypic changes. Experiments revealed that all examined constructs promote neuroectodermal fate while repressing mesoderm formation.

These results indicate that Oct60 plays an important role in the induction and specification of germ layer formation. By cloning and testing these different G.o.F. variants I accomplished to obtain important tools for further dissecting the molecular function of Oct4 homologs in *Xenopus* embryos.

2 Introduction

2.1 Stem cells

Embryogenesis is the fundamental process of differentiation of all tissues from a unicellular zygote. From a single totipotent cell distinct stem cells emerge that will form the three germ layers – ectoderm, mesoderm and endoderm and will later differentiate to over 200 unique cell types (Loebel, Watson et al. 2003; Boyer, Lee et al. 2005). All these events need to be extremely well regulated in the temporo-spatial context of the developing organism.

Stem cells possess two unique characteristics: their self-renewal and differentiation potential. Therefore, they are different from progenitor cells, which can differentiate into mature cell types but are incapable of self-renewing, or somatic cells, which are capable of proliferating but unable to differentiate.

According to their differential potential, stem cells are being subdivided into totipotent, pluripotent and multipotent cells although the boundaries between them are constantly in a state of flux. Totipotent cells hold the indefinite feature of differentiating into all adult and embryonic tissues, including extra-embryonic tissues such as trophoctoderm. Pluripotent stem cells are capable of differentiating into the derivatives of the three germ layers (ectoderm, mesoderm and endoderm) and germ cells. However, they cannot differentiate into certain cell types especially extra-embryonic tissues such as trophoctoderm and are therefore not able to form a viable organism. Multipotent stem cells have an even lower potential and can only differentiate into a limited number of cell types.

The earliest stem cells in ontogeny - the zygote and to some extent early blastomeres - are totipotent. They give rise to somatic stem/progenitor cells and primitive germ line stem cells. At about the neurula stage, tissue and organ specific stem cells emerge. Little is known about the stages of somatic stem cells between the blastocyst stage and the neurula stage (Weissman 1999; Surani, Hayashi et al. 2007; Guo, Huss et al. 2010).

This classification might create the misimpression, that totipotency, pluripotency and multipotency are homogenous and stable states. Contrary to this assumption, basic research has provided more and more data suggesting that they are time dependent characteristics that only represent a snap shot in development. Therefore, there is possibly not one kind of pluripotent cell but more a cell that is passing a pluripotent state with different features at different examined points in time. Pluripotent stem cells might be more heterogeneous in nature then they are most often thought to be and more than one 'state of pluripotency' may exist. (Skottman, Mikkola et al. 2005; Kalmar, Lim et al. 2009; Cherry and Daley 2010)

2.2 Stem cells in medicine

In medicine, great hopes are being pinned on tissue regeneration using stem cells. Stem cell therapies are promising solutions for currently unmet medical needs. They would open new prospects for the replacement of damaged or aged cells for example in patients with degenerative illnesses like age-related functional defects, haematopoietic and immune system disorders, heart failure, chronic liver injuries, Alzheimer, diabetes type I, multiple sclerosis, Parkinson's disease as well as aggressive or recurrent cancer. Additionally they could be useful as gene ferries for gene therapy (Weissman 2000; Lian, Chow et al. 2010).

Numerous recent investigations carried out with *ex vivo* expanded and/or differentiated embryonic -, fetal-, and umbilical cord stem cell-derived fully functional progeny as well as adult stem cells have provided accumulating evidence supporting their potential use for the treatment of numerous disorders (Barrilleaux, Phinney et al. 2006; Bryder, Rossi et al. 2006; Mimeault and Batra 2006; Behfar, Perez-Terzic et al. 2007; Ringden 2007; van Vliet, Sluijter et al. 2007; Wu, Boyd et al. 2007)

In 1997 transplantation experiments have shown that neural precursor cells generated *in vitro* can participate in the development of embryonic rat brain. After the transplantation of glial precursor cells into the ventricle of myelin-deficient rat brains, the formation of new myelin sheets could be observed in various brain regions (Brustle, Spiro et al. 1997). However, even more than 10 years later, there are only very few fields in medicine – e.g. cardiac infarction - where stem cells are used for clinic therapies. Even there, the results are not satisfying. Implementation in clinical settings requires extensive safety studies and a solution to immunogenic issues in particular. In the therapy of Parkinson's disease the use of stem cells also did not prove to be successful. Precursors of dopaminergic neurons in cultured mesencephalic cells obtained from human embryos, aborted 7–8 weeks after conception, were transplanted to Parkinson's disease patients. Some of the implanted cells survived and differentiated. Improvement rates were low and in 15% of the patients, spontaneous dyskinesias developed as a disabling complication (Freed, Greene et al. 2001).

Taken together, stem cells and their progeny constitute cells with an enormous therapeutic potential to treat and even cure diverse genetic and degenerative disorders, which are currently incurable with other types of treatments. For a fundamental understanding of human development and to achieve the goal of clinical application, it is essential to understand the molecular mechanisms underlying the pluripotency of ES cells. Especially for clinical application, it is important to make better use of the distinctions between the pluripotent and differentiated states. ES cells need to be differentiated efficiently into a specific lineage and undifferentiated ES cells have to be eliminated from the differentiated cells. Further improvement might be achieved

by reprogramming adult cells. These cells could enable the production of patient-specific cell types, which are fully immune-compatible with the original donor. Furthermore, induced stem cells from patients suffering from diverse diseases could be differentiated into the cell type targeted by the disease and thereby providing *in vitro* models for studying pathogenesis as well as pharmacological and toxicological testing (Arsenijevic 2005; Teo and Vallier 2010).

2.3 Molecular mechanisms involved in the maintenance of pluripotency

Embryonic development is based on repression and activation of a vast number of cell-type specific genes but only a small number of transcription factors are thought to be responsible for their regulation. One property of ES cells is their continuous self-renewal, which requires that the unique transcriptional profile of the pluripotent state is maintained. Both transcriptional regulation and epigenetic regulation play pivotal roles in maintaining the existing transcriptional profile and controlling the plasticity of the transcriptional profile (Surani, Hayashi et al. 2007). Additionally, micro-RNAs (miRNAs) are emerging as regulators of pluripotency (Chen and Daley 2008).

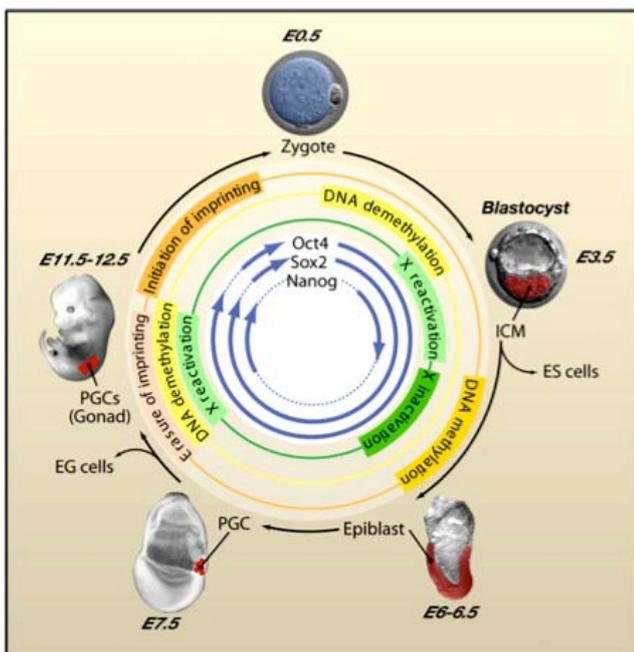


Figure 1: Genetic and epigenetic regulation of pluripotency during mouse development.

Oct4, Sox2 and Nanog are being inherited maternally and are expressed within the pluripotent tissue (indicated in red).

Early germ cells exhibit epigenetic and transcriptional states that are associated with pluripotency. The figure depicts the main epigenetic changes occurring during the critical stages of development. The figure was modified from Surani, Hayashi et al. (2007).

2.3.1 Transcriptional regulation of pluripotency

The existence of pluripotency factors has been hypothesized for a long time. To test this idea, some early studies compared global transcriptional profiles of ES cells and their differentiated

derivatives (Ivanova, Dimos et al. 2002; Ramalho-Santos, Yoon et al. 2002; Sato, Sanjuan et al. 2003). Interestingly, the results of these studies varied significantly and revealed an overlap of only about 1,5% (Evsikov and Solter 2003; Fortunel, Otu et al. 2003). Nevertheless, a few individual genes were successfully identified as critical pluripotency factors.

The two homeodomain transcription factors Oct4 and Nanog were the first proteins identified as essential for both early embryonic development and pluripotency maintenance in ES cells (Nichols, Zevnik et al. 1998; Chambers, Colby et al. 2003).

Another transcription factor that is thought to be involved in the regulation of pluripotency is Sox2. It belongs to the Sox (Sry-related HMG box-containing) gene family and is expressed in preimplantation embryos and in ES cells in a similar manner as *Oct4* (Yuan, Corbi et al. 1995; Collignon, Sockanathan et al. 1996). Later in development, Sox2 is again coexpressed with *Oct4* in postmigratory primordial germ cells (Collignon, Sockanathan et al. 1996). Sox2 is also expressed in other stem cells and precursor cells during development, including neural stem cells. Sox2-null mutant embryos cannot give rise to embryonic or trophectoderm lineages and are therefore not viable (Yuan, Corbi et al. 1995; Botquin, Hess et al. 1998).

Oct4, Nanog and Sox2 share a substantial fraction of their target genes. Interestingly, these three factors control each others transcription in a regulatory circuit. Many targets of Oct4, Sox2 and Nanog encode key transcription factors for differentiation and development, and are transcriptionally inactive in ES cells. Oct4, Sox2 and Nanog also control genes that are transcriptional active in ES cells and thought to be involved in the maintenance of pluripotency. These three factors are therefore involved in blocking differentiation and maintaining the pluripotent state (Boyer, Lee et al. 2005; Loh, Wu et al. 2006). It should be noted, however, that the phenotypes of Oct4, Nanog and Sox2 knockout mice are not identical, which indicates also non-redundant functions (reviewed by (Boiani and Scholer 2005)).

In addition to Oct4, Sox2 and Nanog, other factors required for pluripotency have been identified, including Sall4, Dax1, Essrb, Tbx3, Tcl1, Rif1, Nac1 and Zfp281 (Loh, Wu et al. 2006; Wang, Rao et al. 2006).

Sall4, a member of the spalt-like protein family, interacts with Nanog and co-occupies Nanog and Sall4 enhancer regions. Thus, Sall4 regulates Nanog expression as well as the expression of ES cell-specific genes. Sall4 also regulates Oct4 expression by binding to the Oct4 promoter (Wu, Chen et al. 2006; Zhang, Tam et al. 2006). Essrb and Rif1 are primary targets of both Oct4 and Nanog (Loh, Wu et al. 2006). Starting with Nanog interacting proteins, a complicated transcriptional regulatory network in mouse ES cells has been constructed with Oct4 and Nanog placed in key positions and many of the genes encoding proteins in the interaction network are targets of Nanog and/or Oct4 (Wang, Rao et al. 2006).

The importance of the Oct4 and Sox2 is underscored by recently published results of the groups of Thompson (Wisconsin, USA) and Yamanka (Kyoto, Japan). Both groups independently

accomplished to reprogram somatic cells to cells that behave like embryonic stem cells. Oct4, Sox2, c-Myc and Klf4 together can reprogram mouse embryonic and adult fibroblast cells to a pluripotent state (Takahashi and Yamanaka 2006; Okita, Ichisaka et al. 2007; Wernig, Meissner et al. 2007). These same four factors have also been proven capable of reprogramming human dermal fibroblasts (Takahashi, Tanabe et al. 2007). The group of Thompson showed that Oct4, Sox2, Nanog and Lin28 were sufficient to establish pluripotent cells from human somatic cells (Yu, Vodyanik et al. 2007). The induced pluripotent stem cells proliferate indefinitely in culture and differentiate into all tissues that are necessary to generate a live mouse. Though the proteins that were upregulated for reprogramming differed between both groups (Oct4, Sox2, Klf4 and c-Myc used by the group of Yamanaka in contrast to Oct4, Sox2, Nanog and Lin28 used by the group of Thompson), both found out that two factors are essential: Sox2 and Oct4 (Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007). These data suggest that Oct4 might be one of the key regulators to maintain and even induce stem cell pluripotency.

2.3.2 Epigenetic regulation of pluripotency

One feature of stem cells is their degree of self-renewal by which they can maintain the undifferentiated state through multiple cell divisions. But how is this undifferentiated state maintained, based on the fact that all cells of one organism possess the identical genome? The underlying principle of this cellular memory is most likely of epigenetic nature.

Conrad Waddington coined the term epigenetics, when he defined it 1942 as “the branch of biology, which studies the causal interactions between genes and their products, which bring the phenotype into being” (Waddington 1942). In 1957, he published the epigenetic landscape (Waddington 1957). It describes the differentiating cell as a marble rolling along a hilly landscape. At each branch, the marble can choose between two ways. Thereby, it will have made several binary choices until it reaches the bottom of the landscape (for review see (Slack 2002)).

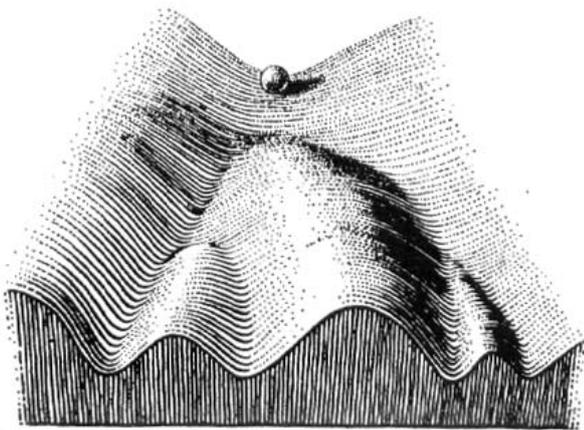


Figure 2: The epigenetic landscape
(Figure taken from Waddington 1957)

From a more modern point of view the term 'epigenetics' describes heritable changes in genome function that are not due to changes in the nucleotide sequence of the DNA. Chromatin is subjected to various forms of epigenetic regulation that influence the transcriptional activity. This process includes chromatin remodeling, histone modifications, histone variants and DNA methylation. In general, trimethylation of lysine 9 and lysine 27 of histone 3 (H3K9 and H3K27) correlate with inactive regions of chromatin, whereas H3K4 trimethylation, and acetylation of H3 and H4 are associated with active transcription, and DNA methylation generally represses gene expression (Jenuwein and Allis 2001; Lachner and Jenuwein 2002; Santos and Dean 2004; Kouzarides 2007). To maintain pluripotency in ES cells, genes whose upregulation leads to differentiation should be inactive whereas pluripotency factors should be expressed. Polycomb group proteins (PcG) play important roles in silencing these developmental regulators. The PcG proteins function in two distinct Polycomb Repressive Complexes, PRC1 and PRC2. Genome-wide binding site analyses have been carried out for PRC1 and PRC2 in mouse ES cells and for PRC2 in human ES cells (Boyer, Plath et al. 2006; Lee, Jenner et al. 2006). The genes regulated by the PcG proteins are co-occupied by nucleosomes with trimethylated H3K27. These genes are transcriptionally repressed in ES cells and are preferentially activated when differentiation is induced. Many of these genes encode transcription factors with important roles in development. Interestingly, the pluripotency factors Oct4, Sox2 and Nanog co-occupy a significant fraction of the PcG protein regulated genes (Boyer, Plath et al. 2006; Lee, Jenner et al. 2006). These data suggest that the PcG proteins may facilitate pluripotency maintenance by suppressing developmental pathways.

Beyond the specific regulations of development-related genes, ES cells exhibit unique chromatin features. Chromatin proteins in ES cells are hyperdynamic and bind loosely to chromatin. Upon differentiation, the hyperdynamic proteins tend to be immobilized on chromatin. Fewer heterochromatin foci are detected in ES cell nuclei and they seem to be more diffuse than those in differentiated cells (Meshorer and Misteli 2006).

Hyperdynamic binding of structural chromatin proteins seems to be a functionally important hallmark of pluripotent ES cells. This feature appears to contribute to the maintenance of plasticity in undifferentiated ES cells and to the establishment of higher-order chromatin structure. Additionally "bivalent domains" – containing both the histone H3K4 as well as the histone H3K27 trimethylation mark – were described in mouse ES cells (Bernstein, Mikkelsen et al. 2006). These "bivalent domains" are thought to keep cell-type specific genes repressed while keeping them poised for activation during differentiation. It was found that ES cell chromatin is enriched in active marks such as methylation H3K4 and acetylation of H3 and H4 whereas marks that are associated with repression of gene expression are diminished. Differentiation is associated with decrease in H3K4 trimethylation as well as an increase in H3K9 methylation and therefore

represents a transcriptionally less-permissive chromatin state (Lee, Hart et al. 2004; Meshorer, Yellajoshula et al. 2006).

The highly dynamic and transcriptionally permissive chromatin environment in ES cells may facilitate rapid transcriptional profile alternations upon differentiation and allow various transcriptional profiles to be established.

Pluripotency factors and epigenetic control seem to engage in cross talk with one another in order to maintain pluripotency. It has been reported that Oct4, Sox2 and Nanog regulate genes that encode components of chromatin remodeling and histone modifying complexes, such as SMARCAD1, MYS3 and SET (Boyer, Lee et al. 2005). Pluripotency factors also interact with histone modifying enzymes and chromatin remodeling complexes. Nanog and Oct4 interact with the histone deacetylase NuRD (P66b and HDAC2), polycomb group (YY1, Rnf2 and Rybp) and SWI/SNF chromatin remodeling (BAF155) complexes (Wang, Rao et al. 2006). Additionally, the genes of pluripotency factors are subjected to epigenetic regulation. One example for the epigenetic regulation of pluripotency factors are the histone demethylases Jmjd1a and Jmjd2c, which are positively regulated by Oct4. By demethylating H3K9Me2 at the promoters Jmjd1a positively regulates pluripotency-associated genes, such as Tcf1, Tcf2l1 and Zfp57 (Loh, Zhang et al. 2007). Additionally, Jmjd2c positively regulates Nanog expression by removing H3K9 trimethylation marks at the Nanog promoter. Depletion of Jmjd1a or Jmjd2c results in ES cell differentiation (Loh, Wu et al. 2006; Loh, Zhang et al. 2007).

2.4 Oct-proteins

Oct proteins are transcription factors that belong to the family of POU proteins. The acronym POU derives from the names of three mammalian transcription factors:

- pituitary-specific **Pit-1**
- octamer-binding proteins **Oct-1** and **Oct-2**
- neural **Unc-86** from *Caenorhabditis elegans*.

All Oct proteins contain a DNA binding POU domain that binds to the octamer motif ATGCAAAT, which was first found in the promoter of immunoglobulin genes. This binding sequence is found in the promoters and enhancers of many ubiquitously expressed and cell specific genes (Boheler 2005). The POU domain consists of two highly conserved subdomains – a “POU specific domain” (POU_s) and a C-terminal “POU homeodomain” (POU_{HD}) that are joined by a flexible linker structure (Herr, Sturm et al. 1988; Herr and Cleary 1995) (Klemm, Rould et al. 1994).

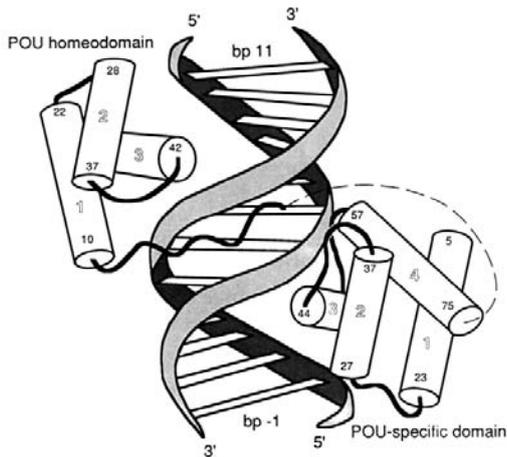


Figure 3: Binding of the POU homeodomain and the POU specific domain to the octamer motif.

POU_S domains bind to the first (ATGCAAAT), POU_{HD} domains to the second half (ATGCAAAT) of the octamer motif. The figure was modified from Klemm, Rould et al. (1994).

The mammalian genome contains typically three Oct proteins:

- **Oct1**: present in all eukaryotic cells
- **Oct2**: present in cells of the immune and nervous system
- **Oct4**: expressed in germ cells, totipotent and pluripotent cells during embryonic development

2.5 The role of Oct4 in mouse early development

2.5.1 Protein structure of Oct4

Oct4, also known as Oct3, is a mammalian transcription factor expressed by early embryonic cells and germ cells. It belongs to the group of POU V transcription factors. The protein consists of 325 amino acids (Scholer, Dressler et al. 1990) (Rosner, Vigano et al. 1990). The 160 amino-acid-long binding domain can be subdivided into the POU_S domain, measuring 75-amino-acids and the 60-amino-acid long carboxy-terminal POU_{HD} domain that are joined by a flexible linker region (Scholer 1991; Verrijzer and Van der Vliet 1993; Herr and Cleary 1995). The amino acid sequence of human OCT4 is 87% identical to that of the mouse, and the genomic organization, with regard to intron–exon boundaries is the same (Takeda, Seino et al. 1992; Abdel-Rahman, Fiddler et al. 1995).

2.5.2 Expression profile of Oct4

The gene *POU5F1* encodes the Oct4 protein. The *Oct4* gene has been found only in mammalian species. The gene maps to human chromosome 6 in a region homologous to mouse chromosome 17 (Takeda, Seino et al. 1992).

The expression of the *Oct4* gene is regulated by two enhancers – a distal and a proximal enhancer - linked to a single promoter (Nordhoff, Hubner et al. 2001). The distal enhancer region contributes to Oct expression in preimplantation embryos, ES cells, and embryonic germ cell, whereas the proximal enhancer regulates expression stage-specific and is active in the epiblast of mouse embryos (Yeom, Fuhrmann et al. 1996; Ovitt and Scholer 1998; Gidekel and Bergman 2002). Downregulation of Oct4 appears to be tightly controlled at several levels (Boheler 2005). Experiments indicate that epigenetic changes are involved in the regulation of Oct4. Analysis of the DNA methylation status of mouse *Oct4* gene upstream region revealed that the *Oct4* enhancer/promoter region is DNA hypomethylated, while the corresponding nucleosomes are hyperacetylated in ES cells compared to trophoblast stem cells. Furthermore, *in vitro* methylation suppresses *Oct4* enhancer/promoter activity (Hattori, Nishino et al. 2004). *In vivo*, repression of the *Oct4* gene locus is associated with increased DNA methylation and changes in chromatin structure in the regulatory region (Ben-Shushan, Pikarsky et al. 1993).

Oct4 mRNA and protein are detectable in unfertilized oocytes, and the protein is localized to the pronuclei following fertilization. As is typical of most maternal mRNAs, *Oct4* mRNA levels drop dramatically after fertilization. Nevertheless, Oct4 protein is detectable in the nuclei of 2-cell embryos. Zygotic *Oct4* expression is activated prior to the 8-cell stage, when levels of both mRNA and protein increase significantly in the nucleus. Expression of Oct4 is abundant and uniform in all cells of the embryo through the morula stage (32–64 cells) (Scholer, Hatzopoulos et al. 1989; Rosner, Vigano et al. 1990; Palmieri, Peter et al. 1994).

The first differentiation step occurs when the trophectoderm splits from the inner cell mass. Here the Oct4 expression stays restricted to the pluripotent tissue – the inner cell mass. Oct4 expression is then restricted to the primitive ectoderm although it is transiently expressed in high levels in the primitive endoderm (Palmieri, Peter et al. 1994). In the primitive ectoderm, Oct4 expression persists through day 7,5 p.c. in the presomitic mesoderm, decreasing anteriorly to posteriorly as somites form (Scholer, Dressler et al. 1990). The phenomenon of stem cell-specific expression can be observed during the whole development. Oct4 is being expressed in totipotent and pluripotent lineages and is being downregulated as soon as tissues differentiate (Scholer, Hatzopoulos et al. 1989). In contrast to the immediate downregulation of Oct4 in trophectodermal and somatic lineages, Oct4 protein levels are increased initially in cells of the premigratory hypoblast (Palmieri, Peter et al. 1994; Herr and Cleary 1995). The transient upregulation in the primitive endoderm, in contrast to the downregulation in the trophectoderm and other tissues, suggest that it is not a repression of *Oct4* that results in differentiation but more the continuous level of Oct4 expression that prevents differentiation. In adult mice, Oct4 is only expressed in germ cell precursors (Ovitt and Scholer 1998).

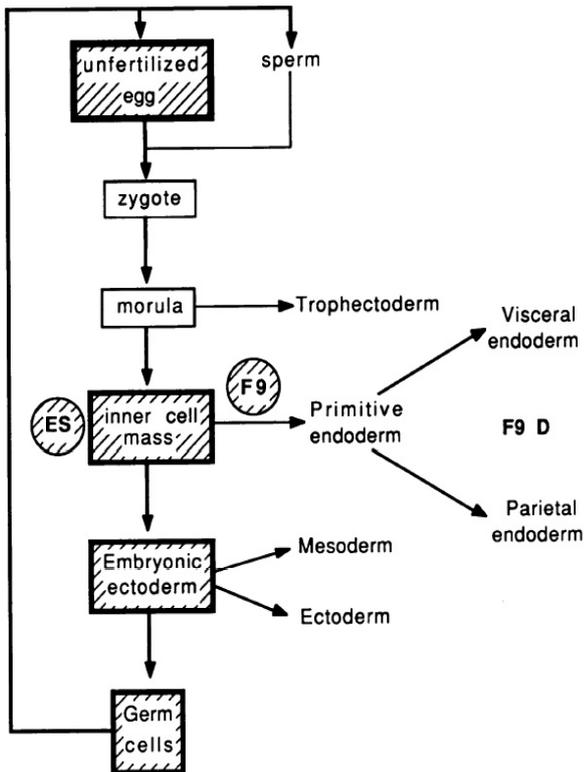


Figure 4: Germline-specific expression of Oct4

Boxes: cells and tissues representing the germline; hatched boxes and circles: cells and tissues expressing Oct4; in tissues and cells that are not framed, expression of Oct4 is not detectable or strongly reduced; expression of Oct4 was not investigated in the zygote and in the morula. The figure was modified from Scholer et al. (1990).

Oct4 expression can be detected in many germ cell tumors and somatic tumors such as lung cancer, breast cancer, bladder cancer etc. (Atlasi, Mowla et al. 2007; Chen, Hsu et al. 2008; Wang, Meng et al. 2010). Activation of Oct4 in epithelial tissues of adult mice results in dysplastic growth. These dysplastic tumors are dependent on continuous Oct4 expression (Hochedlinger, Yamada et al. 2005). Dysplastic lesions show an expansion of progenitor cells and increased β -catenin transcriptional activity. In the intestine, Oct4 expression causes dysplasia by inhibiting cellular differentiation in a manner similar to that in embryonic cells. Data indicates that adult progenitor cells remain competent to respond to Oct4 protein, suggesting Oct4 to be a potent proto-oncogene (Hochedlinger, Yamada et al. 2005). Also in cultured embryonic stem cells, the Oct4 expression correlates with the differentiation potential.

These findings are supported by knock out experiments of Oct4 in mouse embryos. Oct4 deficient embryos develop until the blastocyst stage, but when the first differentiation step occurs – when the trophectoderm splits from the ICM – they are unable to maintain the pluripotent cells of the ICM and differentiate into trophectoderm. This demonstrates that Oct4 expression is essential for the maintenance of potency during early development (Nichols, Zevnik et al. 1998). Overexpression of more than 1,5-fold of Oct4 induces differentiation to primitive endoderm or mesoderm (Niwa, Miyazaki et al. 2000). Thus a critical amount of Oct4 expression is necessary to maintain stem cell pluripotency. Up and down regulation induces divergent developmental programs. Oct4 expression is therefore associated with the undifferentiated or differentiating

state - *in vivo* as well as *in vitro*. Thus, proliferation, differentiation, and migration are three processes in which Oct4 might be involved during the formation of germ layers.

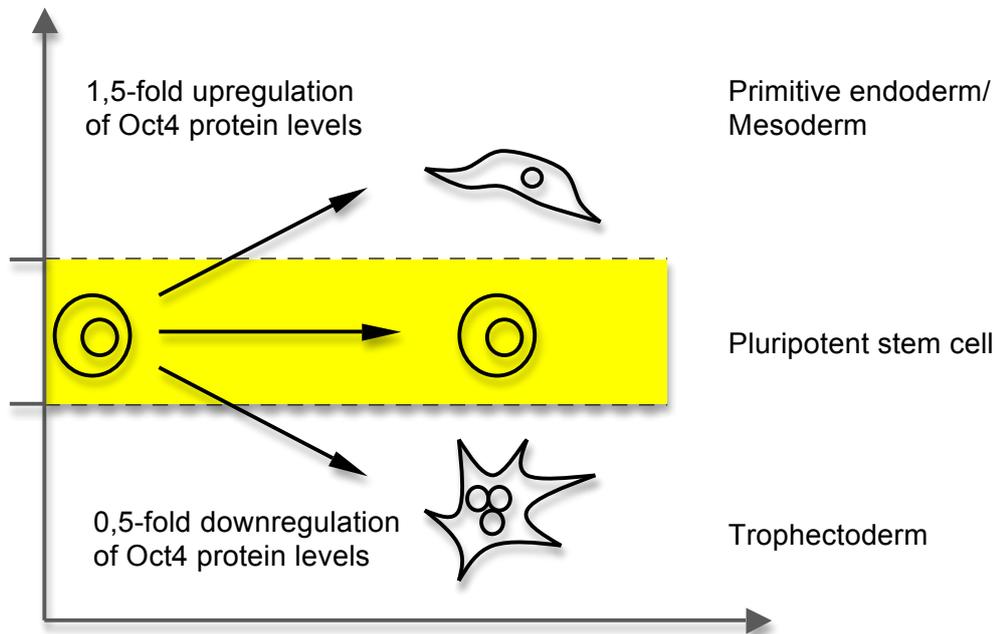


Figure 5: For the maintenance of pluripotency Oct4 levels need to be well regulated.

If Oct4 protein levels are upregulation more than 1,5-fold, differentiation is triggered into primitive endoderm and mesoderm. Downregulation of more than 0,5-fold, results in the formation of trophoblasts. The figure was modified from Niwa, Miyazaki et al. (2000)

2.5.3 Target genes of Oct4

The Oct4 protein carries out a dual function, acting *in vivo* both as a transcriptional activator as well as a transcriptional repressor (Ben-Shushan, Thompson et al. 1998).

Recent studies used a functional genomic approach in a human ES cell line to identify Oct4 dependent genes. More than 1,000 genes were identified, including targets regulated directly by Oct4 either positively (e.g. *Nanog*, *Sox2*, *Rex1*, *LEFTB*, *LEFTA/EBAF*, *DPPA4*, *THY1*, and *TDGF1*) or negatively (e.g. *Cdx2*, *EOMES*, *BMP4*, *Tbx18*, *FGF8*, *Brachyury [T]*, *Dkk1*, *HLX1*, *GATA6*, *ID2*, and *DLX5*), as well as targets for the Oct4-associated stem cell regulators Sox2 and Nanog.

This data suggest involvement in multiple signaling pathway, e.g. through Dkk1, which is a negative regulator of Wnt signaling, BMP4 that belongs to the transforming growth factor- β (TGF- β) family and FGF8 that is part of the fibroblast growth factor signaling (Kawabata, Imamura et al. 1998; Niehrs 1999). These pathways are implicated in regulating human ESC differentiation. Furthermore, a number of differentially expressed genes that are involved in epigenetics, chromatin remodeling, apoptosis, and metabolism were identified (Babaie, Herwig et al. 2007).

Wnt signaling plays an important role in early development and is involved in axis formation (Harland and Gerhart 1997). It was shown that activation of the Wnt/ β -catenin signaling pathway can cause embryonic stem cells to remain pluripotent under conditions that would normally induce differentiation (Kielman, Rindapaa et al. 2002; Hao, Li et al. 2006; Takao, Yokota et al. 2007).

Recently, published data highlighted the importance of a cross talk between Oct4 and the Wnt/ β -catenin signaling pathway. Oct4 facilitates proteasomal degradation of β -catenin by specific interaction. Thereby downregulation of Oct4 results in increased β -catenin levels leading to enhanced Wnt/ β -catenin signaling. Reduction of β -catenin levels was also achieved by overexpression of *Xenopus* Oct25 (Abu-Remaileh, Gerson et al. 2010).

2.6 *Xenopus laevis* as a model organism

Since Oct proteins are known to influence early development, it is favorable to study their function in a model organism that develops extra-uterine. Additionally, injection techniques must be established for injecting RNA into the egg cell. For the evaluation of observed phenotypes, it is advisable to choose a model organism whose early development is well studied from a macroscopic and molecular point of view.

The African clawed frog *Xenopus laevis* has been one of the most favored model organisms for vertebrate experimental embryology over the past decades. Major insights into early embryogenesis like signaling events important for body axis determination or germ layer formation were obtained from studies with *Xenopus* (for review see (Heasman 2006)).

Regarding embryological studies, the major advantage of *Xenopus* over other model organisms, like the mouse, is that the embryos develop extra-uterine. High numbers of eggs can be obtained from one female frog and can be fertilized *in vitro* at the same time and thus offer the synchrony of clutch. These features allow the performance of large-scale experiments. Existing fate maps enable the prediction of cell fate for each cell of the early embryo making it possible to manipulate selectively specific parts of the embryo. Embryos are relatively large (1-2 mm in diameter), develop rapidly and can be cultured in semi-sterile conditions what makes them easy to be manipulated e.g. by RNA injections.

These features make it an ideal model organism for further evaluating the molecular functions of *Xenopus* Oct proteins *in vivo*.

2.6.1 Early development of *Xenopus laevis* embryos

Figure 6 shows an overview of the *Xenopus* life cycle. The embryos are staged according to the normal table by Nieuwkoop and Faber (Nieuwkoop and Faber 1994). After fertilization, twelve subsequent cleavage divisions take place. In this cleavage phase, cells divide very rapidly every 30min. The cell cycle during this first phase consists of only S-phase and M-phase. Transcription does not start until 4000-cell stage, the so-called mid-blastula transition (MBT) hence a maternal pool of mRNAs is essential for early pattern formation. In *Xenopus*, the future germ layer domains are specified early by asymmetric localization of maternal mRNAs such as Vg1, VegT and Wnt11 which provide a blueprint for development by a specific positioning in the oocyte (Joseph and Melton 1998; Xanthos, Kofron et al. 2001; Dupont, Zacchigna et al. 2005; Tao, Yokota et al. 2005).

At MBT, the cell divisions slow down to about 60-90min and the cell cycle is separated into the four phases G1, S, G2 and M. During this stage of development, cells are already committed, but not yet determined to their future germ layer. During blastula stage a cavity called the blastocoel develops. The mid-blastula embryo has three regions, the animal cap (which forms the roof of the blastocoel), the equatorial or marginal zone (the walls of the blastocoel) and the vegetal mass (the blastocoel floor). Gastrulation starts at about 10 hours post fertilization (hpf). During this phase, major cell rearrangements occur. A blastopore is formed through which the endoderm and mesoderm invaginate. The invaginated dorsal mesoderm induces development of the neural plate in the overlying ectoderm. Through this process, the basic body plan of the tadpole is established. A fundamental aspect in the development of all triploblastic organisms is that cells have to maintain a non-committed state prior to gastrulation.

At about 15hpf, the neural tube folds up, which gives rise to the brain and the spinal cord. During those stages, the organs are also formed. Organogenesis mainly takes place from 21hpf onwards. The tadpole hatches at the age of about 48hpf. After about 38 days, changes in the environment, such as nutrient levels, induce the secretion of thyroid hormone. This leads to metamorphosis, which results in effects like the tail destruction, lung enlargement and ossification of the skull. The frogs are sexually mature after about 2 years. This summary is in essence taken from books written by Nieuwkoop and Wolpert (Nieuwkoop and Faber 1994; Wolpert 1998).

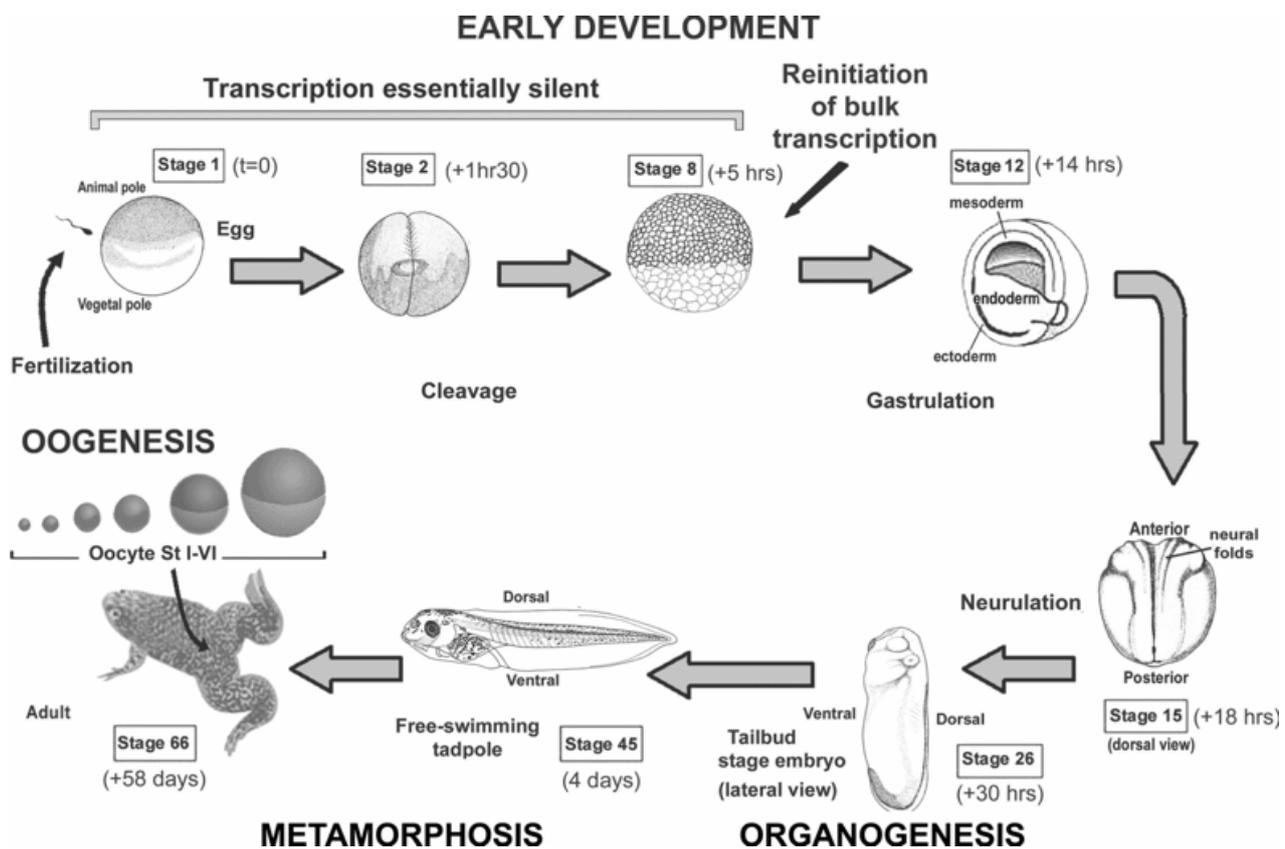


Figure 6: *Xenopus laevis* development.

Illustrated are the major steps in *Xenopus laevis* development. Numbers in parentheses indicate the timing after fertilization. The figure was adapted from Mereau, Le Sommer et al. (2007)

2.6.2 From totipotent cells to multicellular organisms - lineage commitment and differentiation in *Xenopus laevis* embryos

The development of a multicellular organism is a tightly controlled spatial and temporal process. It starts with a single, totipotent cell — the fertilized egg. Through subsequent cell divisions, the zygote gives rise to a complex organism. The developmental process leading from a totipotent cell to specialized cell types is called differentiation. This requires multiple successive events that result in the commitment of cells and finally leads to differentiated cell types. The process of commitment is subdivided into two stages. First, cells are specified – a step that is still reversible. A cell or a tissue is defined as specified, when it can differentiate autonomously in a neutral environment. The second step of commitment is called determination. Cells are considered to be determined, when they can differentiate autonomously even when placed in a non-neutral environment.

Competence – the ability of cells to respond to an inducing signal – and induction – the change in behavior of a group of cells – are important mechanistic principles of the development from a totipotent zygote to a multicellular organism (for detailed information see (Gilbert 2006)). A

fundamental aspect in the development of all triploblastic organisms is that cells have to maintain a non-committed state prior to gastrulation.

Although all mid-blastula cells are pluripotent, explants of the animal cap of *Xenopus* embryos form ectodermal derivatives in culture, while equatorial explants form mesoderm and vegetal explants form endoderm, indicating that they are already specified (Heasman, Wylie et al. 1984). Embryonic cells prior to gastrulation have the capacity to respond to various signaling pathways indicating that cell fate is not determined (Snape, Wylie et al. 1987; Gardner and Beddington 1988). The fate of ectoderm is reversible up to the early gastrula stage. When labeled single animal pole blastomeres from *X. laevis* blastulae are transplanted into the blastocoel of late blastula host embryos, the daughter cells of the transplanted blastomeres contribute to all three germ-cell layers. This data indicates, in agreement with the transplantation and induction experiments, that cells are still pluripotent at early gastrula stage. At later stages, the fate of the transplanted animal cells becomes restricted solely to ectoderm (Snape, Wylie et al. 1987).

At about the same time of development, endodermal cells become restricted to endoderm when transplanted (Heasman, Wylie et al. 1984; Snape, Wylie et al. 1987).

The marginal zone is specified as mesoderm early in the development process by factors located after cortical rotation in the marginal zone (e.g. FGF and BMP), without induction by the endoderm. Isolated marginal zones from *X. laevis* have the capacity for self-differentiation from the early blastula stage onwards, which shows that mesoderm is determined before the mid-blastula transition (Nakamura, Takasaki et al. 1978).

Although the temporo-spatial pattern of lineage commitment and differentiation has been extensively studied in *Xenopus laevis*, the understanding of molecular mechanisms controlling the precise timing of these events remains incomplete. Given the high degree of evolutionary conservation among vertebrates, *Xenopus laevis* offers an attractive alternative to investigate mechanisms of pluripotency and lineage-commitment in a more accessible non-mammalian model organism.

2.7 Oct4 homologs in *Xenopus laevis* embryos

In *Xenopus* embryos three POU factors belonging to subclass V are known: Oct25 (Hinkley, Martin et al. 1992), Oct60 (Whitfield, Heasman et al. 1993), Oct91 (Frank and Harland 1992). In the last years, a number of publications have addressed the issue whether Oct4-related POU V proteins in non-mammalian vertebrates have conserved functions. First evidence for this came from the demonstration that *Xenopus* Oct4-related proteins can partially substitute for Oct4 in mouse cells and thereby prevented these ES-cells from differentiation. Moreover POU V proteins

are known to regulate similar genes in ES cells and *Xenopus* embryos and different POU V proteins are capable of rescuing the *Xenopus* POU V knockdown phenotype (Morrison and Brickman 2006).

These experiments indicate that the unique ability of Oct4 to maintain ES cell pluripotency is derived from an ancestral function of this class of proteins.

2.7.1 Sequential homologies of *Xenopus* and mouse Oct proteins

The three *Xenopus* Oct4 homologs are similar in size - ranging from 47.3 to 49.5 kDa - and amino acid composition (Hinkley, Martin et al. 1992). Furthermore, they have a high degree of sequence homologies compared to Oct4. Comparing mouse and *Xenopus* Oct proteins, identity is immediately apparent in the POU_s and POU_h domains. Outside these regions there exists only weaker homology. The predicted protein sequences are more highly related to each other than they are to the sequences of other POU-domain proteins.

The POU domains of *Xenopus* Oct proteins display approximately 70% amino acid identities relative to each other, the complete sequence approximately 35% with Oct91 and Oct25 showing a higher degree of sequence homologies than Oct60 compared to Oct25 and Oct91. Comparing the POU domains of mouse Oct4 and *Xenopus* Oct proteins, homologies range from 57,7% (Oct60) to 66% (Oct91) (Morrison and Brickman 2006). Comparing the complete protein sequence, homologies are around 32%.

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: POU_s+h, POU specific domain and POU homeodomain; M, mouse Oct4; 91, Xlpou91; 60, Xlpou60; 25, Xlpou25; D, zebrafish Pou2.

Table 1: Protein sequence alignment of mammalian Oct4, *Xenopus* and Zebrafish POU proteins.

The Table was modified from Morrison and Brickman (2006).

2.7.2 Expression profiles of *Xenopus* Oct proteins

Oct25 and Oct91 are expressed zygotically and transcripts are detectable in gastrula and neurula stage embryos. Oct25 is expressed from late oocyte to early neurula stage showing a maximum of zygotic transcripts during the early gastrula stage (Hinkley, Martin et al. 1992).

Oct91 is activated at early gastrula stage and rises to peak expression levels at late gastrula (Frank and Harland 1992). Both proteins are expressed in the animal and marginal zone. They continue to be expressed throughout the process of gastrulation but only in cells that have not

undergone involution. Expression is downregulated as cells begin the process of involution and commitment to their germ layer fate (Morrison and Brickman 2006).

The expression pattern of Oct60 is unique among the *Xenopus* POU-genes. Oct60 is expressed maternally and is first detectable in stage V oocytes. It accumulates rapidly following fertilization with a peak at mid-blastula transition. The expression is restricted to the animal hemisphere in both unfertilized oocytes and early cleavage stage embryos and remains in the animal and marginal zones of the embryo.

In the blastula, when Oct25 and Oct91 begin to accumulate, Oct60 is being downregulated. Oct60 protein is detectable until midgastrula (Hinkley, Martin et al. 1992; Whitfield, Heasman et al. 1993; Whitfield, Heasman et al. 1995).

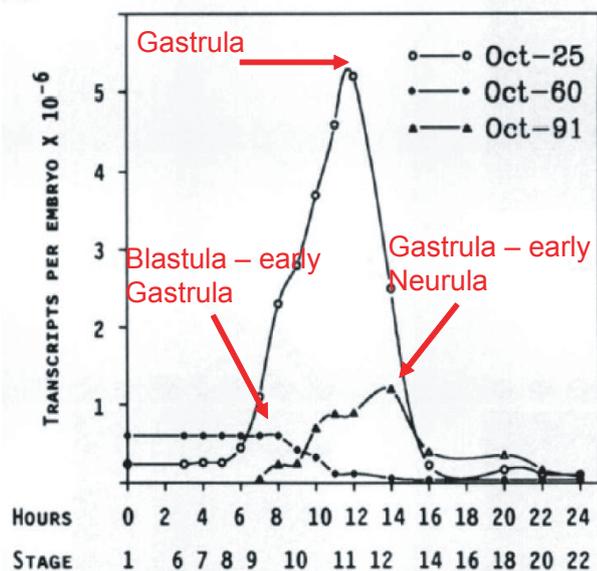


Figure 7: Temporal expression of *Xenopus* Oct-proteins.

Number of transcripts per embryo, expressed relative to time after fertilization and stages according to Nieuwkoop and Faber. RNase-resistant transcripts were quantified with a Betascanner and normalized to the results obtained with measured amounts of synthetic RNA. The figure was modified after Hinkley, Martin et al. (1992).

2.7.3 State of experiments on *Xenopus* Oct protein function

Xenopus Oct4 homologs were first described in 1992 and since then a considerable effort has been undertaken to investigate their functions (Frank and Harland 1992; Hinkley, Martin et al. 1992; Whitfield, Heasman et al. 1993). Recent work suggests that *Xenopus* POU-V proteins play a role in multiple signaling pathways involved in germ layer formation and patterning. Research has highlighted their role in VegT, FGF and BMP4 signaling.

VegT is a maternally expressed T-box transcription factor that is localized in the vegetal pole in full-grown oocytes and cleavage stages. Its depletion results in defects of primary germ layer formation and in loss of zygotic FGF and TGF- β /nodal expression (Zhang, Houston et al. 1998; Kofron, Demel et al. 1999). Maternal VegT is known to play a key role in the formation of mesoderm and endoderm (Xanthos, Kofron et al. 2001).

Overexpression studies indicate a possible role for Oct60 and Oct25 in the inhibition of genes activated by VegT and β -Catenin. Cao et al. suggested that Oct25, VegT and Tcf3 might interact with each other and form repression complexes on promoters of VegT and β -Catenin target genes and thereby repress mesendodermal germ layer induction and patterning. These results were obtained by overexpression of Oct25 and Oct60 in the vegetal pole of the embryo. Since Oct proteins are expressed in the animal hemisphere, while VegT is expressed in the vegetal half, further investigations are needed to figure out whether the interaction with VegT and Tcf3 is of biological relevance. Within the ectoderm, it was shown that Oct25 promotes neural fate by upregulating neuroectodermal genes, which prevent differentiation of neural progenitors into neurons (Cao, Knochel et al. 2004; Cao, Siegel et al. 2006; Cao, Siegel et al. 2007).

During development, the cellular responsiveness to fibroblast growth factor (FGF) changes. In blastula stages FGF induces mesoderm, whereas at gastrula stages neuroectoderm is being induced. Oct91 was recently shown to switch FGF function from a mesoderm inducing to a neural inducing signal at the beginning of gastrulation. Ectopic Oct91 expression inhibits FGF induction of *Xbra*, an early mesodermal marker, while neural tissues are unaffected. Knockdown of Oct91 expression induces *Xbra*, while *Sip1* and *Churchill*, two genes regulating neural competence, are strongly downregulated. Morphant ectodermal explants show an extended mesodermal responsiveness from blastula to gastrula stages. These experiments suggest, that Oct91 is required for a proper temporal response to FGF signals (Snir, Ofir et al. 2006).

Bone morphogenetic proteins (BMPs) and the related ligands activin/nodal are members of the TGF- β family that play essential roles in the specification and subsequent patterning of the germ layers (Massague 2000; Kishigami and Mishina 2005). In the ectoderm of early *Xenopus* embryos, BMP promotes epidermal differentiation by inducing expression of the *Msx*, *Dlx*, *Vent* and *Id* families of transcription factors that are responsible for inhibition of neural differentiation (Ladher, Mohun et al. 1996; Feledy, Beanan et al. 1999; Luo, Matsuo-Takasaki et al. 2001)

Oct25 inhibits the competence of ectodermal tissues to respond to BMP. BMP plays an important role in the regulation of ectodermal fate by inducing epidermis at the expense of neural tissue during gastrulation.

Oct25 overexpression in the ectoderm after the blastula stage suppresses early BMP responses of ectodermal cells downstream of BMP receptor activation and promotes neural induction while suppressing epidermal differentiation. In contrast, inhibition of Oct25 function in the prospective neuroectoderm results in expansion of epidermal ectoderm at the expense of neuroectoderm.

Oct proteins seem to have far reaching functions in coordinating dynamically embryonic induction events and germ layer formation. Investigations so far have mainly concerned the function of

Oct91 and Oct25. Much less is known about the function of Oct60. Still further efforts have to be made to find out whether *Xenopus* Oct proteins also control pluripotency comparable to mouse Oct4 and in particular by which mechanisms this is achieved. Furthermore, it has to be elucidated whether the three Oct4 paralogs carry out redundant or non-overlapping functions.

2.8 Objectives

The POU transcription factor Oct4 is one of a few key regulators of cellular pluripotency in mammals. In *Xenopus*, three Oct4-related genes have been identified, which are maternally and zygotically expressed from fertilization to neurulation. As shown by previous work, they can partially substitute for Oct4 in mouse ES cells. Furthermore, recent work implicates these proteins in multiple signaling pathways during germ layer formation, providing a conceptual framework for further analysis of their functions during frog development.

The goal of this work was to obtain tools for evaluating the function of *Xenopus* Oct proteins concerning the maintenance of pluripotency and germ layer formation. Therefore, I generated dominant activating and repressing gain of function variants for all three wild type *Xenopus* Oct proteins. Protein expression was verified *in vitro* as well as *in vivo* and their transactivating functions were evaluated in a luciferase assay *in vivo*.

Additionally, I performed overexpression studies of Oct60 and its newly generated gain of function variants. Several phenotypes were observed and analyzed by *in situ* hybridizations.

3 Materials and Methods

3.1 Laboratory Equipment

The subsequent laboratory equipments were used. The companies are put in brackets.

CCD camera: ProGres C14 (Zeiss)

Centrifuges: Eppendorf centrifuge 5417C (Eppendorf); Omnifuge 2.0 RS (Haereus); Sorvall RC-5B (Du Pont), Micro 22R (Hettich Zentrifugen), Optima LE-80K Ultracentrifuge (Beckman Coulter), PicoFuge (Stratagene)

Developer: Curix-60 (Agfa)

Glass injection needles: Glass 1BBL W/FIL 1.0 mm (World Precision Instrument).

Injector: Pli-100 (Digitimer Ltd.).

Incubator: Driblock DB1 and DB20 (Teche).

Luminometer: Lumat LB 9501, EG&G Berthold

Microneedle Puller: P-87 (Sutter Instrument).

Micromanipulator: Mm-33 (Science Products).

Microscopes: Stereomicroscopes Stemi SV6, Stemi SV11 (Zeiss), MZFCIII (Leica),

Nylon membrane: Hybond™ N (Amersham).

Ph-Meter: pH-Meter 761 Calimatic (Knick)

Pipettes: Pipetman, Gilson (2µl, 20µl, 200µl, 1000µl)

Software: Photoshop CS2 (Adobe); Illustrator CS2 (Adobe); MacVector 7.1 (Oxford Molecular Group); Office 2004 for Mac (Microsoft), Endnote 9.0 (Thomson), ABI Prism Primer Express (Applied Biosystems)

Sonicator: Bioruptor™ (Diagenode)

Spectrophotometer: Nanodrop ND-1000 (PeqLab)

3.2 Reagents

3.2.1 Chemicals

Agar (Difco); Agarose (Gibco/BRL); Ampicillin, Streptomycin, Bacto trypton, Yeast extract (Difco); Chicken serum, lamb serum (Gibco/BRL); Human chorionic gonadotropin (Sigma); Levamisol (Vector Laboratories), QIAzol (Qiagen)

The subsequent fine- and bio-chemicals were ordered at the following companies:

Fluka, Merck, Sigma-Aldrich, Roth and Biomol.

3.2.2 Enzymes and proteins

The following enzymes were ordered at the companies put in brackets:

Alkaline phosphatase (Roche); BSA fraction V, Chymostatin, Leupeptin, Pepstatin (Sigma); Klenow enzyme (Roche); Restriction endonuclease with 10x restriction buffer system (New England Bio Labs, Roche, Fermentas); RNaseA (Sigma); RNasin (Promega); T3, T7 and SP6 RNA polymerase with 5x incubation buffer (Promega); Taq DNA polymerase with 10x PCR buffer (NEB), Advantage 2 Polymerase Mix (BD Bioscience Clontech), Proteinase K (Sigma); RNase free DNase I (NEB) and Pre-stained protein molecular weight standard (Sigma), Precision Plus Protein Prestained Standard (Biorad)

3.3 Nucleic acids

3.3.1 Size standard

1kb ladder: GeneRuler™ 1kb DNA ladder (Fermentas)

The DNA ladder yields the following 14 discrete fragments (in base pairs): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250.

100bp ladder: GeneRuler™ 100bp DNA ladder plus (Fermentas)

The DNA ladder yields the following 14 discrete fragments (in base pairs): 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100.

3.3.2 Oligonucleotides

Oligonucleotides were synthesized by Biomers.

Morpholino oligonucleotides were ordered from Gene Tools.

3.3.2.1 Oligonucleotides for cloning

Oligos for PCR-cloning of *oct60* into the pCS2⁺-MT6 vector:

LM 01	5'- C ^{XhoI} TCT CGA GCA ATG GAC CAG CCC ATA -3'	sense; 25-mer
LM 02	5'- CC ^{XbaI} TCT AGA TCA GCC GGT CAG GAC -3'	reverse; 23-mer

Oligos for PCR-cloning of *oct25* into the pCS2⁺-MT6 vector:

LM 03	5'- C ^{XhoI} TCT CGA GCA ATG TAC AGC CAA CAG -3'	sense; 25-mer
LM 04	5'- CC ^{XbaI} TCT AGA TCA GCC AAT GTG GC -3'	reverse; 22-mer

Oligos for PCR-cloning of *oct91* into the pCS2⁺-MT6 vector:

LM 05	XhoI 5'- C <u>TCT CGA GCA</u> ATG TAT AAC CAA CAG -3'	sense; 25-mer
LM 06	XbaI 5'- CC <u>TCT AGA</u> CTA GTT GCC TTG GTT AC -3'	reverse; 25-mer

Remarks: Primers LM 01,02,03,04,05,06 amplify the *oct* cDNA so that it can be cloned into the pCS2⁺-MT vector downstream of the *enR/vp16/gr* domain. XhoI and XbaI can be used for cloning.

Oligos for PCR-cloning of the *gr* repression domain into the pCS2⁺-MT6 vector:

RR 149	StuI MluI SpeI 5'-GA <u>AGG CCT ACG CGT ACT AGT</u> CCC TCT GAA AAT-3'	sense; 32-mer
LM 07	XhoI 5'- CTC <u>TCG AGC</u> CTT TTG ATG AAA C -3'	reverse; 29-mer

Oligos for PCR-cloning of the *vp16*- activation domain into the pCS2⁺-MT6 vector:

RR 177	StuI SpeI 5'-GA <u>AGG CCT ACT AGT</u> ACG GCC CCC CCG ACC GAT-3'	sense; 31-mer
LM 08	XhoI 5'- C CGC <u>TCG AGC</u> CCC ACC GTA CTC GTC AAT - 3'	reverse; 28-mer

Oligos for PCR-cloning of *oct60* into the pCS2⁺-MT6-enR/vp16 vector:

LM 09	XhoI 5'- C <u>TCT CGA GCA</u> GGA ATG ACC CTT GAG GA- 3'	sense; 27-mer
LM 10	XbaI 5'- CC <u>TCT AGA</u> CTA GGA CAT TCT GAA TTT GC - 3'	reverse; 28-mer

Oligos for PCR-cloning of *oct25* into the pCS2⁺-MT6-enR/vp16 vector:

LM 11	XhoI 5'- C <u>TCT CGA GCA</u> GTT CCC AGC GAA TCA GAA - 3'	sense; 28-mer
LM 12	XbaI 5'- CC <u>TCT AGA</u> CTA GGG CAT TCC CTG ACG CTT - 3'	reverse; 29-mer

Oligos for PCR-cloning of *oct91* in pCS2⁺-MT6-enR/vp16 vector:

LM 13	XhoI 5'- C <u>TCT CGA GCA</u> GCC CCT AAT TCT GGG GAG - 3'	sense; 28-mer
LM 14	XbaI 5'- CC <u>TCT AGA</u> CTA ATA GGG GTA CAC CTG GCG - 3'	reverse; 29-mer

3.3.2.2 Antisense morpholino oligonucleotides

All antisense morpholino oligonucleotides were designed according to Morrison et al., 2006 and provided by Gene Tools.

oct60-MO	5'-GTACAATATGGGCTGGTCCATCTCC-3'
oct25-MO	5'-ACATGGTGTCCAAGAGCTTGCAGTC-3'
oct91-MO	5'-GTAGGTCTGTTGGTTATACATGATC-3'

3.3.3 Plasmids

Unless stated otherwise, plasmids were constructed and cloned in our lab.

3.3.3.1 Vectors for cloning

pCS2+MT6 (Rupp, Snider et al. 1994): The vector backbone is from pBluescript II KS+ pCRII-TOPO-vector (Invitrogen)

3.3.3.2 Plasmids for in vitro transcription

Plasmid	PCR primers for amplification	Enzymes used for cloning	Linearized by	Polymerase by
pCS2 ⁺ -MT-EnR			SacII	SP6
pCS2 ⁺ -MT-VP16	RR 177 & LM 08	StuI, XhoI	NotI	SP6
pCS2 ⁺ -MT-GR	RR149 & LM 07	StuI, XhoI	NotI	SP6
pCS2 ⁺ -MT-Oct60	LM 01 & LM 02	XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-Oct25	LM 03 & LM 04	XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-Oct91	LM 05 & LM 06	XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-EnR-Oct60	LM 09 & LM 10	StuI, XhoI, XbaI	SacII	SP6
pCS2 ⁺ -MT-EnR-Oct25	LM 11 & LM 12	StuI, XhoI, XbaI	SacII	SP6
pCS2 ⁺ -MT-EnR-Oct91	LM 13 & LM 14	StuI, XhoI, XbaI	SacII	SP6
pCS2 ⁺ -MT-VP16-Oct60	LM 09 & LM 10	StuI, XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-VP16-Oct25	LM 11 & LM 12	StuI, XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-VP16-Oct91	LM 13 & LM 14	StuI, XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-GR-Oct60		StuI, XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-GR-Oct25		StuI, XhoI, XbaI	NotI	SP6
pCS2 ⁺ -MT-GR-Oct91		StuI, XhoI, XbaI	NotI	SP6

3.3.3.3 Plasmids for dig-labeled RNA in situ hybridization probes

RNA antisense probe	Plasmids linearized by	Polymerase
<i>Cardiac actin</i>	PvuII	SP6
<i>Endodermin</i>	EcoR1	T7
<i>N-β-tubulin</i>	BamH1	T3
<i>Xbra</i>	EcoR1	T7

3.3.3.1 Plasmids for the luciferase assay

6w-tk-luc: The 6w enhancer contains 6 copies of oligonucleotides with an octamer binding motif from the mouse Ig heavy chain gene enhancer (Tomilin, Remenyi et al. 2000). The promoters are linked to the luciferase reporter gene. The promoter was fused with the *luciferase* gene.

6xMORE-tk-luc: The *more* promoter is a palindromic Oct recognition element (ATGCATATGCAT) that mediates assembly of stable Oct homodimers and heterodimers and leads to strong transcriptional activation. The promoter was fused with the *luciferase* gene.

Both plasmids were kindly provided by the lab of Hans Schöler.

3.4 Handling of bacteria

Preparations of competent cells and transformation have been performed according to standard methods (Sambrook et al., 1989).

3.4.1 Bacteria strains

Summary of *Escherichia coli* strains

Strain	Genotype	Company
BL21(DE3)	<i>B F- dcm ompT hsdS(rB- mB-) gal (DE3)</i>	Novagene
One Shot E. coli	<i>F-φ80lacZΔM15Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K,m_K) phoA supE44thi-1 gyrA96 relA1 tonA</i>	Invitrogene
JM110	<i>rpsL (Strr) thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB) [F' traD36 proAB lacIqZΔM15]</i>	Stratagene
XL1Blue	<i>F'::TN10 proA⁺B⁺lac^d Δ(lacZ)M15/recA1 end A1 gyrA96(Nal^R) thi hadR17 (r_K⁻m_K⁻) glnV44 relA1 lac</i>	Stratagene

3.5 Antibodies

3.5.1.1 Antibodies for *in situ* Hybridization

Sheep anti-Digoxigenin Fab fragment coupled with alkaline phosphatase (Roche);

3.5.1.2 Antibodies for Western Blot analysis

Primary anti-myc-tag-antibody: **c-Myc 9E10 (WB 1:100), monoclonal** (Evan, Lewis et al. 1985)

Secondary antibody: peroxidase-conjugated AffiniPure Goat-Anti Mouse IgG (H+L) (1:10000) (Dianova)

3.6 Molecular biological methods

3.6.1 Solutions

AB-buffer: 80% TBSX, 15% heat-inactivated lamb serum, 5% *Xenopus* egg extract.

AP-Buffer: 100mM trichlorethane Tris/HCl 9.5; 100mM NaCl; 50mM MgCl₂

Bleaching solution: 1% H₂O₂; 5% Formamid; 0.5x SSC

DEPC-H₂O: ddH₂O with 0.1% Diethylpyrocarbonat (DEPC) agitated at 23°C over night and autoclaved afterwards.

10mM DIG NTP mixture: 10mM CTP, GTP, ATP, 6.5mM UTP and 3.5mM Dig-11-UTP.

Hybridizing solution: 5x SSC, 50% formamide, 1% Boehringer block, 0.1% Torula RNA, 0.01% Heparin, 0.1% Tween-20, 0.1% CHAPS, 5mM EDTA.

Lamb Serum: Heat-inactivated lamb serum (30 min with 56°C), stored at -20°C.

MEMFA: 0.1M 3-(N-Morpholino)-propanesulfonic acid (MOPS), 2mM EGTA, 1mM MgSO₄, 3.7% formaldehyde pH 7.4

Paraformaldehyde: 4% paraformaldehyde in PBSw

PBS: 137mM NaCl, 2.7mM KCl, 8mM Na₂HPO₄, 1.7mM KH₂PO₄ pH 7.2

PBSw: 1xPBS, 0.1% Tween-20

PCI: 50% phenol, 48% chloroform, 2% isoamyl alcohol.

Proteinase K: 10µg/ml Proteinase K in PBSw

20xSSC: 3M NaCl, 0.3M sodium citrate (pH 7.0 at 23°C).

TBS: 50mM trichloroethylene (Tris)/HCl, 150mM of NaCl (pH 7.5 at 23°C).

TBSX: 1xTBS, 0.1% Triton X-100 (pH 7.5 at 23°C).

TE: 1mM EDTA, 10mM of Tris/HCl (pH 8.0 at 23°C).

TBE: 100mM Tris/HCl, 83mM borate, 0.1mM EDTA (pH 8.6 at 23°C).

***Xenopus* egg extract for *in situ* hybridization:** unfertilized eggs were dejellied with 2% cysteine, washed 3 times, 1 volume of PBS was added. Then they were lysed by 10 strokes

of a Dounce homogenisators, and centrifuged (7500xg, Sorvall Rc-5b, rotors SS-34, 10000rpm, 4°C, 10min). The supernatant was transferred into a fresh centrifuge tube and recentrifuged twice under the same conditions. The supernatant was aliquoted and stored at -20°C.

3.6.2 Isolation of nucleic acids

3.6.2.1 Mini-preparation with Qiagen kit

Plasmid DNA mini-preparations were carried out using the QIA prep spin Miniprep kit (250) (Cat.No. 27106).

3.6.3 Analysis and manipulation of nucleic acids

3.6.3.1 Cloning methods

The cloning of DNA has been performed according to standard methods (Sambrook et al., 1989).

3.6.3.2 Gel electrophoresis of nucleic acids

DNA or *in vitro* synthesized RNA was isolated in horizontal agarose gel. Depending upon fragment size, one to two percent TBE agarose gels were used. After electrophoresis the gels were photographed. 1kb or 100bp DNA ladder was used as size standard.

3.6.3.3 Isolation of DNA fragments from agarose gel

In order to isolate DNA fragments after electrophoresis from agarose gel, the appropriate bands were cut out under long-wave UV light. The DNA was extracted from the gel with QIAquick gel extraction kit (250) (Cat.No. 28706).

3.6.4 Polymerase chain reaction (PCR)

3.6.4.1 PCR amplification of DNA fragments for cloning

The reaction was accomplished in a total volume of 50µl. The reaction mixture contained 100ng template DNA, 25pmol each primer, 0.5mM dNTPs, 1U Advantage Taq Polymerase (Invitrogen) or Taq polymerase (NEB) and 1x of the supplied buffer. The program was 95°C 30 sec, x°C 30sec (annealing temperature depended on the primers used), 68°C/72°C 1min/kb, 30 cycles. The PCR products were digested with the suitable endonuclease and separated on agarose gel. Subsequently, the desired DNA fragment was isolated.

3.6.5 *In vitro* transcription

3.6.5.1 *In vitro* transcription for microinjection

Capped mRNAs for microinjection were *in vitro* transcribed with RNA polymerase. Reactions were set up as following: in a total volume of 50µl, 4µg linearized plasmid DNA, 1x of the supplied transcription buffer, 0.5mM dNTPs, 2.5mM RNA cap structure analogue, 10mM DTT, 20U RNasin and 40U Sp6 or 60U T3 or T7 RNA Polymerase. The reaction was incubated for 4 hours at 37°C. Subsequently, the template DNA was digested with 10U RNase free DNaseI for 30min at 37°C. The RNA was purified with the RNeasy Kit Mini (Qiagen) (Cat.No.74104). The concentration of the RNA was determined by NanoDrop ND-1000 spectrophotometer (Peglab).

3.6.5.2 *In vitro* transcription of dig labeled RNA probes

Plasmids were linearized and antisense RNA was generated by *in vitro* transcription. The reactions were set up in a total volume of 50µl as following: 4µg linearized plasmid DNA, 1x of the supplied transcription buffer, 0.1mM Dig-NTPs, 20U RNasin and 20U SP6 or T3 or T7 RNA Polymerase. The reactions were incubated at 37°C for 4h and purified with the RNeasy Kit Mini (Qiagen).

3.6.6 RNA *in situ* hybridization

The embryos were fixed in fresh MEMFA for 1.5-2 hours and washed afterwards with PBS 3x5min. The dehydration of the embryos was performed over a period of one hour by replacing the PBS subsequently with 100% ethanol. The lipid membranes were dissolved overnight at -20°C in 100% ethanol. The embryos were rehydrated through a 75, 50, 25% ethanol series in PBSw. Each ethanol step was incubated for 5min at room temperature. Afterwards 3 washes with for 5min with PBSw were performed. The solution was then changed to Proteinase K in PBSw and incubated for 20min at 17°C, followed by a short rinse with PBSw. Again two washes for 5min each were performed with PBSw. After the Proteinase K digest the embryos were refixed with paraformaldehyde for 20min. A short rinse with PBSw was performed followed by subsequent washing in PBSw for 5x5min. The PBSw was replaced with hybridization solution (50% PBSw: 50% hybridization solution; 100% hybridization 3min each step). 0.5ml of fresh hybridization solution was added to each vial and incubated at 65°C for 1h to inactivate endogenous phosphatases. The embryos were then prehybridized at 60°C for 2-6h. To 100µl of hybridization solution 30-50ng of RNA probe was added and incubated at 95°C for 2-5min, cooled immediately afterward on ice and added to the embryos in prehybridization solution. The RNA probe was hybridized to the mRNA over night at 60°C. To remove excessive RNA probe, the embryos are washed the following after the hybridization: 2xSSC; 0.1% CHAPS short rinse; 2xSSC;0.1% CHAPS for 20min; short rinse with 0.2xSSC;0.1% CHAPS; 2x for 30min at 60°C in

0.2xSSC;0.1% CHAPS. Prior to the antibody binding the embryos were transferred into TBSX (short Rinse in 50% TBS: 50% 0.2xSSC; 0.1% CHAPS), washed in TBS for 5min and rinsed in TBSx. To block unspecific antibody binding sites, the embryos were incubated in antibody buffer (0.5ml per vial) for 2h at 4°C. In parallel, AP-conjugated anti-DIG antibody (1/5000 diluted) was preabsorbed against *Xenopus* proteins present in antibody solution. 0.5ml of preabsorbed antibody solution was added to the embryos and incubated overnight at 4°C. After antibody binding, the embryos were briefly rinsed with TBSx and washed 6 times for 1h in TBSx. Embryos were shortly rinsed in AP buffer and equilibrated for 15min. AP-buffer was replaced with 0.5ml staining solution and incubated overnight until or to 3 days at 17°C in the dark. The staining reaction was stopped by washing twice in PBS for 10min. If the embryos were over-stained, some color was removed by washing the embryos in 75% ethanol in PBS for 20min. The stain was fixed in MEMFA for 90min. The embryos were bleached in bleaching solution on a light box for 2h. The bleach solution was washed off with PBS 3x5min. For long-term storage, the embryos were transferred to PBSw containing 0.2% azide and kept at 4°C.

3.7 Protein analysis

3.7.1 Solutions

3x Lämmli buffer: 150mM Tris pH6.8, 300mM DTT, 4% SDS, 30% glycerol

Chemiluminescence reagents (ECL): Luminol solution: 0.44g luminol in 10ml DMSO, freeze in 1ml aliquots, store at -20°C; p-coumaric acid: 0.15g in 10ml of DMSO, freeze in 0.44ml aliquots, store at -20°C;

solution 1 (100ml): 10ml 1M Tris/HCl pH 8.5, 1ml luminol, 0.44ml p-coumaric acid;

solution 2 (100ml): 10ml 1M HTris/Cl pH 8.5, 60µl 30% H₂O₂

3.7.2 *In vitro* translation

In vitro translations of proteins were performed with the TNT® SP6 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's protocol.

3.7.3 Protein extraction for Western Blot Analysis

For each condition 5 embryos were collected at the required stage. 50 µl of embryo-lysis-buffer for Western blot analysis were added per embryo (250 µl starting volume/ 5 embryos/ tube). Embryos were lysed by pipetting with a 200µl Pipetman yellow tip and sonication, using the Bioruptor 3x30sec at high level. The cell debris was removed by a centrifugation step for 10min at

4°C at full speed. 250µl of 1,1,2 trichlorotrifluoroethane were added to the supernatant, vortexed and centrifuged for 10min at 4°C at full speed. The upper, aqueous phase containing the protein was separated into a new tube and precipitated by Wessel-Flügge-precipitation. The samples were mixed with 1 vol ddH₂O, 2 vol methanol, 1 vol CHCl₃, vortexed and centrifuged for 5min at full speed at 4°C. The upper phase was discarded and 3 vol of methanol were added to the remaining solution and vortexed. After centrifugation the white protein pellet was air-dried and resolved in 3x Lämmli buffer, incubated for 10min at 95°C, shock frozen with liquid nitrogen and stored at -80°C.

3.7.4 SDS-PAGE and Western Blot Analysis

SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and Western blot analysis were carried out according to standard protocols (Sambrook, Fritsch et al. 1989). After Protein extraction a 10% SDS-polyacrylamide gel was used to resolve the protein with a voltage of 120V/400mA for 90 minutes. Proteins were transferred on to a PVDF membrane by the wet transfer system from BioRad for 60 minutes at 70V/400mA and 4°C. Afterwards the membrane was transferred into 5% milk in PBSw for one hour to block nonspecific binding sides. The membrane was transferred into 5% milk in PBSw with the primary anti-myc-tag-antibody (1:100) and incubated at 4°C overnight. Thereupon the membrane was washed with PBSw three times for 10 minutes each and then incubated with the secondary peroxidase-conjugated Goat-Anti Mouse IgG antibody (1:1000) for two hours at room temperature. The membrane was washed in PBSw three times for 20 minutes each. Signals were detected by enhanced chemiluminescence solution in a relation of 1:1 after exposing the membrane to X-ray film (Super-RX Fuji medical X-ray film). Films were developed according to the manufacturer's protocol.

3.7.5 Luciferase assay

Injected embryos were frozen in liquid nitrogen and stored at -80°C until further analysis. For every condition three samples, consisting of 5 embryos each, were analyzed in parallel. Preparation of cell lysates and measurements were performed using the Dual-Luciferase™ Reporter Assay System (Promega) and a Luminometer (Lumat LB 9501, EG&G Berthold). Lysis buffer (PLB), luciferase assay reagent II (LARII) and stop&glow reagents are part of the Dual-Luciferase™ Reporter Assay Systems kit and were prepared according to the manufacturer's protocol. 50µl of Passive Lysis Buffer (PLB) were added per embryo. Embryos were frozen and thawed two times followed by manual lysis by vortexing and pipetting with a 200µl Pipetman yellow tip. After a 10 minute incubation period on ice the cell lysate was spun for 30 seconds at 4°C full speed. 100µl of LARII solution were pre-dispensed into the luminometer tubes. 20µl of cell lysate was added and mixed by pipetting. Tubes were placed into the luminometer and firefly luciferase

activity was measured. 100µl of stop&glow reagent was added to record the *Renilla* luciferase activity. Means from the three different samples of each condition were assessed and the mean coefficient firefly / renilla activity was calculated.

3.8 Histological methods

3.8.1 Solutions

AP buffer: 100mM Tris/HCl (pH 9.5), 50mM MgCl₂, 100mM NaCl, 0.1% Tween 20, 5mM Levamisole.

AP staining solution: 4.5µl NBT, 3.5µl BCIP in 1ml AP buffer.

A-PBS: 103mM NaCl, 2.7mM KCL, 0.15mM KH₂PO₄, 0.7mM NaH₂PO₄ pH7.5

A-PBS-T: APBS with 0.1% Tween20

Blocking buffer: PBT plus 10% heat inactivated serum

MEMFA: 0.1M MOPS, 2mM EGTA, 1mM MgSO₄, 3.7% formaldehyde (pH7.4 at 23°C), prepare freshly.

PBS: 137mM NaCl, 2.7mM KCl, 8mM Na₂HPO₄, 1.7mM KH₂PO₄ (pH7.2 at 23°C).

PBT: PBS, 2mg/ml BSA, 0.1% Triton-X-100.

X-Gal staining solution: 50mm K₃Fe(CN)₆, 50mm K₄Fe(CN)₆, 25µl Xgal (40mg/ml), 2µl MgCl₂ (1M) per ml PBS

3.8.2 LacZ staining

Embryos were fixed in MEMFA for 1 hour and rinsed with 1xPBS twice for 10 min each. Embryos were stained with X-gal staining solution in the dark at 30°C for approximately 1 hour until staining was completed. The staining reaction was stopped by washing 3 times in PBS for 5 minutes each. For fixation embryos were transferred to MEMFA for another hour, washed in PBS three times for 10 minutes each and stored in methanol at -20°C.

3.9 Embryological methods

3.9.1 Solutions

Cystein: 2% L-Cystein in 0.1xMBS (pH7.8 at 23°C, adjusted with 5M NaOH).

Human Chorionic Gonadotropin (HCG): 1000 I.U./ml HCG in ddH₂O.

MEMFA: 0.1M 3-(N-Morpholino)-propanesulfonic acid (MOPS), 2mM EGTA, 1mM MgSO₄; 3.7% formaldehyde (pH 7.4 at 23°C).

1xModified Barth's Saline (MBS): 5mM HEPES, 88mM NaCl, 1mM KCl, 0.7mM CaCl₂, 1mM MgSO₄, 2.5mM NaHCO₃ (pH 7.6 at 23°C). Add the CaCl₂ before use.

MBS/high salt: 1xMBS with 50mM NaCl

0.1/0,5xMBS/Gentamycin: 0.1/0,5xMBS, 10µg/ml Gentamycin

MBS/CS: 0.8xMBS high salt with 20% chicken serum, 200U Penicillin/ml, 200µg/ml streptomycin stored at -20°C

3.9.2 Experimental animals

Adult wild-type *Xenopus laevis* frogs (Xenopus Express and Nasco) were used. The frogs were kept in tap water with a temperature of 17-19°C and a population density of 5l water per frog. The animals were fed three times per week with Pondsticks Premium brittle (Interquell GmbH, Wehringen).

3.9.3 Superovulation of female *Xenopus laevis*

Egg deposition was stimulated by injection of 500-700 units of human chorionic gonadotropin (Sigma) into the dorsal lymph sac. Egg laying started about 12-18h later.

3.9.4 Preparation of testis

A male frog was anaesthetized in 0.1% 3-Aminobenzoic acid-ethyl-ester in ddH₂O for 30min, cooled down in ice-cold water and killed by decapitation. The two testes were taken from the abdominal cavity by pulling out the yellow fat body, with which they are connected by connective tissues. Until use, the testes were stored in MBS/CS for maximal 7 days.

3.9.5 *In vitro* fertilization of eggs and culture of the embryos

For *in vitro* fertilization a piece of testis was minced in 1xMBS and mixed it with freshly laid eggs. Afterwards the embryos were cultured in 0.1xMBS at 16-23°C in 110mm Petri dish.

3.9.6 Removal of the egg jelly coat

One hour after fertilization or later, the egg jelly coat was removed in 2% cysteine solution pH 7.8 for about 5min with gentle agitation in a conical glass flask. Embryos were washed three times with 0.1xMBS and cultured further in 0.1xMBS at 16-23°C.

3.9.7 Injection of embryos

Injection needles (World Precision Instrument, Inc; glass thin wall W/Fil 1,0mm, 4IN) were pulled from capillaries with the Microneedle Puller (setting: heat:800; pull:35; vel:140; time: 139; Sutter Instrument, model P-87). One injection needle was placed into the holder of the injection equipment (Medical System, model Pi-100). The tip was broken carefully with Dumont tweezers (No 5) and the opening was calibrated until an injection pressure of 30psi produced an output-volume of 5nl in a defined injection time (30ms-1s). The needle was filled with 1-2 μ l nucleotide acid containing solution shortly before the injection. Embryos were injected at two cell stage into the animal hemisphere. Depending on the performed experiment one single blastomere (5nl per embryo) or both blastomeres (10nl per embryo) were injected twice each. After injection, the embryos were incubated in 0.1xMBS containing gentamycin at 16-23°C until the desired developmental stages in a 60mm Petri dish covered with 1% agarose in 0.1xMBS. The saline was changed every day to increase the survival rates of the embryos.

4 Results

4.1 Molecular tools for functional interference with *Xenopus* Oct4 homologs

Oct4 plays an important role in coordinating embryonic induction processes and germ layer formation in mammals. Little is known about the molecular functions of Oct4 homologs in other vertebrates. *Xenopus laevis* offers several advantages in studying developmental functions.

Mouse Oct4 was reported to act as a transcriptional activator as well as a repressor in mammals (Babaie, Herwig et al. 2007). The trans-activating functions of *Xenopus* Oct4 homologs are not fully understood, yet. To gain further insights into the function of *Xenopus* Oct proteins during early embryogenesis, I decided to study overexpression of the wildtype *Xenopus* Oct proteins as well as overexpression of dominant activating and dominant repressing protein variants. The idea of this approach is to discriminate between activating and repressing influences on the expression of target genes and the corresponding phenotypic changes. Assuming that Oct60 functions as an activator as well as a repressor, fusion constructs with activating domains should accentuate the activating aspects and get rid of the repressive effects while vice versa fusion to a strong repressor domain should accentuate the repressive aspects.

Additionally, hormone inducible transcription factor variants were cloned. The structures of the generated *Xenopus* Oct protein variants are shown in Figure 8.

To generate variants of *Xenopus* Oct proteins with activating function, the herpes simplex virus VP16 protein domain was used, which is one of the most potent transcriptional activators known. The VP16 domain consists of 87 amino acids and is a structural component of the virion that activates immediate early viral gene expression (Cousens, Greaves et al. 1989).

For transcription factor variants with repressing functions, the transactivation domain of the *engrailed* (*enR*) gene from *Drosophila melanogaster* was used (Badiani, Corbella et al. 1994; Conlon, Sedgwick et al. 1996; Steinbach, Ulshofer et al. 1998). The VP16 activator domain and the *enR* repression domain respectively were cloned in frame to the 5'-end of the *Xenopus-oct*-cDNAs, to produce the corresponding plasmids.

Furthermore, for controlling gene expression temporally, hormone inducible transcription factor variants were generated. These constructs are useful for analyzing gene function during development, particularly if effects later in development are to be examined. Injection of RNAs into cleavage-stage embryos generally results in immediate translation.

Hormone-inducible systems enable the control of protein expression in a temporal manner due to fusion of hormone-binding domains of steroid receptors and heterologous proteins. In the absence of hormone, the fusion protein is held in an inactive state, most likely due to complex formation with hsp90 (Cadepond, Schweizer-Groyer et al. 1991; Scherrer, Picard et al. 1993). Addition of hormone results in translocation of the fusion protein into the nucleus and its activation (Hollenberg, Cheng et al. 1993; Gammill and Sive 1997). To produce hormone inducible protein variants, the 259 amino acid glucocorticoid receptor domain (GR) was cloned to the 5'-end of the *Xenopus-oct*-cDNAs.

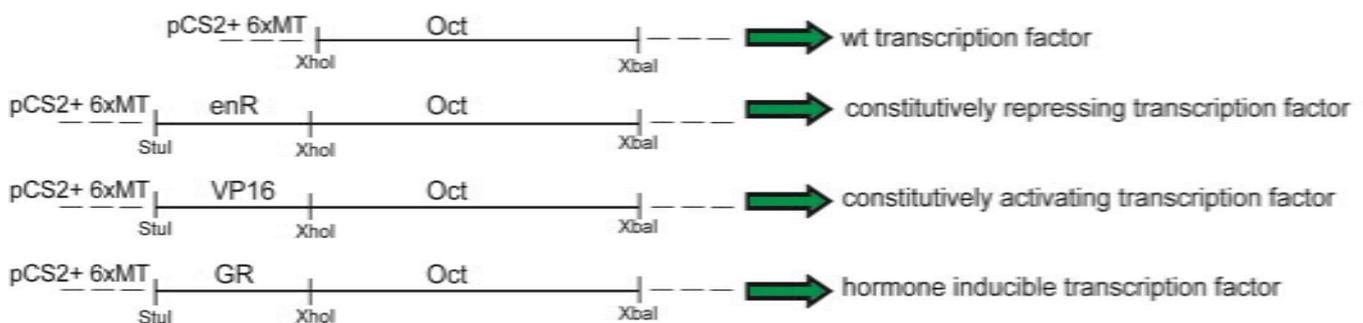


Figure 8: Expression constructs

For the generation of constitutively activating, constitutively repressing and hormone inducible Oct protein variants, the *enR* repressor-, the *vp16* activator- or the glucocorticoid receptor domain (*gr*) was cloned to the 5' end of the *Xenopus oct* cDNA.

The mapped constructs were generated for each of the three *Xenopus oct* cDNAs. The pCS2+MT6 expression vector was used for mRNA expression *in vitro*.

The pCS2⁺-6xMT-vector was used for expression (Figure 9). It was generated for the expression of epitope-tagged fusion proteins in *Xenopus* embryos from either injected RNA or DNA (Rupp, Snider et al. 1994). The vector contains 6 myc tags. After expression these myc epitopes are specifically recognized by the 9e10 monoclonal antibody (Evan, Lewis et al. 1985). Thereby, detection of the overexpressed proteins is possible *in vitro* as well as *in vivo*. An advantage of this approach is that different proteins can be detected with similar efficiency since the same epitope tag and antibody is used for all.

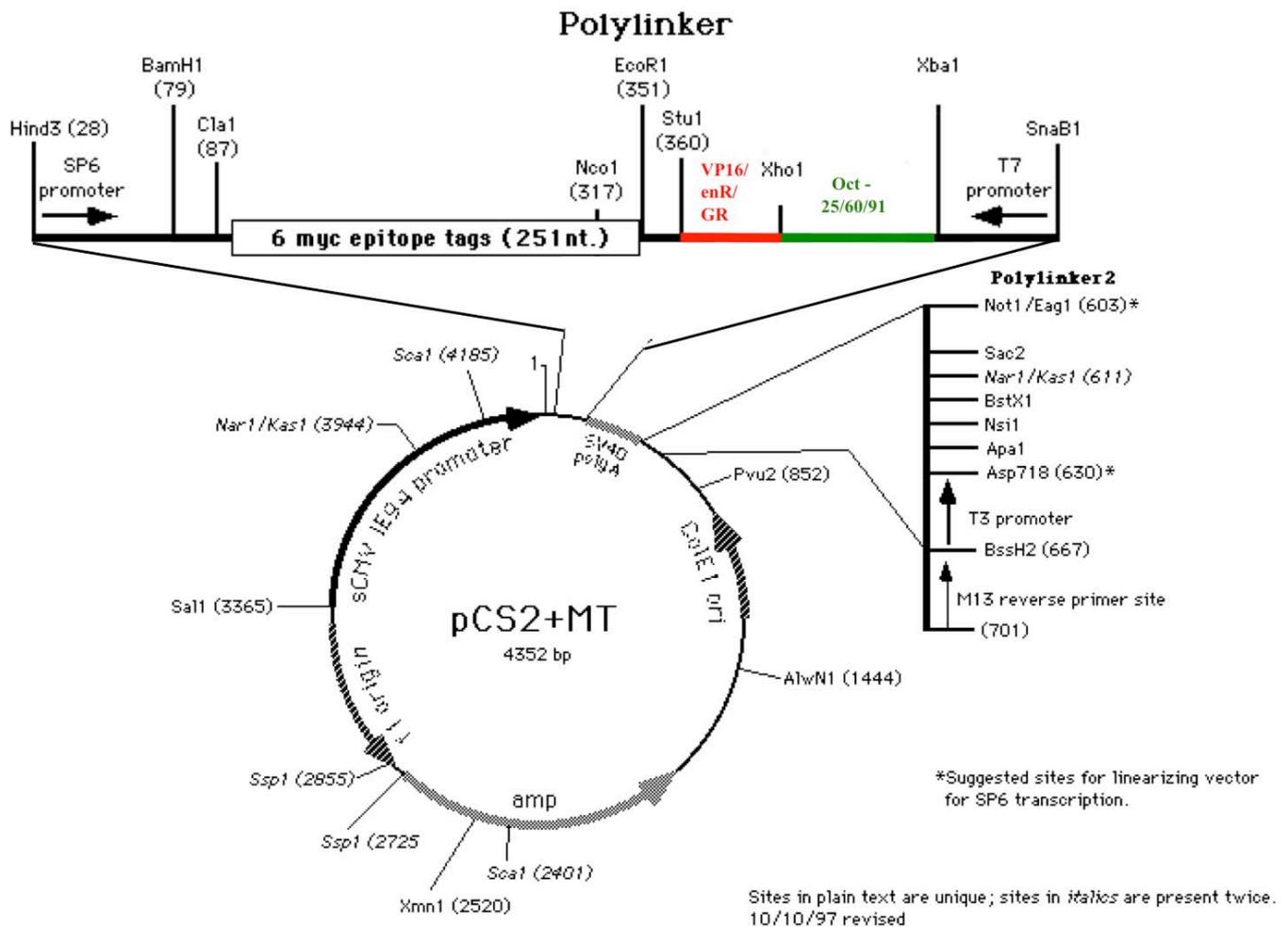


Figure 9: Construction of *Xenopus* Oct G.o.F. variants.

The pCS2⁺-MT6 vector was used as expression system. The 6xMT was fused to the N-terminus. *vp16*, *enR*, *gr* domains and *oct*-cDNAs were cloned into the polylinker. The figure was modified from <http://sitemaker.umich.edu/dlturner.vectors/home>.

Oct-cDNAs were amplified via PCR (for primers see chapter 3.3.2.1), cloned into the dual TOPO vector and sequenced. The verified cDNA-inserts were released by digestion with XhoI and XbaI and subcloned into the pCS2⁺-MT vector.

For generation of the dominant activating, dominant repressing and hormone inducible constructs, the *enR*, the *vp16* and the *gr* domain were amplified (for primers see chapter 3.3.2.1) and subcloned into the TOPO vector for sequencing. They were digested with StuI and XhoI and cloned into the pCS2⁺-MT-vector (Figure 9).

4.2 Verification of protein overexpression *in vitro* and *in vivo*

4.2.1 Cloned Oct variants accumulate in comparable amounts *in vitro*

In toto, 15 constructs were generated. To test the protein expression *in vitro*, a coupled transcription/translation reaction assay with reticulocyte lysates (TNT) was used. The generated proteins were resolved on SDS-polyacrylamide gels and were analyzed by western blot using the anti-myc-tag-antibody (Figure 10). Protein bands migrated slower than according to their estimated size. This is a known phenomenon for MT-proteins. Comparison of the Oct protein bands revealed that MT-Oct60 (57kDa) shows the highest mobility, whereas MT-Oct91 (60kDa) shows the lowest one (Figure 10, band 4a, 5b, 6c). Within the expression domains, the VP16-domain (9,5kDa) migrates fastest, followed by the GR-domain (28kDa). The EnR-domain (32kDa) showed the lowest mobility (Figure 10, band 1a, 2b, 3c). Comparison of protein bands of Oct25, Oct60 and Oct91 shows similar intensities, indicating that their expressed amounts are comparable *in vitro* (Figure 10, band 4a, 5b, 6c). Double bands were observed in the MT-Oct60 and the MT-EnR-Oct60 bands (Figure 10, band 5b, 9c). Their appearance is most likely due to degradation processes or variations in the number of transcribed myc-tags, as it was reported before.

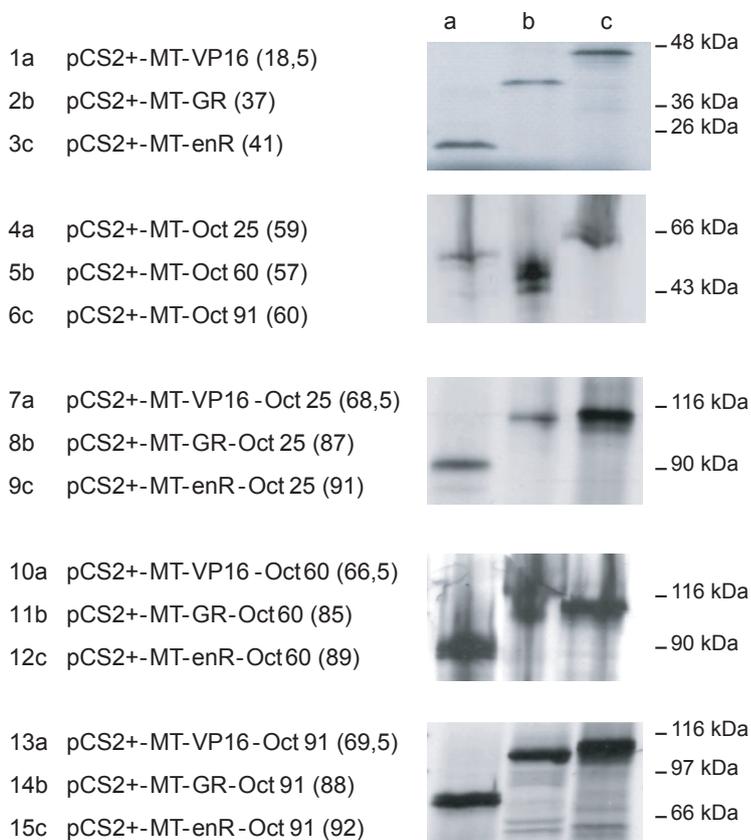


Figure 10: All cloned constructs are expressed in similar amounts *in vitro*

In vitro translation was verified by western blot via the anti-myc-tag-antibody 9E10 (1:100). Western Blot analysis indicates that all generated plasmids are expressed in comparable amounts *in vitro*. Numbers in brackets indicate the estimated molecular weight (kDa). MT-proteins migrate slower than according their estimated size.

4.2.2 Ectopic Oct25, Oct60 and Oct91 accumulate in different amounts *in vivo*

In *Xenopus laevis* embryos three Oct4 homologs – Oct25, Oct60 and Oct91- are known that have different temporal expression profiles. To which degree their functions are overlapping has not been shown, yet. The different temporal expression profiles might suggest a difference in function between the three *Xenopus* Oct proteins.

Considering that the combination of all three *Xenopus* Oct homologs might mimic the mouse Oct4 function best, an equimolar mix of all three constructs was used for the following experiments. Embryos were injected with an *oct-wt-* (consisting of *oct25*, *oct60* and *oct91*), an *enR-* (consisting of *enR-oct25*, *enR-oct60* and *enR-oct91*) or a *vp16-mix* (consisting of *vp16-oct25*, *vp16-oct60* and *vp16-oct91*). Embryos were injected into both blastomeres at two-cell stage. Surprisingly, western blot analysis of protein expression from embryos injected with the *oct-wt-mix* did not show a homogenous accumulation of the three *Xenopus* Oct proteins (data not shown). Only Oct91 protein was detectable. Consequently, *oct25*, *oct60* and *oct91* were injected separately. Western blot analysis revealed that the three Oct proteins are present at different amounts *in vivo* (Figure 11, A). Oct91 accumulates in highest levels, Oct25 in medium levels while Oct60 is hardly detectable. To test whether this might be a RNA specific effect mediated by RNA instability, a TNT was performed with RNA in comparison to DNA (Figure 11, B). The experiments showed that *in vitro* DNA as well as RNA results in comparable protein levels. In conclusion, the stability of the RNAs and the efficiency of transcription and translation of the tested RNAs seem to be comparable *in vitro*, but not *in vivo*.

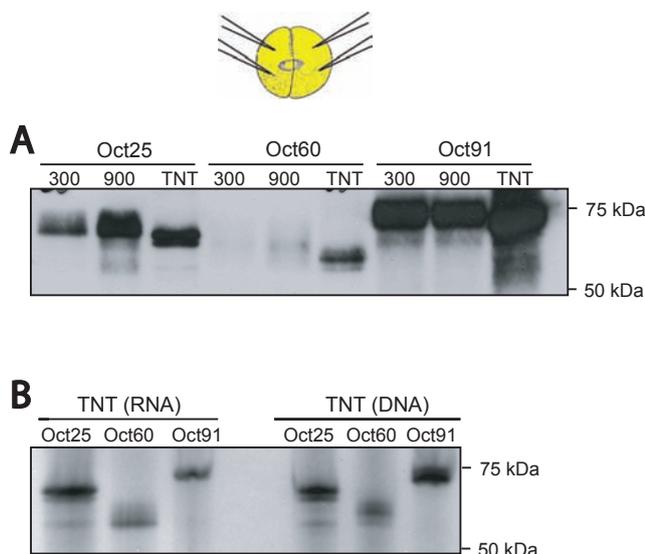


Figure 11: Oct25, Oct60 and Oct91 are enriched in different amounts *in vivo*

A) 300pg or 900pg of the indicated mRNA were injected into the animal pole of both blastomeres at two cell stage. Each lane shows protein extracts from one embryo equivalent detected with the anti-myc-tag-antibody 9E10 (1:100). TNTs are shown for comparison of size. Comparison of protein bands indicates that Oct91 protein accumulates in highest, Oct25 in medium and Oct60 in least amounts.

B) Comparison of protein expression from RNA versus DNA. TNTs with 1 μ g of the indicated RNA or DNA were performed. Comparison of protein expression reveals that both, RNA and DNA are expressed in similar amounts *in vitro*.

To find out whether the observed differences in protein amounts are time dependent, Oct25, Oct60 and Oct91 protein expression was examined at three different developmental stages – NF 7, NF 9 and NF 11 (Figure 12). The results indicate that unequal accumulation is not a time dependent effect. At each examined point in time Oct91 proteins are detectable at highest amounts. Western blot analyses of mix injections confirm the unequal enrichment of the three Oct-proteins at each examined point of time.

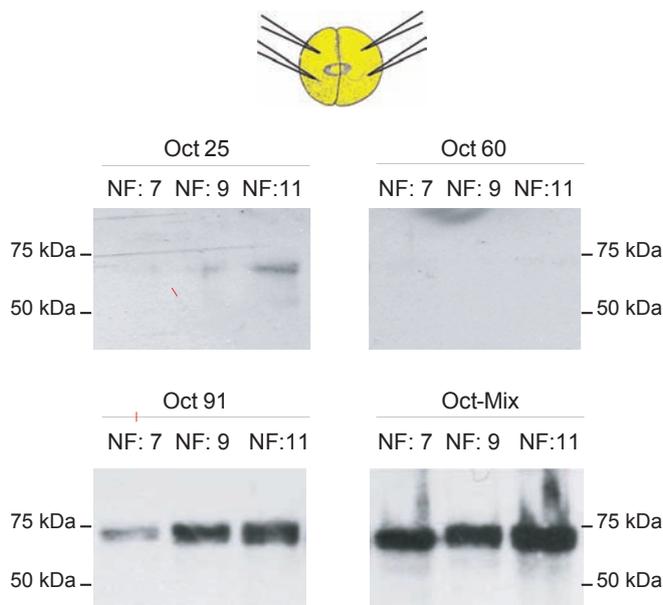


Figure 12: Unequal *in vivo* protein accumulation can be observed at different points of time in development

Embryos were injected with 300pg of the indicated RNA into the animal hemisphere. Embryos, injected with the “Oct-mix”, were injected with 300pg of each of the three *oct*-RNAs. Protein expression was analyzed at three different points in time (NF 7, NF9 and NF11) via western blotting. Each lane contains proteins from one embryo equivalent that were detected via anti-myc-tag-antibody 9E10 (1:100). At each of the examined points in time Oct91 is detected in highest, Oct25 in medium and Oct60 protein in lowest amounts.

The unequal abundance made it impossible to predict whether observed phenotypes were produced by all three Oct-proteins or whether the expression of Oct91 was dominant so that phenotypic changes resulted exclusively from Oct91 function. This might have been particularly true for Oct60, which appeared to be very unstable, and therefore, might have been outcompeted by Oct25 and Oct91. In consideration of these results the mixing injections were stopped.

4.2.3 Injection of *oct60*, *enR-oct60* and *vp16-oct60* mRNA results in comparable protein levels *in vivo*

Oct60 is one of the earliest genes to be transcribed in oocyte development (Whitfield, Heasman et al. 1993). *Oct60* becomes downregulated during gastrulation, when *Oct25* and *Oct91* are beginning to be expressed. Its expression profile distinguishes *Oct60* from the other *Xenopus* *Oct4* homologs. *Oct60* expression correlates with stages of pluripotency and expression ceases when pluripotency is lost. Its expression profile is most consistent with the expression profile of

Oct4. This makes Oct60 exceptionally interesting for further research concerning its function. Therefore, I decided to focus on Oct60.

To find out whether Oct60 and its G.o.F. variants accumulate in comparable amounts *in vivo*, embryos were injected with 900pg/E of *oct60*, *vp16-oct60* or *enR-oct60* into both blastomeres at two-cell stage. Proteins from embryos NF 15 were separated via an SDS-gel. Western blot analysis displayed that all three proteins are present in comparable amounts *in vivo* (Figure 13).

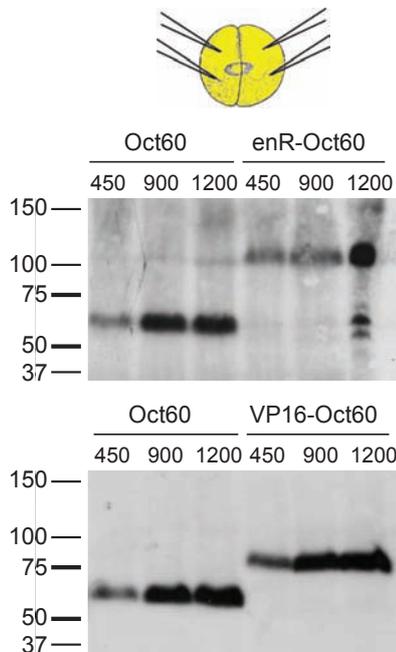


Figure 13: Oct60, EnR-oct60 and VP16-oct60 proteins are detectable in comparable amounts *in vivo*

Embryos were injected with 450pg, 900pg or 1200pg of mRNA into the animal hemisphere as indicated. Each lane shows purified proteins from one embryo equivalent NF15. Proteins were detected with anti-myc-tag-antibodies 9E10 (1:100). Western blot analysis shows that Oct60, EnR-Oct60 and VP16-Oct60 are expressed in comparable amounts *in vivo*.

4.3 Transcriptional activities of wildtype Oct60 and its fusion proteins

It was shown by previous work that murine Oct4 acts *in vivo* as a transcriptional activator as well as a transcriptional repressor (Babaie, Herwig et al. 2007). Little is known about the function of *Xenopus* Oct60. We tested the Oct60 wildtype and its gain of function variants in a transient luciferase reporter assay with two artificial Oct4 sensitive promoters (Figure 14).

The *more* promoter is a palindromic Oct recognition element (ATGCATATGCAT) that mediates assembly of stable Oct homodimers and heterodimers leading to strong transcriptional activation by mammalian Oct proteins. When binding to the *more* promoter, the POU_S and POU_{HD} domains of two different Oct molecules make contact with each half-site (Tomilin, Reményi et al. 2000). The *6w* enhancer contains 6 copies of a natural octamer binding motif from the mouse Ig heavy chain gene enhancer (Botquin, Hess et al. 1998; Tomilin, Reményi et al. 2000). The *more* promoter as well as the *6w* enhancer are linked to the luciferase reporter gene, which provides a quantitative readout for the transactivating activities of the Oct60 protein variants. As a control, a

plasmid containing the *renilla* gene is coinjected. To find out to which degree the luciferase gene is activated, the firefly/*renilla* coefficients are calculated.

Embryos were injected with Oct60 wildtype or its gain of function variants (900pg of *MT6-oct60*, *MT6- ν p16-oct60*, *MT6-enR-oct60* or *gfp* mRNA (control)) in combination with the *more* or *6w* reporter plasmid (100pg) and a plasmid containing the *renilla* cDNA (100pg).

Using the *more* promoter, injection of *oct60* resulted in 35-fold stronger activation of the reporter plasmid compared to the basal activity in *gfp* injected control embryos. In *ν p16-oct60* injected embryos a 730-fold stronger activation compared to the control embryos was observed. *enR-oct60* injection resulted in an 2.42-fold repression compared to basal activity (Figure 14, A). The same set up was performed using the *6w* instead of the *more* promoter (data not shown). Although activation rates were lower with this promoter similar results were obtained. Oct60 resulted in 2,8-fold and VP16-Oct60 induced 57-fold activation. As in the previous assay, *enR-Oct60* injection yielded a 2-fold repression compared to *gfp* injected embryos. This experiment was performed three times on the *more* promoter with comparable results.

The repressing function of EnR-Oct60 was relatively low, compared to the strong activation observed in VP16-Oct60 injected embryos. One problem was the relatively low basal activity of the *more* and *6w* promoters leading to difficulties in visualizing a possible repression by EnR-Oct60. For a better demonstration of the repressing function of EnR-Oct60, we decided to perform a competition assay. Therefore embryos were injected with a mRNA mix, consisting of *ν p16-oct60* and different concentrations of *enR-oct60*. If EnR-Oct60 really has a repressive effect, activation rates induced by VP16-Oct60 should decrease the more *enR-oct60* is coinjected. The luciferase assay was performed using embryos injected with 200pg *ν p16-oct60* mRNA in combination with increasing dosages of *enR-oct60* mRNA. Additionally, activation rates were measured after injecting 1000pg of *enR-oct60* mRNA or 200pg of *ν p16-oct60* mRNA. This experiment was also performed using the *more* promoter (Figure 14, B). 200pg of *ν p16-oct60* alone resulted in 182-fold activation whereas 1000pg of *enR-oct60* led to 2,4-fold repression. Coinjection of 200pg of *ν p16-oct60* with 200pg, 500pg or 1000pg of *enR-oct60* mRNA showed decreasing activation rates of 12-fold, 6-fold and 1,2-fold, respectively. Therefore, EnR-Oct60 was able to repress the strong activation induced by VP16-Oct60 almost up to basal activity. These results confirm that EnR-Oct60 functions as a repressing transcription factor variant.

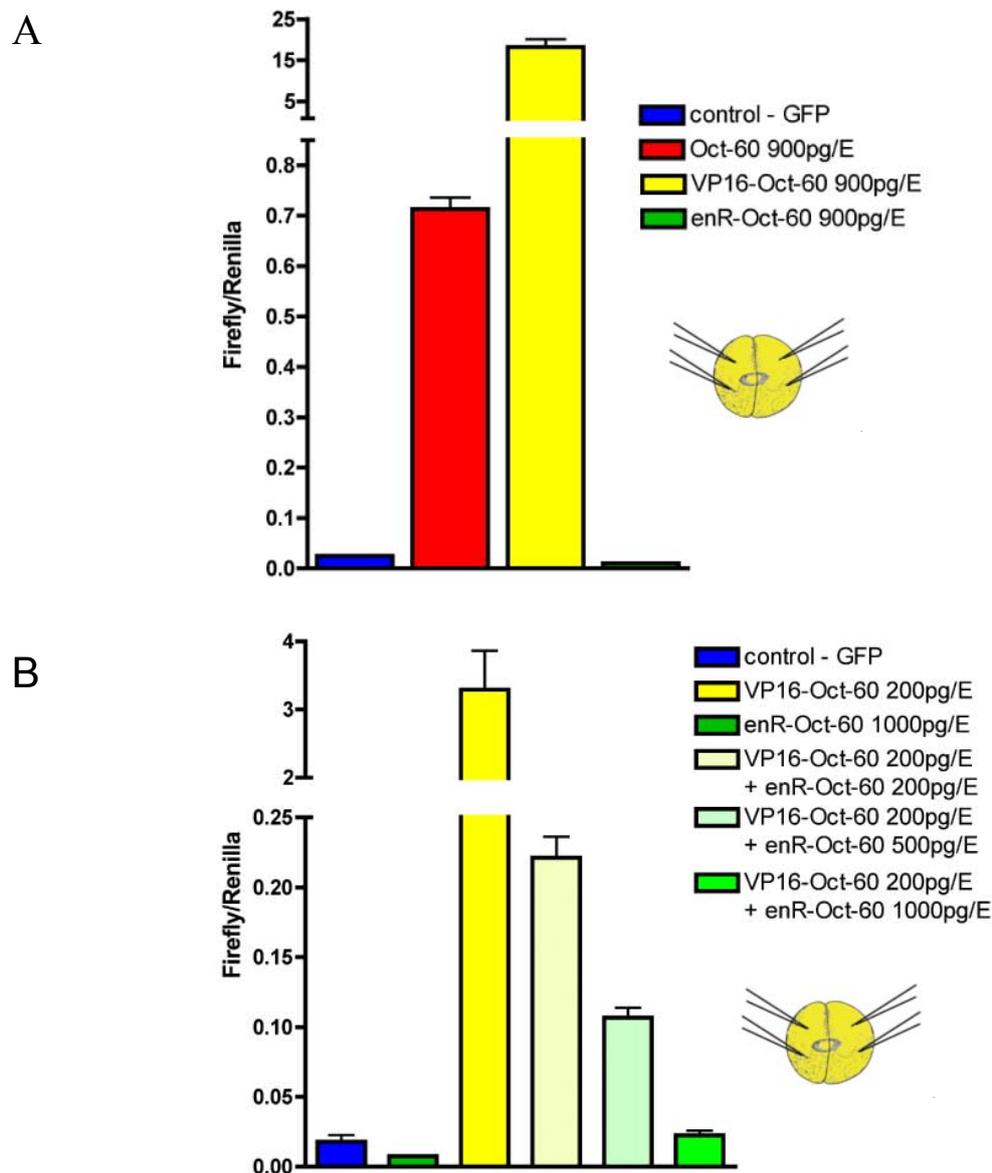


Figure 14: Transactivating activities of Oct60 G.o.F. protein variants

Luciferase assay showing the activating and repressing functions of Oct60 and its G.o.F. variants *in vivo*. The *more* and the *6w* promoters were used as modified palindromic Oct factor recognition elements. Embryos were injected into the animal hemisphere of both blastomeres with 900pg of *oct* mRNA as indicated, 100pg of the reporter plasmid and 100pg of a plasmid containing the *renilla* cDNA.

A) The luciferase assay, using the *more* promoter, shows a 35-fold activation by injected *oct60* mRNA and a 730-fold upregulation by *vp16-oct60* mRNA compared to control embryos. *EnR-oct60* resulted in 2,4-fold repression.

B) The competition assay was performed on embryos that were injected with 1000pg *enR-Oct60* mRNA, 200pg *vp16-oct60* or 200pg of *vp16-oct60* mRNA in combination with 200, 500 or 1000pg of *enR-oct60* mRNA. The *more* promoter was used as an Oct factor recognition element. In addition to the indicated *oct* mRNAs, 100pg of the reporter plasmid and 100pg of a *renilla* containing plasmid were injected into the animal hemisphere of both blastomeres. Data indicates that EnR-Oct60 is able to repress the VP-16-Oct60 mediated activation.

In summary it can be stated that Oct60 acts as a transcriptional activator on both, the *more* as well as the *6w* promoter. Injection of *vp16-oct60* resulted in a more than 20-fold higher activation rate compared to *oct60*. In both cases activation rates of the *more* promoter were roughly 13-fold higher than activation rates of the *6w* promoter. Injection of *enR-oct60* resulted in a 2.42-fold repression of the *more* promoter and a 2-fold repression of the *6w* promoter. In this way we were able to clearly demonstrate the repressing function of enR-Oct60 in a titration assay with VP16-Oct60 performed with the *more* promoter.

Oct60 acts as an activator on both promoters. VP16-Oct60 and enR-Oct60 show the expected activating/repressing functions *in vivo*. It can be concluded that the promoter responses of the *6w* and *more* promoters are specific according to different variants of Oct60.

4.4 Phenotypic changes caused by injection of *oct60* constructs

4.4.1 Oct60 and its G.o.F. variants impair blastopore closure

Next, effects of Oct60 on early embryogenesis were investigated by overexpression studies of *oct60* and its G.o.F. variants.

As it was described for Oct4 before, Oct60 was able to activate the *more* as well as the *6w* promoter in the previous experiments (Botquin, Hess et al. 1998; Tomilin, Reményi et al. 2000). Nevertheless, also repressing functions were described for Oct4 (Ben-Shushan, Thompson et al. 1998; Babaie, Herwig et al. 2007). To gain further insights into the *in vivo* function of Oct60, phenotypes produced by overexpression of Oct60 and its dominant activating and repressing forms were compared.

Embryos were injected with 450pg of *oct60*, *enR-oct60* and *vp16-oct60*, respectively, into the animal hemisphere at two-cell stage into one blastomere. Injection into the animal hemisphere results in protein overexpression in parts of the embryo that contain multipotent precursor cells and express Oct proteins. By injecting only one cell at two-cell stage, mainly one half of the body is affected by the overexpression leaving the “wildtype body-half” as an endogenous “un-injected” control. To tag which body half was manipulated, Alexa dextrane was coinjected. Additionally, control embryos were injected with 450pg *gfp* to distinguish Oct specific phenotypic changes from effects produced by the technique.

During the time of germ layer induction, until NF9, embryos injected with Oct60 and its G.o.F. variants showed no apparent abnormalities compared to controls. At gastrula stage 10,

blastopore formation begins at the exterior of the embryo. The first indentation is gradually evolving to form a complete circle - the line of invagination. Coordinated movements transform the blastula into a multilayered embryo with head, trunk and tail rudiments.

During the process of gastrulation, all embryos injected with *oct60* or its gain of function variants showed problems in blastopore formation. In the majority of embryos, this resulted in incomplete blastopore closure forming mushroom like structures by non-migrating cells (data not shown). Blastopore closure defects were most pronounced in embryos that were injected with *vp16-oct60*. More than 50% of these embryos died around stage 11. Therefore, it was necessary to reduce the injected *vp16-oct60* mRNA dose from 450pg/E to 150pg/E, if phenotypic changes in post-gastrula stages wanted to be observed. This dose reduction resulted in 25% of the embryos showing blastopore closure defects instead of 63% when 450pg/E were injected. Injection of *oct60* affected the blastopore formation in 21%, injection of *enR-oct60* in 28% of the embryos. The highest non-lethal RNA dose was 600pg for *oct60* and *enR-oct60* and 400pg for *vp16-oct60*. Higher doses were lethal, with most embryos dying at gastrula stages. In post-neurula stages the incomplete blastopore closure led to incomplete closure of the posterior dorsal midline of the embryos.

4.4.2 Injected embryos show developmental defects at distinct parts of the body

Embryos injected with *oct60* mRNA showed a severe hyperpigmentation (91%) that most often occurred isolated in the head region and was accompanied by enlargement of the injected head side in 68% of all cases (Table 2, Figure 15, row 3,4). Enlargement of the head as well as the hyperpigmentation appeared most pronounced around stage 26. 24% of the embryos were additionally affected in the trunk region. Hyperpigmentation was transient in most parts. At stage 33, the hyperpigmentation was of slightly reduced intensity, whereas around stage 40 it had almost completely disappeared.

In contrast to control embryos with entirely black eyes and nearly closed choroids fissures, eye development was severely impaired by *Oct60* overexpression in 71% of the embryos. Less than 30% of the embryos showed normal eye formation. Most embryos showed no pigmented retinal structure and no visible protrusion of the eye vesicle at stage 33. This seemed to be rather due to a delay in development than to an absence of the eye anlage. At stage 40, incomplete eye formation was visible that presented in the majority as partially pigmented optic cups. The degree of the eye pigmentation seemed to be almost normal compared to control embryos. However, the retinal ring did not seem to be fully developed in form. If present, the size of the optic vesicle seemed normal at the injected side. Also the lens formation seemed to be strongly impaired. The degree of impairment was reaching from a reduction in size to almost not detectable lens structures.

Overexpression of EnR-Oct60 induced hyperpigmentation in 88% of the embryos. This phenotype however differed from the Oct60 phenotype in localization and pattern (Table 2, Figure 15, row 5,6). EnR-Oct60 affected the trunk region in 88% whereas the head region was only affected in 29%. In contrast, in *oct60* injected embryos the head region was predominantly affected in 91%. These embryos showed hyperpigmentation combined with an enlargement of the affected side of the head. The hyperpigmentation produced by Oct60 was uniformly spread over the body. In contrast to this, the hyperpigmentation produced by EnR-Oct60 appeared localized, restricted and protruding. Hyperpigmentation induced by overexpression of *enR-Oct60* did not evenly affect the head region as it was observed in *oct60* injected embryos. Instead, hyperpigmentation was locally enriched, resembling a spot or line.

Additionally, hyperpigmentation in *enR-oct60* injected embryos was accompanied by bulge formation in more than 80%. These bulges occurred in hyperpigmented areas only, ranging in shape from “tentacle like” to “double axis” like structures. Bulge formation varied in size and position. Prominence of bulge formation differs, ranging from only slightly elevated hyperpigmented areas to vast outgrowth mimicking a secondary body axis. Bulge formation was scored at stage 21-24 at its maximum extensions. At stage 40, the hyperpigmentation and the outgrowth were partially retained but did not disappear completely in most cases. The outgrowth became less prominent and the hyper-pigmented areas were severely reduced in size and intensity.

Embryos injected with *enR-oct60* showed protruding optic vesicles at stage 33. In most cases no pigmented retinal structures were observed. At stage 40, 68% of the injected embryos showed defects in retinal formation indicated by reduced pigmentation of the retina. Additionally, the optic vesicle was reduced in size in about one third of the embryos. Beside the impaired formation of the retinal pigment epithelium, lens formation was disturbed.

Overexpression of VP16-Oct60 disturbed predominantly the formation of the head region (Table 2, Figure 15, row 7,8). In 96%, an enlargement of the injected side of the head was observed at stage 24. Around stage 26, no optic vesicle had formed visibly on the injected side. In 96%, impaired eye formation was observed on the injected side of the head at stage 33. Neither a protrusion of the optic vesicle nor a pigmentation of retinal structures was visible. At all points of time, the neural defects were more prominent in *vp16-oct60* injected embryos than in *oct60* or *enR-oct60* injected embryos. At stage 40, about one third of the embryos was still showing neither a protrusion of the optic vesicle nor a pigmentation of retinal structures. One third of the embryos developed eyes that were severely reduced in size and pigmentation. The rest of the embryos showed mildly impaired eyes. Additionally, all embryos that were injected with *vp16-oct60* showed reduced formation of the dorsal fin at stage 33.

In control embryos, melanophores appear for the first time dorsally on the head at stage 33/34. Melanophores also arrange on the pronephros and in a horizontal row along the trunk, beginning ventrally at the pronephros along the axial musculature. Melanophores, visible in control-injected embryos at stage 33, were absent in most embryos that were injected with Oct60 and its gain of function variants. This indicates a delay in development since they developed partially until stage 40. Cement gland, gut structures as well as proctodeum formation appeared to be normal in all injected embryos.

Phenotypes	Control	Oct60	EnR-Oct60	VP16-Oct60 (450pg/E)	VP16-Oct60 (150pg/E)
Gastrulation defects (NF 11)	11/83 (3) 13%	21/98 (3) 21%	29/103 (3) 28%	29/46 (3) 63%	19/75 (3) 25%
Truncated body axis (NF 36)	4/43 (2) 9%	27/56 (2) 48%	38/53 (2) 66%	11/11(2) 100%	42/49 (2) 86%
Eye defects (NF 36)	0/43 (2) 0%	40/56 (2) 71%	36/53 (2) 68%	11/11 (2) 100%	47/49 (2) 96%
Bulge formation in the trunk region (NF 26)	0/86 (3) 0%	0/78 (3) 0%	51/65 (3) 78%	0/26 (3) 0%	0/54 (3) 0%
Thickening of the head region (NF 26)	0/86 (3) 0%	69/78 (3) 88%	0/65 (3) 0%	26/26 (3) 100%	48/54 (3) 89%
Delayed melanophore formation (NF 36)	0/43 (2) 0%	71/78 (2) 91%	39/53 (2) 74%	11/11 (2) 100%	44/49 (2) 90%
Hyperpigmentation affecting the head (NF 26)	0/86 (3) 0%	71/78 (2) 91%	19/65 (3) 29%	22/26 (3) 85%	41/54 (3) 76%
Hyperpigmentation affecting the trunk (NF 26)	0/86 (3) 0%	19/78 (2) 24%	57/65 (3) 88%	0/26 (3) 0%	0/49 (3) 0%
Embryos without phenotypic changes (NF 26)	80/86 (3) 93%	7/78 (3) 9%	8/65 (3) 12%	0/26 (3) 0%	0/49 (3) 0%

Table 2: Representative percentages of observed phenotypic changes produced by injection of Oct60 and its G.o.F. variants

Embryos were injected with either 450pg of *oct60*, *enR-oct60* or *vp16-oct60* mRNA or 150 pg of *vp16-oct60* mRNA into one blastomere at two cell stage. Control embryos were injected with 450pg of *gfp* RNA. Phenotypic changes observed at different developmental stages (NF11, NF26, NF36) were counted. Total numbers and percentages are shown. The numbers in brackets indicate the numbers of experiments from which embryos were obtained.

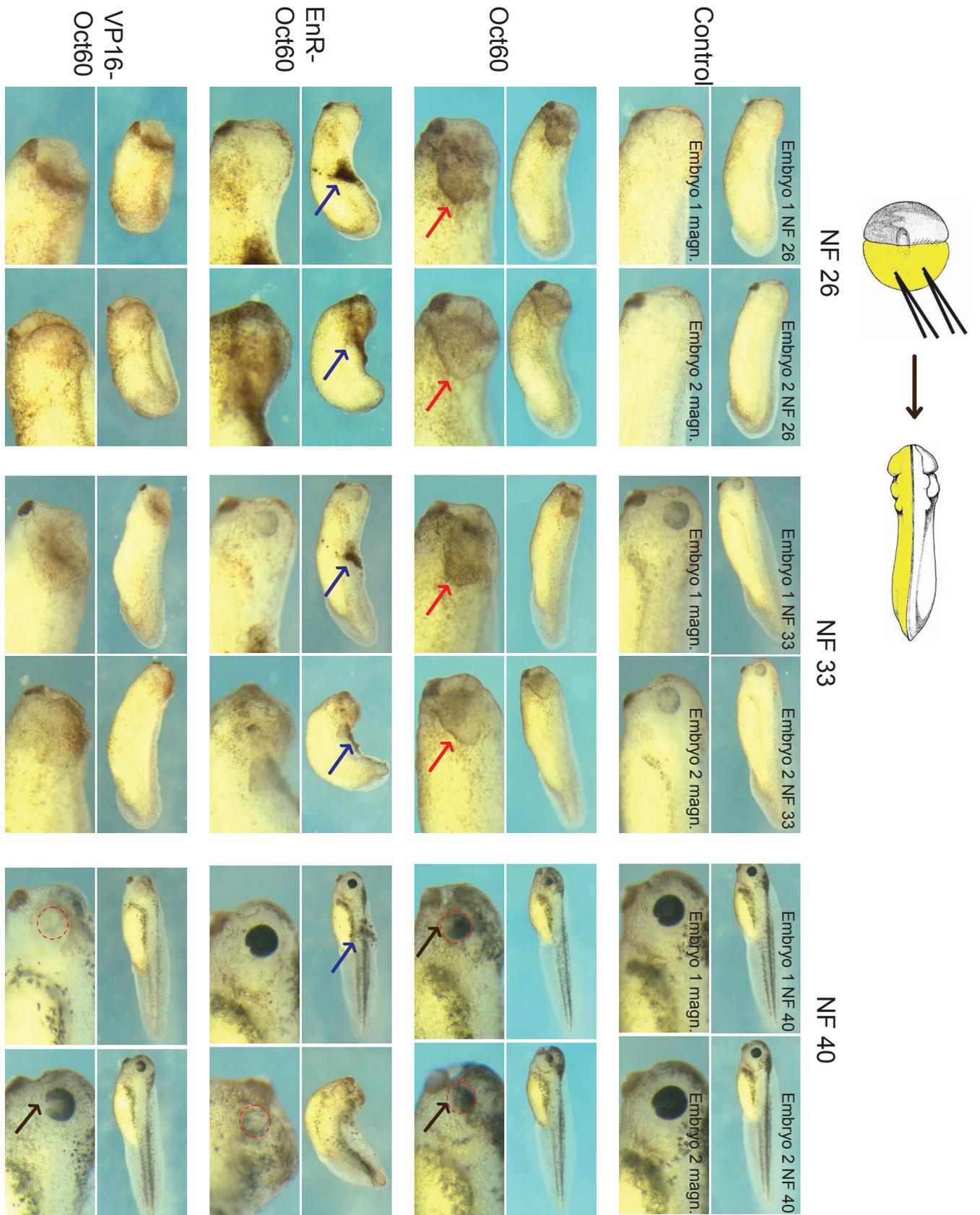


Figure 15: Overexpression of Oct60 and its G.o.F. variants

Phenotypic changes observed by overexpression of Oct60 and its G.o.F. variants. Embryos were injected with either 450pg of *oct60* or *enR-oct60* mRNA or 150pg of *vp16-oct60* mRNA into one blastomere at two-cell stage, as indicated. Control embryos were injected with 450pg of *gfp* RNA. All embryos shown here were injected into the left body half. The first row of every condition shows two representative embryos at three points in development (NF 26, NF 33, NF 40). The second row shows a magnification of the head region. Red arrows indicate pigmented bulges in the head region. Blue arrows indicate hyperpigmented bulges at the trunk region and black arrows point to closure defects of the retinal pigment epithelium.

4.4.3 Injected embryos develop a shortened, specifically curved body axis

Overexpression of Oct60 and its G.o.F. variants induced the formation of a shortened body axis compared to wild type embryos. This was most pronounced in *vp16-oct60* injected embryos, of which 86% showed severe truncation. 48% of the *oct60* and 66% of the *enR-oct60* injected embryos showed slight reduction in body length (Table 2). In control embryos, tail bud formation begins at stage 24. After a slow initial phase, the process of tail bud expansion accelerates at stage 28. At stage 36, the tail bud reaches a length of approximately one fourth of the body length. Embryos, injected with 450pg of *vp16-oct60*, appeared ventralized and showed severely diminished tail bud formation in more than 60%. In these embryos, body length was reduced to 50 to 75% of control embryos (15 embryos measured). The severity of this phenotype was dose-dependent. These changes were not as severe in *enR-oct60* and *oct60* injected embryos. In these embryos, body length varied from 75 to 100% compared to control embryos.

Injection into both blastomeres resulted in a kink like dorsal flexure in the majority of embryos. This phenomenon also occurred in about 5-10% of embryos that were injected into one blastomere.

Control injected embryos responded to touch stimulation with muscle contraction from stage 24 on. At stage 26, embryos injected with *oct60* and its gain of function variants showed no or strongly impaired movements in reaction to stimulation with tweezers. At stage 36, also *oct60* and *enR-oct60* injected embryos reacted adequately to stimuli. Embryos, injected with 450pg of *vp16-oct60*, were most severely affected. Their truncation hindered them in performing normal movements, like swimming. Upon stimuli, they reacted with cramp like, rapid motions.

Beside the abnormalities described above, embryos, injected into one cell at two-cell stage, showed a specific curvature (Figure 16). The curvature was counted at stage 24. Three hours prior to enumeration, the remaining vitellin membranes were removed. This allowed the embryos to stretch and straighten from their constricted position within the vitellin membrane.

More than 50% of the embryos, injected with *oct60* mRNA, showed a curvature directed towards the injected side. Embryos that were injected with *vp16-oct60* RNA also showed a curvature to the injected side in more than 70%. The curvature showed its maximum in the anterior region of the embryo. In contrast to this, *enR-oct60* injected embryos curved to the non-injected side in more than 65%. In these cases, the maximum of the curvature was in the trunk region. Control injected embryos were equally curved to the injected or non-injected side, whereas the majority of uninjected embryos was not curved (more than 70%). This curvature disappeared partially in most of the cases as embryos developed. It was most prominent around NF 24-26.

In summary, it can be stated that embryos, injected with *oct60* and its G.o.F. variants, show defects at distinct parts of the body. Oct60 and VP16-Oct60 seem to affect predominantly the head region whereas *enR-Oct60* injected embryos show also an affection of the trunk region. Most prominent changes include hyperpigmentation, disturbance of the formation of the head and a specific curvature of the main body axis. Phenotypic changes are transient and improve during development. Some of these defects like the inappropriate eye and melanophore formation seem to reflect developmental retardation.

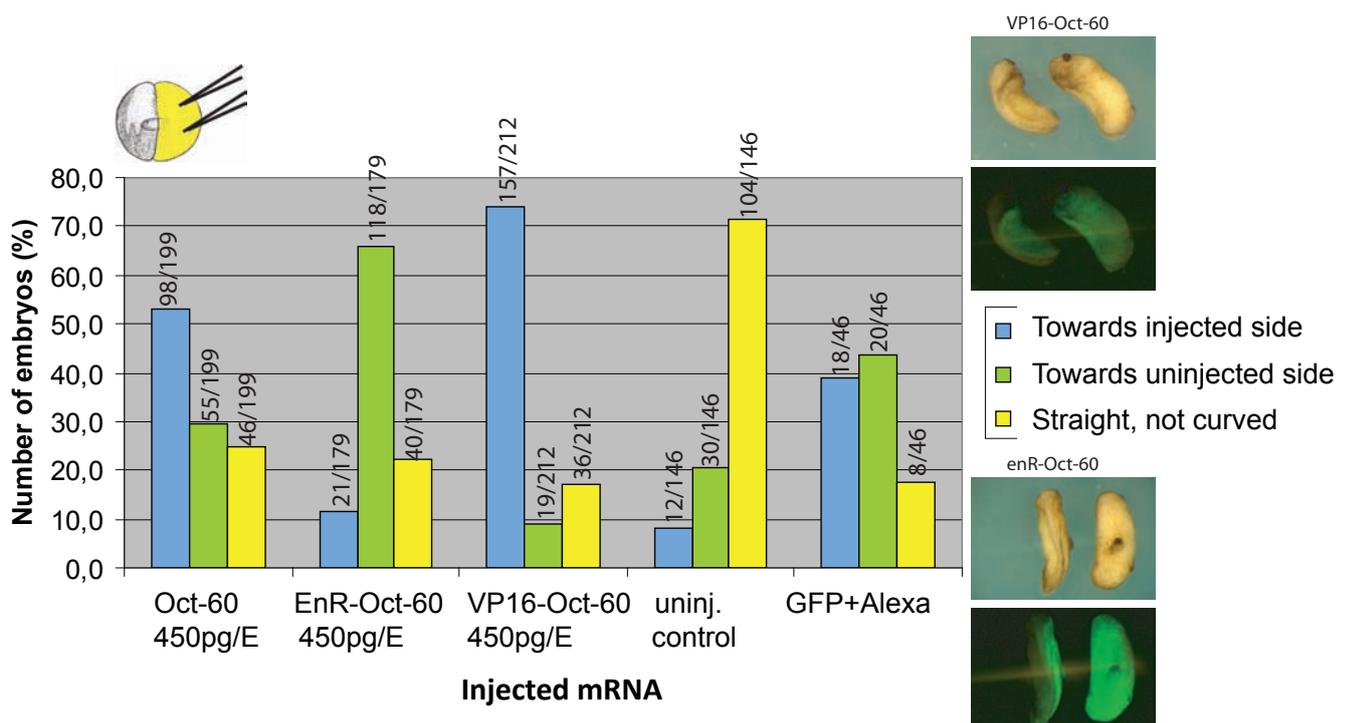


Figure 16: Oct60 and its G.o.F variants cause curvature of the main body axis

Embryos were injected with 450pg of mRNA into one blastomere at two cell stage, as indicated. The curvature was counted at stage 24. Three hours prior to enumeration the remaining vitellin membranes were removed. The majority of embryos injected with *oct60* was curved to the injected side (more than 50%). The same phenomenon was observed in more than 70% of *vp16-oct60* injected embryos. In contrast, overexpression of *EnR-Oct60* led to a curvature to the non-injected side in more than 65%. Uninjected control embryos were mainly not curved at this stage of development. Embryos that were injected with 450pg of *gfp* mRNA were curved to the injected or non-injected side in similar percentages.

4.5 Molecular analysis of interference phenotypes

Malformation in the head region, pigmentation defects, trunk outgrowth and a curved body axis were the most prominent phenotypic changes. To further investigate the nature of the observed phenotypes, *in situ* hybridizations were performed.

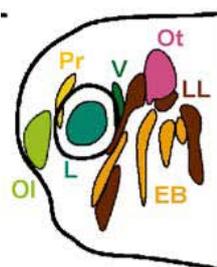
Whole mount RNA *in situ* hybridization is used for detection of localized, specific mRNAs in fixed embryos. Compared to other techniques for analyzing gene expression like RT-PCR its advantage is the additional spatial resolution up to single cell level.

Embryos were injected with 450pg of *oct60*, *enR-oct60* or 150pg of *vp16-oct60* mRNA, respectively into the animal region of one cell at two-cell stage. 100pg of *lacZ* mRNA were coinjected to detect the injected region. Control embryos were injected with 100pg of *lacZ* RNA. Early gastrula stage embryos (NF 10,5-11,5) and tadpole stages (NF 28) were used for staining.

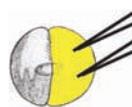
4.5.1 Neuroectodermal interference

Head defects, observed most prominently in *vp16-oct60* and *oct60* injected embryos, and hyperpigmentation, observed in *enR-oct60* and *oct60* injected embryos, indicate disturbance of neuroectodermal tissues. To analyze the gene expression pattern of the late neuroectodermal marker *n-β-tubulin*, *in situ* hybridizations were performed on embryos stage 28 (Figure 17). This experiment was repeated three times with comparable results on embryos from different injection rounds.

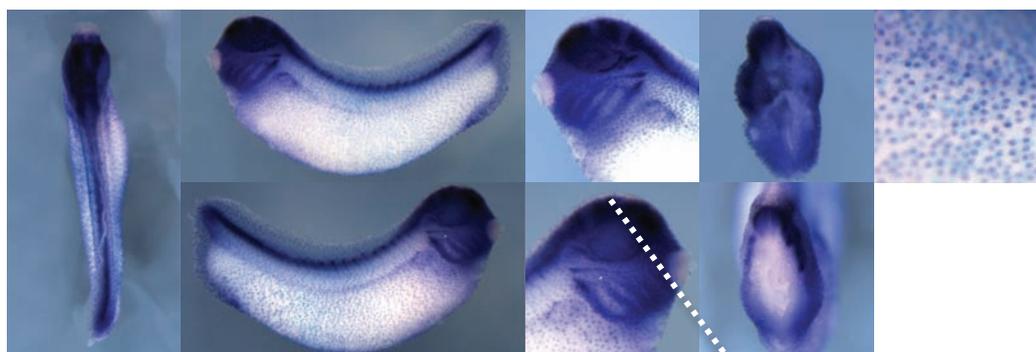
Injection of Oct60 and its G.o.F. variants resulted in severe disturbance of the *n-β-tubulin* expression pattern. In control embryos, *n-β-tubulin* expressing domains, placodes, the eye region, the brain and the notochord were clearly defined. In embryos, injected with Oct60 and its G.o.F. variants, the *n-β-tubulin* expressing domain was broadened. This was most prominent in *vp16-oct60* injected embryos. The different anatomic structures were hardly distinguishable (Figure 17, column 3). Epibranchial placodes were not detectable on the injected sides. Especially in *enR-oct60* injected embryos, ectopic *n-β-tubulin* expressing cells were detectable towards the trunk region. The *n-β-tubulin* staining seemed to almost affect the whole broadened head domain in *oct60* and *vp-16-oct60* injected embryos. Ectopic *n-β-tubulin* expressing cells were found in the vicinity of the trunk outgrowth of *enR-oct60* injected embryos but could not be found within the actual outgrowth. Interestingly, *lacZ* positive cells were concentrated in these trunk bulges (Figure 17, row 5, column 5).



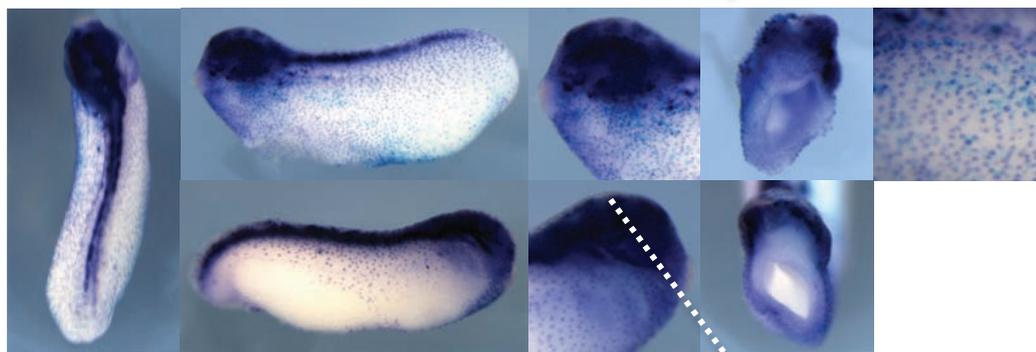
EB = epibranchial placodes;
 LL = lateral line placodes;
 Ot, = otic placode or vesicle;
 OI = olfactorial placodes;
 L = Lens
 Pr = profundal placode
 V = trigeminal placode



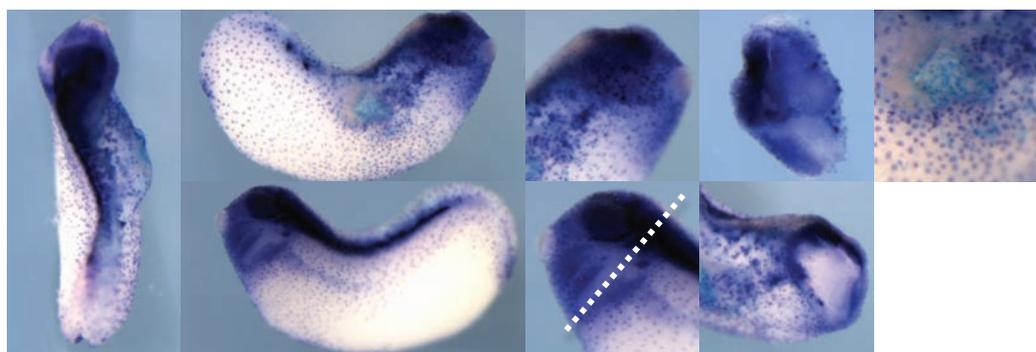
Control



Oct60



EnR-
Oct60



VP16-
Oct60

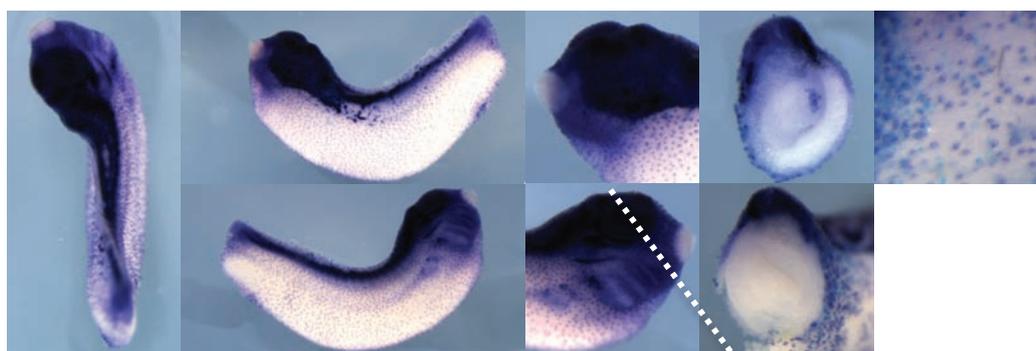


Figure 17: The expression pattern of the neural marker $n\text{-}\beta\text{-tubulin}$ is strongly disturbed by overexpression of Oct60 and its G.o.F. variants

Embryos were injected with 450pg of *oct60* or *enR-oct60* or 150pg of *vp16-oct60* mRNA respectively. Additionally, 100pg of *lacZ* mRNA were injected. Control embryos were injected with 100pg of *lacZ* mRNA only. Embryos were fixed at stage 28 and stained for *n- β -tubulin* and *lacZ*. For each injected mRNA, pictures of one representative embryo are shown. Column 1 and 2 show an overview of the embryo with the upper row in column 2 showing the injected, the lower row showing the non-injected body half. Column 3 shows a magnification of the head region. Column 4 shows a cross section as indicated in column 3 by the white, dashed lines. The upper picture shows the anterior part, the lower picture the posterior part of the embryo. Column 5 shows a magnification of the trunk region. Light blue represents *lacZ* lineage trace, dark blue indicates *n- β -tubulin* expression. The model showing placode formation was taken from Schlosser and Ahrens (2004).

4.5.2 Mesodermal interference

The specifically curved main body axis, produced by injection of *oct60* and its G.o.F. variants, suggests alteration in the process of convergence-extension. To test whether this results from changes in mesoderm expression and to figure out whether the observed bulges in the *enR-oct60* injected embryos were formed by mesodermal tissue, we analyzed the expression of the early mesodermal marker *xbra* and the late mesodermal marker *cardiac actin* (Figure 18 A,B). *Xbra* is a T-box transcription factor that is expressed from stage 10 on until tadpole stage. *Xbra* can be used as a pan-mesodermal marker for *in situ* hybridization. Cardiac actin is expressed in both, the cardiac and skeletal muscle, in later stages of development.

In situ* hybridization for *xbra revealed a disturbed expression pattern produced by Oct60 and its G.o.F. variants. *In situ* hybridizations were repeated 4 times with comparable results on embryos from different injection rounds. Embryos, injected with *oct60*, *enR-oct60* or *vp16-oct60*, showed a disruption of the wt *xbra* ring (Figure 18, A). As shown by *lacZ* lineage tracing, *xbra* mRNA was ablated only when the injected mRNAs reached into the suprablastoporal region, where *xbra* is induced. This suggested a cell-autonomous inhibition of *Xbra* transcription by the injected Oct60 proteins. Additionally, in 2 of 4 *in situ* hybridizations ectopic *xbra* expression was observed in addition to the disrupted wt expression in *oct60* and *enR-Oct60* injected embryos. The ectopic *xbra* stain was located towards the animal region (data not shown). In *vp16-oct60* injected embryos, no ectopic *xbra* expression was observed, but a severe disruption of the *xbra* ring was visible.

In situ* hybridization for *cardiac actin showed a disruption of somite formation by Oct60 and its G.o.F. variants (Figure 18, B). Most severe effects were seen in embryos injected with *enR-Oct60* and *vp-16-Oct60*. Interestingly predominantly the anterior somites were affected. Posterior somites showed an almost normal development. Anterior somites featured reduced *cardiac actin* expression as well as disturbed pattern formation.

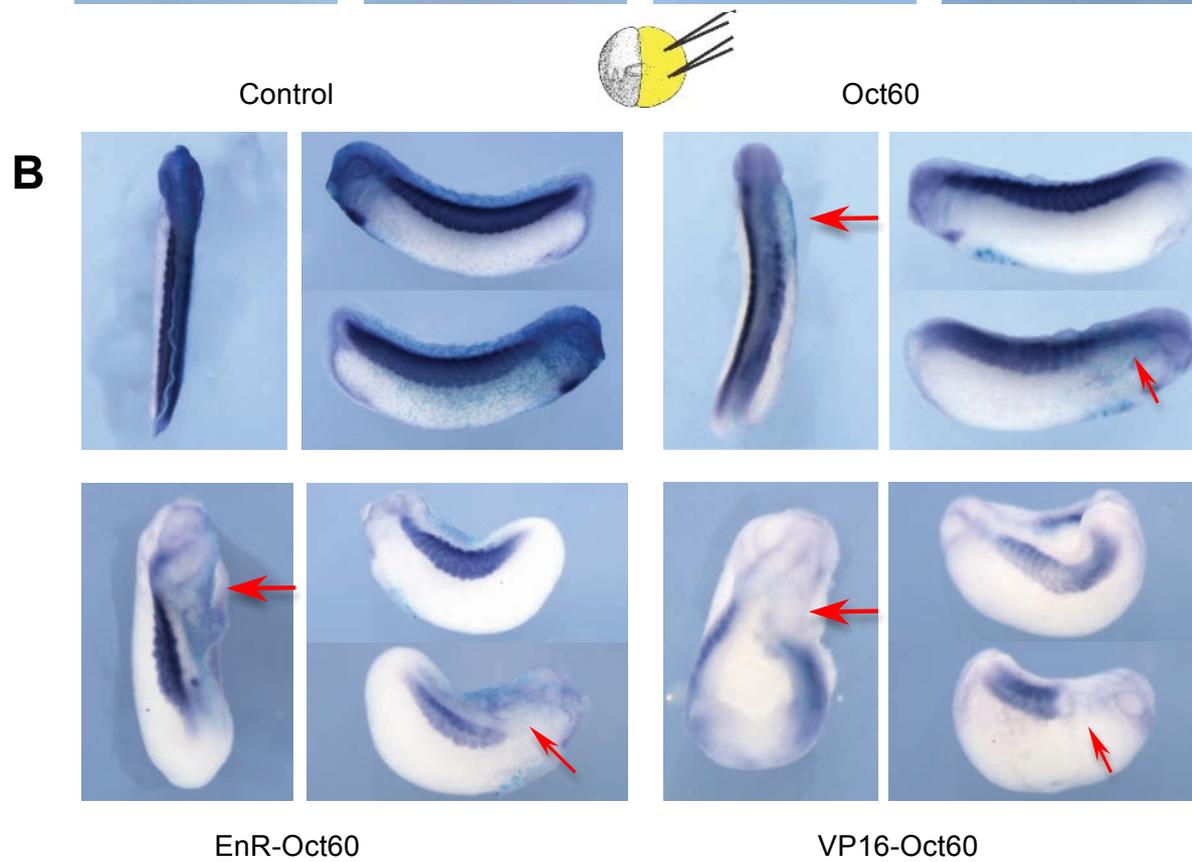
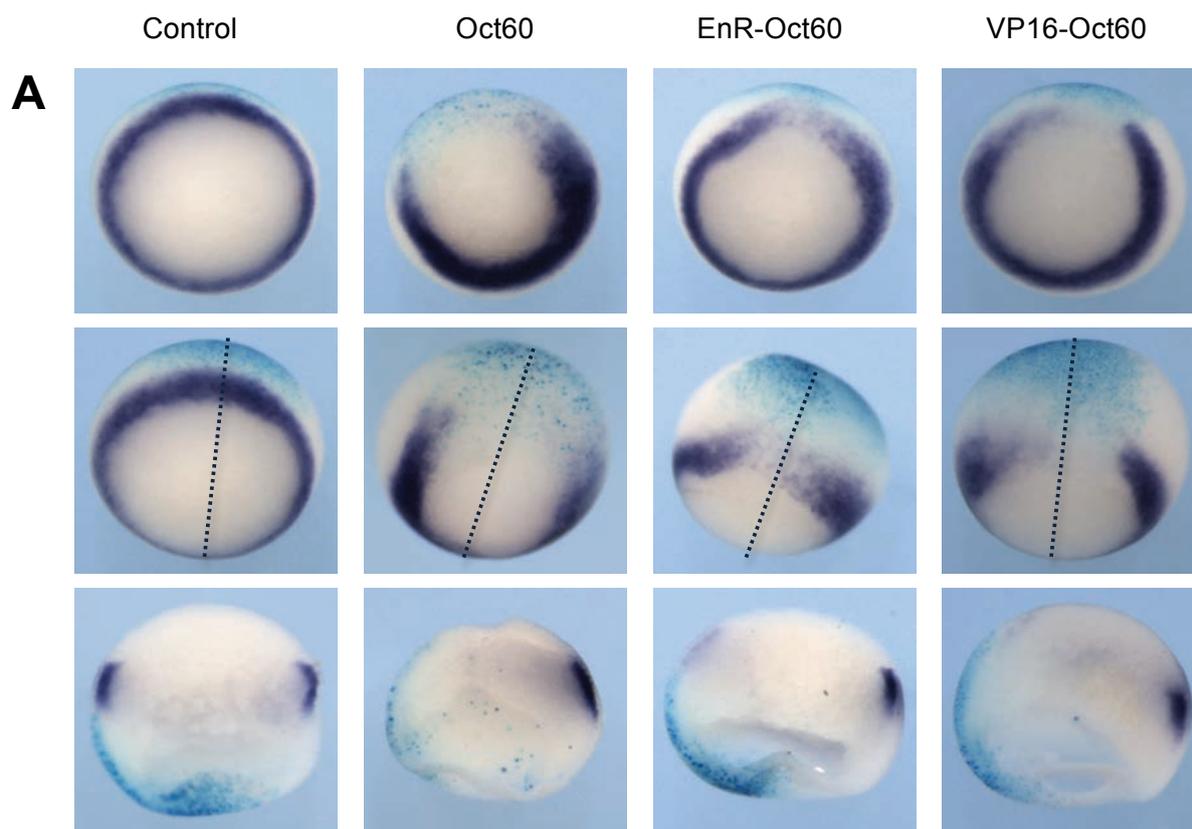


Figure 18: Injection of *oct60*, *enR-oct60* and *vp16-oct60* leads to disturbed development of mesodermal tissues in early embryos

In situ hybridizations were performed on gastrula stage embryos (NF10) and on tailbud embryos (NF 28) that were injected at two-cell stage into one blastomere as indicated. *LacZ* mRNA was coinjected. The *lacZ* stain indicates the injected region and is visible in light blue. One representative embryo is shown for each injected mRNA.

A) *Xbra in situ* hybridization shows a disruption of the *xbra* ring by all injected mRNAs. Row 1 and 2 show the injected embryo from different angles of view. Row 3 shows cross sections of the same embryo, as indicated in row 2 by black dashed lines.

B) *Cardiac actin in situ* hybridization indicated that Oct-60 and its G.o.F variants disturb the formation of somites (indicated by red arrows). The upper row shows the uninjected body side, the lower row shows the injected one. Especially in *oct60* and *vp16-oct60* injected embryos, hardly any *cardiac actin* staining is visible in the injected area. In *enR-oct60* injected embryos a diminished stain is visible.

4.5.3 Endodermal interference

In situ hybridization for the endodermal marker *endodermin*, performed on embryos stage 26 and 36 that were injected with *oct60* and its G.o.F. variants, showed no changes in the expression pattern (data not shown).

5 Discussion

Reprogramming of adult cells was first described in 2006, when the group of Shinya Yamanaka's team at Kyoto University, Japan, succeeded in inducing pluripotent stem cells from mouse fibroblasts (Takahashi and Yamanaka 2006). In 2007, the group of James Thomson created induced pluripotent stem cells from adult human cells (Yu, Vodyanik et al. 2007). Both groups came to the conclusion that two proteins are essential for reprogramming: Sox2 and Oct4. Lately, it was shown that Oct4 alone is sufficient to induce pluripotent stem cells from adult cells (Kim, Sebastiano et al. 2009). Apparently, adult cells are still sensitive to Oct4 expression.

In mammalian normogenesis, Oct4 expression is extremely well regulated. In early development, alterations in the expression level of mouse Oct4 lead to differentiation into extra-embryonic tissue or early endoderm (Niwa, Miyazaki et al. 2000).

Though the importance of Oct function in cell reprogramming and differentiation is evident, the molecular mechanisms by which Oct4 affects the pluripotent state and the transition from pluripotency to cellular determination is still largely unknown.

All the experiments named above were performed in mammals. POU V proteins have also been found in multiple amphibians e.g. *zebrafish* (DrPOU2), *axolotl* (AmOct4) and *Xenopus* (Frank and Harland 1992; Hinkley, Martin et al. 1992; Whitfield, Heasman et al. 1993; Burgess, Reim et al. 2002; Bachvarova, Masi et al. 2004). Nevertheless, the question, whether the role of Oct4 in maintaining pluripotency and its ability to reprogram adult cells is conserved or mammalian-specific has not been answered yet. In *Xenopus laevis*, three Oct4 homologs are known – Oct25, Oct60 and Oct91. The aim of this study was to find out more about Oct protein function including their role in maintaining the pluripotent state and their influence on cell fate decisions and differentiation in *Xenopus laevis* early development. By cloning dominant activating and dominant repressing Oct constructs for all three *Xenopus* Oct proteins, I obtained important tools for further evaluating and comparing Oct protein function.

5.1 G.o.F. protein variants

5.1.1 Studying Oct protein function: advantages of using gain-of-function variants

It was shown by previous work that mouse Oct4 acts as a transcriptional activator as well as a transcriptional repressor *in vivo* (Babaie, Herwig et al. 2007). Whether *Xenopus* Oct60 shares similar trans-activating functions is unknown. To investigate this question, we decided

to generate dominant negative and constitutively active transgenes to convert the transcriptional gene regulation of Oct transcription factors into dominant-activating and dominant-repressing activities.

Classical analysis of protein function is performed by gene mutation studies. This approach is subject to restrictions, since gene mutations often implicate early embryonic lethality. Moreover, related factors might compensate for mutated genes. Alternative approaches to bypass these drawbacks include downregulation of protein expression and overexpression studies. Several means can be used for this including wild-type DNA or RNA, antisense RNA or morpholino antisense oligonucleotides.

Furthermore, antimorphs display important tool to investigate transcription factor functions. Antimorphs are mutant alleles, which act in the opposite direction to normal alleles.

Their use offers several advantages compared to overexpression and loss-of-function studies. Loss-of-function approaches only reflect the unique contribution of a mutated or silenced gene. Especially when looking at the three Oct4 homologs in *Xenopus*, other Oct proteins might compensate, if one Oct protein is deficient. This might result in less severe phenotypic changes.

By comparing phenotypic changes that are produced by overexpression of the wildtype-protein with dominant-activating and -repressing protein variants, conclusions can be drawn with respect to protein function and molecular regulatory networks – if the effect produced by injection of wildtype-RNA resembles the activating G.o.F. variant, the wildtype-transcription factor must be an activator and vice versa.

5.1.2 Construction of G.o.F. Oct protein variants

Several general requirements have to be fulfilled when generating G.o.F. constructs: an expression system has to be used that assures the transcription of high amounts of polyadenylated RNA for injection. After injection, RNA has to be translated efficiently. Proteins should be detectable by antibodies to confirm protein expression *in vitro* as well as *in vivo*. Since antibodies feature different binding activities it is favorable, if all proteins can be detected by the same antigenic epitope. Thus, protein expression can be compared most accurately.

We decided to use the pCS2⁺-MT6 vector, which fulfills the mentioned requirements best. The pCS2⁺-MT6 vector was designed for generating epitope-tagged fusion proteins and allows the production of DNA as well as RNA for the injection into *Xenopus* oocytes (for further information, see <http://sitemaker.umich.edu/dlturner.vectors/home>).

Transcription factors regulate transcription of specific genes to control cellular functions. This is achieved through binding to target DNA sequences, followed by interactions with components of the basal transcriptional machinery. These two capacities can be structurally and functionally separated into distinct DNA-binding and effector domains. The DNA-binding domain ensures nucleotide sequence-directed localization to target genes. The effector domain interacts with one or more components of the basal transcription complex, either directly or indirectly via bridging cofactors, to mediate activation or repression of transcription (Vickers and Sharrocks 2002; Chandler and Werr 2003).

Multiple regulatory domains for the generation of G.o.F. protein variants are known. We decided to use the VP16 activation domain as well as the Engrailed repression domain.

The VP16 activation domain from the *herpes simplex* virus has often been used in *Xenopus* to generate dominant activating protein variants. For example, the homeodomain repressor Xoptx2 reduces eye size under VP16 activation (Zuber, Perron et al. 1999) and the *Xenopus* repressor GOOSECOID under VP16 activation upregulates gene expression in the dorsal marginal zone and leads to embryos with severe axial defects (Ferreiro, Artinger et al. 1998). The Engrailed (EnR) homeodomain protein originates from *Drosophila melanogaster* and plays an important role in organizing the segmented body plan. EnR is an active transcriptional repressor (Pownall, Isaacs et al. 1998; Chandler and Werr 2003). Similar to the activator domain of the VP16 protein, the EnR effector domain can be fused to transcription factors, so that the targeting function of the transcription factor is combined with the effector function of the EnR domain. Thus, the transcription factor keeps its targeting function, resulting in DNA sequence-specific binding to target genes, but its effect on transcription is dominated by the VP16 or EnR effector domain, resulting in either transcriptional activation or repression of target genes (Jaynes and O'Farrell 1991).

We decided to fuse the 6xMT domain to the N-terminus of our G.o.F. variants. C-terminal fusion also seems to work but the N-terminal fusion is the most common and best-studied way. The *vp16* and *enR* effector domains as well as the *gr* domain were digested with *StuI* and *XhoI*, *Xenopus-oct*-cDNAs were digested *XhoI*, *XbaI*. Effector domains were cloned at the 5'-end of the *Xenopus oct* cDNAs into the polylinker (Figure 8,9).

Injection of mRNAs into cleavage-stage embryos generally results in immediate translation. Experiments suggest that over the first 24h approximately 50% of injected mRNA stays intact (Colman and Drummond 1986). Endogenous *Xenopus oct* mRNAs are mainly detectable until stage 14. Therefore, a prolongation of Oct protein expression can be achieved by injecting *oct* mRNAs.

5.2 Oct proteins are detectable in different amounts *in vivo*

Work performed by Morrison and Brickman suggests that the ability of mouse Oct4 to maintain pluripotency is derived from an ancient POU V function. The three *Xenopus* Oct proteins are able to rescue knockdown of Oct4 to various extents. *Xenopus* Oct-proteins most likely play overlapping but distinct roles during embryogenesis (Morrison and Brickman 2006). We therefore argued that overexpression of all three *Xenopus* Oct proteins might mimic Oct4 function best. Nevertheless, injection of the three *Xenopus* Oct mRNAs did not result in comparable protein amounts *in vivo* (Figure 11). This finding was in contrary to results from *in vitro* studies, where all three Oct mRNAs were detectable in comparable amounts (Figure 10). Differences in RNA stability were excluded *in vitro*; nevertheless, this cannot exclude different half-lives *in vivo*. Regulatory mechanisms like RNAi or binding of proteins, which may affect mRNA stability, are unlikely since our synthetic mRNAs lack UTRs.

Xenopus Oct proteins are expressed at different time points in development. Therefore, I tested whether differences in protein expression are time-dependent. If the mRNA stability is the determining factor, comparable amounts of protein are expected in early development, whereas differences in protein amounts should increase in later development. This does not seem to be the case. The examination of MT-Oct protein levels revealed that this phenomenon is time independent. At each examined point of time MT-Oct91 was detected at highest, MT-Oct25 at intermediate, and MT-Oct60 in lowest levels (Figure 12). Still, a regulation among the injected Oct-proteins themselves could be a possible explanation. Recent experiments performed in our lab indicate that endogenous Oct proteins regulate the expression of each other (data not shown). Whether this regulation occurs at the DNA, RNA or protein level has not yet been examined. The ultimate reason of the unequal accumulation levels remains uncertain. Due to different protein expression levels, we stopped the combined injections of all three *Xenopus* Oct mRNAs and decided to concentrate on Oct60 alone.

5.3 Validation of the biological activities of Oct fusion proteins

By generating G.o.F. Oct protein variants, heterologous protein domains were fused to wild-type *Xenopus* Oct proteins. To confirm, that both, the DNA binding domains of the Oct proteins as well as the VP16/enR effector domains function in the expected manner, we performed a luciferase assay on two different promoters. With this assay I intended to test the activity of MT-Oct60 proteins on these promoters. Both promoters are strongly activated

by Oct4 and other mammalian POU proteins, but there is no published data on a possible activation by Oct60 (Niwa, Masui et al. 2002; Takebayashi-Suzuki, Arita et al. 2007). DNA-binding domains in POU proteins are highly conserved between mammals and *Xenopus laevis*. Nevertheless, sequence identity in the POU-specific- and POU-homeodomains comparing Oct60 and Oct4 are lower (57,7%) than between Oct25/Oct91 and Oct4 (65,3/66%) (Morrison and Brickman 2006).

Reporter gene analysis represents a sensitive tool to study the activity of transcription factors. We used a reporter plasmid encoding firefly luciferase that has enzymatic activity, placed under the control of transcription factor response elements. If transcription is activated, luciferase is expressed, which emits light when a suitable substrate is added. The light output can then be quantified using a luminometer and the intensity of light emitted is proportional to the level of luciferase expression.

Two different promoters were tested: the *more* and the *6w* promoter. The *more* (More palindromic Oct1 Responsive Element) promoter is an optimal artificial palindromic Oct binding site (ATGCATATGCAT). Several members of the Oct family including Oct4 were found to bind cooperatively as homo- and heterodimers to the consensus *more* (Tomilin, Reményi et al. 2000). When binding to the *more* promoter, the POU_S and POU_{HD} domains of two different Oct molecules make contacts with each half-site (Tomilin, Reményi et al. 2000). The *6w* enhancer contains 6 copies of oligonucleotides with a natural octamer binding motif from the mouse Ig heavy chain gene enhancer (Botquin, Hess et al. 1998; Tomilin, Reményi et al. 2000).

Oct60 by itself was found to be a strong activator of both, the *6w*, as well as the *more* promoter. Injection of *vp16-oct60* mRNA resulted in an even stronger activation, which was more than 20-fold stronger than Oct60 mediated activation (Figure 14 A). Since the basal activity of the *more* and the *6w* promoter, which probably includes activation by endogenous Oct proteins, was low, the activity of EnR-Oct60 could not be visualized. However, coinjection of *enR-oct60* with *vp16-oct60* resulted in a strong, EnR-Oct60 dependent repression on both promoters (Figure 14 B).

Based on the reporter assays, native *Xenopus* Oct60 acts, similar to Oct4, as a transcriptional activator with regards to its direct binding to the promoters via the octamer motif. EnR-Oct60 and VP16-Oct60 are able to bind to both promoters, indicating that the fused effector domains do not disturb DNA binding function. Both effector domains act in a dominant fashion and show the expected transcriptional activities.

5.4 Embryonic phenotypes

5.4.1 Penetrance and expressivity

Phenotypes, observed by injection of *oct60* and its G.o.F. variants, showed a range of severity within each injection round as well as varieties in penetrance and expressivity, when comparing different injection rounds.

A wider range in expressivity was observed in *enR-oct60* and *vp16-oct60* injected embryos than in embryos that were injected with *oct60*. To give an example - changes in eye development reached in *vp16-oct60* injected embryos from absent eyes to normal appearing eyes that were slightly retarded in development. *Oct60* injected embryos showed almost the same degree of malformation comparing embryos from one injection round. One possible explanation for this are differences in the location and depth of penetration of the injection needle, resulting in different allocation of the mRNA so that various parts of the embryo are affected to different degrees. Different mRNA distributions were also observed when embryos were stained for *lacZ*.

Additionally, differences in expressivity and penetrance were observed comparing different injection rounds. This is most likely due to variations in the injection dosage. When injecting the embryos, the needle is manually broken and calibrated through visual examination. This might add up to small errors when measuring the concentration of RNA. From studies with Oct4, it is known that the observed phenotypic changes are highly dose dependent (Niwa, Miyazaki et al. 2000).

Vp16-oct60 injected embryos were affected most severely, especially by gastrulation defects, head defects and defects in epithelial layers. This made it necessary to reduce the injected mRNA dose when post-gastrula stages should be obtained. One possible explanation is the massive activation of target genes, which was more than 20fold higher than the activation observed by Oct60 in the luciferase assay. Even when reducing the injected dose from 450pg to 150pg, the percentage of embryos surviving until post-gastrula stages was lower than in *oct60* and *enR-oct60* injected groups.

5.4.2 Oct60 and VP16-Oct60 produce similar phenotypic changes

Phenotypic changes affected predominantly the head and eye region in *oct60* and *vp16-oct60* injected embryos. *EnR-oct60* injected embryos revealed a bulge like thickening within

the trunk region. Additionally, a specific curvature of the main body axis was observed (Figure 16). Comparing the different Oct60 variants, the phenotype produced by Oct60 resembles more the one produced by injection of *vp16-oct60* than the one produced by EnR-Oct60 – though they are not identical. Both, *oct60* and *vp16-oct60* injected embryos were curved predominantly towards the injected body half and phenotypic changes affected especially the head region, resulting in thickening of the head. Hyperpigmentation was more prominent in *oct60* injected embryos, affecting a large proportion of the injected body half. Also *enR-oct60* injected embryos displayed defects in head development that were less severe. The most prominent findings in these embryos were hyperpigmented bulge-like outgrowths in the trunk regions (Table 2, Figure 15).

In summary, the injected constructs produced different phenotypes, with *oct60* and *vp16-oct60* injected embryos sharing multiple characteristics.

5.4.3 Perturbed formation of anterior structures

As outlined above, injection of *oct60* and its neomorphic protein variants resulted in severe defects of the head- and eye formation. Affection of the retinal pigment epithelium as well as the lens was macroscopically detectable. Although, the same structures were affected by all injected mRNAs, the morphology of eye defects differed in appearance and shape. Inhibition of lens formation was most pronounced in embryos that were injected with *oct60*. The only structure that was macroscopically detectable was the dorsal retinal structure. In *enR-oct60* injected embryos, eye formation was less disrupted. Noticeable was a microphthalmia and reduction of the retinal pigmentation in these embryos. Additionally, the optic fissure was not clearly detectable and often seemed to be positioned more towards the cement gland compared to control embryos. *Vp16-oct60* injected embryos showed no detectable eye structures or microphthalmia in very high percentages.

A bulge-like thickening of the head was observed in *oct60* and *vp16-oct60* injected embryos. In contrast to *vp16-oct60* injected embryos, head bulges in *oct60* injected embryos were strongly hyperpigmented. These bulges were most prominent around stages 24-26, and were strongly reduced with regard to size and pigmentation following differentiation.

Other POU proteins are known to perturb development of neural and epidermal structures when misexpressed: *XIPOU2*, a member of the class III POU domain family, is expressed initially in the Spemann's organizer and later in discrete regions of the developing nervous system in *Xenopus laevis*. *XIPOU2* is a target gene of the neural inducer noggin and plays an important role in neural determination. *XIPOU2* is capable of inducing neural specific genes such as *ncam* or *n-β-tubulin*. Misexpression of *XIPOU2* in the epidermis causes a

direct switch in cell fate from an epidermal to a neuronal phenotype (Witta, Agarwal et al. 1995; Matsuo-Takasaki, Lim et al. 1999). Similar to XLPOU2, overexpression of Oct60 resulted in ectopic expression of the neural marker n- β -tubulin. Whether these findings result from a common mechanism in inducing neural gene expression remains uncertain and is subject to further research.

5.4.4 Pigmentation defects

Oct60 and *enR-oct60* injected embryos displayed severe hyperpigmentation at distinct parts of the body. Injection of *oct60* predominantly resulted in hyperpigmentation affecting the head whereas injection of *enR-oct60* produced hyperpigmented bulges at the trunk region. Hyperpigmentation was visible earlier in development than the endogenous pigmentation indicating premature production of melanin. In contrast to the premature formation of ectopic pigmentation, the normal pigmentation was retarded in embryos injected with *oct60* and its G.o.F. variants. At stage 33, when control embryos displayed melanophores, embryos injected with *oct60* and its G.o.F. variants showed no normal pigmentation. At stage 40, the pigmentation had almost normalized indicating that the absence of pigmentation at stage 33 represented a developmental delay.

Interestingly, the ectopic hyperpigmentation, especially the one on the outgrowth of *enR-oct60* injected embryos, did not disappear when embryos were bleached after *in situ* hybridizations. This made it difficult to judge the staining of *in situ* hybridizations in the affected body parts. Preliminary experiments, performing cross-sections through the trunk outgrowth, were suggestive of a thickened epidermis in this region. Further experiments have to be carried out to confirm these findings. Nevertheless, a thickened epidermis could explain the experienced bleaching problems. Hyperpigmentation has never been observed in animal caps, to my knowledge.

While the epidermis of the frog tadpole is uniformly pigmented, a few organs stand out by a higher concentration of pigment. These include the cement gland, the hatching gland, and the dorsal region (roof plate) of the neural tube. In vertebrates, melanocytes are derived from multipotent neural crest cells (Dushane 1934). In amphibians, three types of pigment cells - black melanophores, yellow xanthophores, and silvery iridophores - differentiate from neural crest cells (Epperlein, Löfberg et al. 1996). On completion of neurulation, the neural crest lies dorsal to the neural tube and immediately below the epidermis. Transparent melanoblasts migrate from this position to their definitive locations in the embryo, where they differentiate and give rise to a species-specific larval pigment pattern (Macmillan 1976).

Therefore, the black shape of the observed hyperpigmentation might be either due to an ectopic formation of melanin-producing cells, an inhibition of migration or a local overproduction of melanin. However, the vast increase in pigmented areas in the injected embryos makes it unlikely that they are caused only by inhibition of neural crest cell migration. Degrees of survival, proliferation, and differentiation of melanophores are other features that have to be considered. Ectopic-pigmented cells displayed a punctuated shape. Therefore, they differ from normal melanophores, which are of dendritic nature.

Ectopic pigmentation was described previously in large scale functional screens (Grammer, Liu et al. 2000; Chen, Voigt et al. 2005) in conjunction with a variety of genes including the transcription factors ESR6, Slug, Forkhead, Hairy 2B, Sox2, and Sox21 (Deblandre, Wettstein et al. 1999), a diacylglycerol binding protein essential for synaptic vesicle maturation (Munc13) (Augustin, Rosenmund et al. 1999), a sequence specific regulator of pre-mRNA splicing (Tra-2 a) (Tacke, Tohyama et al. 1998), and a zinc-binding protein of unknown function (Chp-1) (Brancaccio, Menini et al. 2003).

Additionally, ectopic expression of the Gli1 transcription factor, a regulator of sonic hedgehog signaling, causes ectopic pigmented outgrowth within the trunk region that resembles those observed in *enR-oct60* injected embryos (Dahmane, Lee et al. 1997). This pathway has been implicated in the induction of human cancers. Interestingly, the formation of Gli1-induced pigmentation defects seen in *Xenopus* may be analogous to the formation of human melanomas (Dahmane et al., 1997). The above named proteins that are implicated in the formation of ectopic pigmentation might serve as starting points for further evaluating the molecular mechanism underlying ectopic pigmentation induced by Oct60.

In mouse, ectopic expression of Oct4 in epithelial tissues results in dysplastic growths that are dependent on continuous Oct4 expression. Tumors are fully reversible when Oct4 expression is stopped (Hochedlinger, Yamada et al. 2005). A similar phenomenon was observed in *enR-oct60* injected embryos – here, the pigmented bulges disappear when the injected mRNA decays. Whether hyperpigmentation observed in embryos injected with *oct60* and its G.o.F. variants reflect dysplastic lesions remains uncertain to date.

5.4.5 Bulge formation in the trunk region

Several attempts have been made to determine the nature of the observed bulges by *in situ* hybridization. *Cardiac actin*, *endodermin* and *n-β-tubulin* did not stain the bulges, indicating that they are not composed of differentiated mesodermal, endodermal or neuronal tissues. Staining with the early mesodermal marker *xbra* was observed in two embryos at stages 14

and 26 within the bulges (data not shown). Unfortunately, this observation could not be reproduced in further experiments. At NF 24/26, *xbra* is hardly expressed anymore in the embryo (Smith, Price et al. 1991; Tadano, Otani et al. 1993). Thus, ectopic *xbra* mRNA in Oct60 perturbed embryos may represent a rather transient feature of a retarded differentiation state. This may explain difficulties in detecting *xbra* expression in the trunk bulges.

Other possible causes for difficulties in characterizing the underlying tissue might be that the bulges consist of disorganized tissue, tissues we did not test for or might be retarded in the process of differentiation. One problem was the strong hyperpigmentation affecting the region of the bulges that was not reactive to bleaching and therefore made it difficult to evaluate any underlying *in situ* staining. Future studies will attempt to identify the fate and the molecular mechanism of the observed pigmented bulges.

5.4.6 Shortened and specifically curved main body axis

Phenotypic changes produced by injection of *oct60* and its gain of function variants included a shortened and specifically curved main body axis. Embryos, injected with *oct60* and *vp16-oct60*, curved mainly to the injected side, whereas injection of *enR-oct60* resulted in a curvature to the non-injected side (Figure 16).

In general, a curved anteroposterior body axis forms, when the left and right body axis elongate at different rates. This can be caused by different cell proliferation activities or different morphogenic activities, in particular convergent extension.

In the process of convergent extension, cell intercalation is narrowing tissue along one axis while elongation occurs along a perpendicular axis by cellular movements (Keller, Davidson et al. 2000). Therefore, a curvature towards the injected side is consistent with an inhibition, while a curvature away from the injected side is consistent with an increase in convergent extension movements on that side.

Alterations can be produced by quantitative as well as qualitative changes in this process: on the one hand, alteration of cell fate can result in up or downregulation of cells that can undergo convergent extension. On the other hand, a change in cell behavior can inhibit or boost the ability to undergo convergent extension.

Convergent extension involves multiple cell types. During *Xenopus* development, the presumptive posterior hindbrain and spinal cord undergo convergent extension movements in parallel with similar movements that occur in the underlying mesoderm (Keller, Davidson et al. 2000; Gilbert 2006). Therefore, a gain or loss of these tissues can result in changed extends of convergent extension. This can also be caused by changes in the cell behaviour or features like polarity decisions in the tissues mentioned above. Hereby, it is important to

keep in mind that convergent extension is not a passive event, but rather an active, autonomous morphogenetic process (Keller and Danilchik 1988; Elul and Keller 2000; Keller, Davidson et al. 2000).

Noncanonical Wnt signaling is a common mechanism that controls convergent extension in both, mesodermal and neural tissues (Deardorff, Tan et al. 1998; Medina, Reintsch et al. 2000; Wallingford and Harland 2001). Disruption of Wnt signaling results in changes in the process of convergent extension (Wallingford and Harland 2001; Yokota, Kofron et al. 2003). Interestingly, mouse Oct4 as well as *Xenopus* Oct4 homologs were recently describes to modulate Wnt/ β -catenin signaling (Abu-Remaileh, Gerson et al. 2010).

The fact that the observed curvature differences dissolve during further development indicates that the higher/lower extend of convergent extension can be compensated. This can be achieved by active or passive mechanisms: One possible hypothesis is that the limited or accelerated degree of convergent extension becomes normal again and compensates. Besides this also an increased tension and traction that is due to the curvature might act as a stimulus for the shorter side to elongate.

With regard to the shortened body axis, phenotypic changes are most consistent with a ventralization of the embryos. Recently published data suggests that this is most likely due to inhibition of Wnt/ β -catenin signaling. Interestingly, ventralization was also observed by overexpression of mouse Oct4 in *Xenopus* embryos (Abu-Remaileh, Gerson et al. 2010). Therefore, both, the curvature of the body axis as well as the ventralization, could be explained by modulation of Wnt signaling.

5.4.7 Transient nature of phenotypic changes

A common feature of the observed phenotypes was their transient nature. Bulge formation in the head as well as in the trunk region diminished in size, hyperpigmentation normalized, the curvature of the main body axis became less, eye formation was retarded but eyes became visible even though often dysplastic in the process of development and the truncated body axis improved gradually. Most phenotypic changes were most distinctive around stage 24-28 and improved during the following differentiation. At stage 40, characteristics like the strong hyperpigmentation, the bulge formation in the head and the truncated main body axis had almost normalized completely. The trunk bulges and the curvature of the main body axis became less prominent. At stage 40, eyes were visible but still retarded and/or malformed. Also characteristics such as the strongly reduced motor reaction to exogenous stimuli that was observed in earlier stages normalized completely up to stage 40. This might suggest

that the alterations in differentiation are to a large degree reversible. A likely cause of such improvement would be the turnover of the injected RNA. It was described previously that the majority of injected mRNAs is present up to stage 28, when phenotypic changes are most prominent, and has turned over by stage 40, when changes have partially normalized (Harland and Misher 1988; Gove, Walmsley et al. 1997). This decay of RNA might explain the transient nature of the observed phenotypic changes. Therefore, Oct60 might induce a transient arrest in cell differentiation. This implicates that cells are able to undergo differentiation as soon as Oct60 expression ceases.

5.5 Oct60 promotes neuroectodermal fate while repressing mesoderm formation

5.5.1 Oct60 and its G.o.F. variants produce a broadened, non-organized *n-β-tubulin* expressing domain and disturb placode and eye formation

To gain further insights into the molecular nature of the observed phenotypic changes, *in situ* hybridizations were performed with general markers for neuroectodermal, mesodermal and endodermal differentiation (Figure 17, 18).

No changes were found in the expression of the endodermal marker *endodermin* (data not shown). Expression levels of the neural marker *n-β-tubulin* and the mesodermal markers *xbra* and *cardiac actin* were strongly altered by injection of *oct60* and its G.o.F. variants.

In situ hybridization with the late neural marker *n-β-tubulin* revealed a broadening of the *n-β-tubulin* expressing domain on the injected side of *oct60*, *enR-oct60* as well as *vp16-oct60* injected embryos. *N-β-tubulin* expressing cells were detectable at almost the whole broadened head region. Development of eyes and placodes was strongly inhibited. Placodes were not detectable on the injected side in most cases. Interestingly, Oct60 and its G.o.F. variants produced similar ectopic *n-β-tubulin* expressing domains. Broadening of the *n-β-tubulin* domain was most prominent in *vp16-oct60* injected embryos. Taken together, it appears that neural *n-β-tubulin* expressing cells are being formed but not organized in a proper manner.

The fact that *n-β-tubulin* seems to be upregulated in the head region by all injected *oct60* constructs was surprising. Though changes observed within the head region displayed similarities between the injected constructs, differences were observed within the trunk region. Ectopic expression of *n-β-tubulin* was observed within the anterior trunk region mainly in *enR-oct60* injected embryos. *N-β-tubulin* did not stain the trunk bulges observed in

enR-oct60 injected embryos. *N-β-tubulin* expressing cells that are usually equally spread over the whole trunk region spared the region of the trunk outgrowth. In contrast, *lacZ* positive cells were highly concentrated within these bulges. Possible explanations include cell migration to the region of bulge formation, or a higher rate of cell proliferation in this area. High cell proliferation rates are also found during embryonic development and in neoplastic lesions. Interestingly, Oct4 is known to be involved in both processes. Multiple tumors were found to express Oct4 protein (Atlasi, Mowla et al. 2007; Chen, Hsu et al. 2008; Wang, Meng et al. 2010). Whether the observed bulges display a tumor-like lesion remains uncertain.

5.5.2 Oct60 and its G.o.F. variants inhibit the expression of early and late mesodermal markers

Strong downregulation of *xbra* and *cardiac actin* expression was observed in embryos overexpressing Oct60 and its G.o.F. variants (Figure 18). Therefore, *Xenopus* Oct60 and its G.o.F. variants all direct cell differentiation in a similar manner. They are promoting neuroectodermal fate, while repressing mesodermal differentiation. This result was surprising because it cannot be explained by transcriptional regulation. Both direct as well as an indirect transcriptional regulation should produce regulatory effects in opposite directions.

One possible explanation might be that regulation does not occur on the level of transcription. First evidence that Oct proteins can influence protein levels on a post-transcriptional level came from recently published data suggesting that mouse Oct4 forms a physical complex with β -catenin in the nucleus. Thus, Oct4 facilitates proteasomal degradation of β -catenin. The same effect was also described for *Xenopus* Oct25 (Abu-Remaileh, Gerson et al. 2010). Regulatory effects on a post-transcriptional level could explain similarities observed in the regulation of mesodermal and neural markers between Oct60 and its G.o.F. variants. The neomorphic protein variants are only able to fulfill their additional activating and repressing functions, if regulation occurs on a transcriptional level as it was demonstrated in the luciferase assay. In this case differences between the G.o.F. variants should be detectable. Effector domains will not fulfill their functions, if proteins are regulated post-transcriptionally. This could explain that there is no inverse regulation of mesodermal and neuroectodermal markers by Oct60 G.o.F. variants.

Our G.o.F. variants represent interesting tools for the further analysis of regulatory pathways controlled by *Xenopus* Oct proteins. By comparing regulatory effects of activating and repressing transcription factor variants, it might be possible to differentiate between transcriptional and non-transcriptional regulation.

5.6 Does Oct60 and its G.o.F. variants produce tumor-like lesions in *Xenopus* embryos?

The molecular nature of the bulges observed in the trunk region, the hyperpigmentation and the broadening of the head domain remain unexplained. Further studies are being performed to clarify these questions. One similarity between the observed phenotypic changes is that all of them possess features of dysplastic growth: high amounts of *lacZ* expressing cells were found in the trunk outgrowth of *enR-oct60* injected embryos that might reflect a high cell proliferation rate. The same process could possibly explain proliferation of melanin producing cells and *n-β-tubulin* expressing cells in the head region. Tumors produced by ectopic expression of Oct4 were shown to be dependent on Oct4 expression and were fully reversible when Oct4 expression was ceased (Hochedlinger, Yamada et al. 2005). Similar to this, phenotypic changes observed by overexpression of Oct60 and its G.o.F. variants were transient to various degrees. Hyperpigmentation defects vanished almost completely in most cases by stage 40, showing its maximum around stage 26. Also bulges in the trunk region and the broadening of the head domain disappeared partially by stage 40. This might indicate that phenotypic changes are dependent on the continuous expression of Oct60 and its G.o.F. variants and cease when synthetic mRNAs are degraded.

5.7 Recent development in the field of *Xenopus* Oct research

Among the *Xenopus* Oct proteins, the best-studied one is probably Oct25. The group of Walter Knöchel from the University of Ulm has done most of the work on the molecular functions of Oct25. Their work shows multiple similarities but also differences compared to my work. When comparing my results with theirs, one has to keep in mind, that their injections were targeted differently. While the group of Walter Knöchel examined phenotypic changes produced by ectopic expression of Oct protein within the vegetal region of the developing embryo, we injected *oct60* and its G.o.F. variants into the animal hemisphere. Thereby, I attempted to interfere with Oct proteins within their normal territory of expression.

Knöchel et al. were able to show that Oct25 binds to the Xvent2B promoter and stimulates transcription of the Xvent-2B gene. Thereby, overexpression of Oct25 leads to upregulation of BMP4 and Xvent genes (Cao, Knochel et al. 2004). Furthermore, Oct25 and Oct60 were shown to inhibit activin/nodal and FGF signaling pathways (Cao, Siegel et al. 2006).

Interestingly, phenotypes observed by overexpression of Oct25 show multiple similarities compared to phenotypic changes observed by Oct60 and its G.o.F. variants: overexpression

of Oct25 resulted in gastrulation defects, truncation of the embryos, loss of head structures including brain and eyes, loss of somite structures and no extension of the tail. Additionally, they observed an accumulation of darkly pigmented cells at the lateral or ventral sides of the embryos that resembles the one observed in *enR-oct60* injected embryos (Cao, Knochel et al. 2004). Similar to our results they demonstrated downregulation of the mesodermal markers *xbra* and *cardiac actin* (Cao, Knochel et al. 2004). In animal caps, injected with *oct25*, they observed an upregulation of the neuroectodermal genes *sox2*, *sox3*, *geminin* and *zic1* but no expression of the differentiated primary neuron specific *n-β-tubulin* gene at stage 26. These findings indicate that Oct25 promotes neural fate by upregulating early neuroectodermal genes but suppresses terminal differentiation of neurons (Cao, Siegel et al. 2006). Is this function of Oct25 different to the influences of Oct60 on neuroectodermal differentiation we observed? *In situ* hybridization on embryos stage 28 that were injected with *oct60* and its G.o.F. variants showed a broadening of the *n-β-tubulin* expressing domain with no visible eye structures and placodes. Though differences in functions seem to be evident between Oct25 and Oct60 at first glance, the findings might be conformable with a common role in neuroectodermal regulation. Differentiation might be prevented during early stages when Oct proteins are upregulated. An inappropriate differentiation might occur later, resulting in the observed broadened *n-β-tubulin* expressing domain. It will be interesting to compare the expression of *n-β-tubulin* during early development between control embryos and embryos injected with Oct proteins to determine, whether expression of *n-β-tubulin* starts asynchronous.

Downregulation of the endodermal marker *sox17* by injection of *oct25*, *oct60* and *oct91* was described by the group of Walter Knöchel (Cao, Siegel et al. 2006). Our experiments showed no alteration in the expression of the late endodermal marker *endodermin*. Whether these differences result from the different mode of injection or different endodermal markers examined remains uncertain.

More recent studies of Knöchel et al. showed that Oct25 inhibits the expression of VegT and β -Catenin target genes through complex formation. VegT and β -Catenin are both expressed in the vegetal half of the developing embryo and are thought to play a key role in mesoderm induction by activating transcription of Xnrs (Cao, Siegel et al. 2007).

Interestingly, similar to our results, both, dominant activating as well as repressing fusion proteins of Oct25 produced a downregulation of the mesodermal marker *xbra* (Cao, Siegel et al. 2008). Whether this unidirectional regulation is due to post-transcriptional regulation, as mentioned above, remain to be evaluated.

5.8 Outlook

Future studies will attempt to identify the molecular mechanisms underlying the observed phenotypic changes. It will be particularly interesting to investigate the fate of the trunk bulges as well as the head defects with regards to neoplastic growth. Therefore, molecular studies including *in situ* hybridizations and RT-PCR have to be performed. Hereby, animal cap assays might serve as additional *in vitro* models. Furthermore, the Rupp laboratory is planning to perform cross sections for pathological assessment.

At present, Oct60 knockdown experiments, performed by injection of morpholino oligonucleotides, are being carried out in our lab. Comparison of knockdown and overexpression phenotypes will provide further information on the activating and repressing functions of Oct60. Recently published data suggests that Oct proteins are able to regulate protein expression at the post-transcriptional level (Abu-Remaileh, Gerson et al. 2010). Non-transcriptional regulation might explain the unidirectional regulation of various differentiation markers observed in *in situ* hybridizations. Further studies should attempt to evaluate the level of protein regulation that is responsible for the observed molecular changes.

Furthermore, dominant activating and repressing protein variants of Oct25 and Oct91 have been cloned. Comparison of the three *Xenopus* Oct4 homologs will be interesting to find out to which degree they share redundant functions.

Due to time limitations we could not perform experiments using the GR-fusion constructs. These constructs are important tools to further analyze *Xenopus* Oct protein function. Like mammalian Oct4, *Xenopus* Oct4 homologs are mainly expressed in early development. Recent experiments performed in mice and human fibroblasts suggest that Oct4 is sufficient to reprogram differentiated cells (Takahashi and Yamanaka 2006; Okita, Ichisaka et al. 2007; Takahashi, Tanabe et al. 2007). Additionally, Oct4 expression was detected in several neoplastic lesions such as lung cancer, bladder cancer and breast cancer (Atlasi, Mowla et al. 2007; Chen, Hsu et al. 2008; Wang, Meng et al. 2010). These findings suggest that differentiated cells are still reactive to mammalian Oct protein expression. Thus, it will be interesting to examine the effects of de novo *Xenopus* Oct protein expression later in development using the generated GR-fusion constructs.

To conclude, it should be pointed out that the unutilized Oct25, Oct91 as well as GR-constructs represent important tools with untapped potential. Their use will hopefully contribute to the resolution of further unanswered questions concerning Oct protein function.

6 Abbreviations

bp	base pairs
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
ddH₂O	double-distilled water
DNA	deoxyribonucleic acid
e.g.	exempli gratia, for example
et al.	et alii, and others
etc.	et cetera
g	gram
GFP	green fluorescent protein
G.o.F.	gain of function
GST	glutathione S-transferase
h	hour
hpf	hours post fertilization
IP	immunoprecipitation
l	liter
kDa	kilodaltons
min	minutes
M	molar
MBT	mid-blastula transition
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
NF	<i>Xenopus</i> developmental stages according to the normal table of staging of <i>Xenopus laevis</i> (Daudin) after (Niewkoop and Faber 1994)
ng	nanogram
nm	nanometer
NTPs	nucleotide triphosphate mixture containing adenosine, guanine, uridine and cytosine
PCR	polymerase chain reaction
pg	picogram
pg/E	picogram per embryo
pmol	picomol
RNA	ribonucleic acid

rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gelelectrophoresis
sec	seconds
WB	Western blot analysis
µg	microgram
µl	microliter
µM	micromolar

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Functional Analysis of *Xenopus* Oct4 related Factors, Laura Michel, Ralph Rupp (Poster „Deutsch-Italienischen *Xenopus* Konferenz“, 2007, Como, Italien)

Developmental phenotypes produced by *Xenopus* Oct60 gain-of-function protein variants, Laura Michel, Ralph Rupp (Poster "Internationale *Xenopus* Konferenz", 2008, Trier)

Embolization of a large inferior vena cava aneurysm: The use of coils and the Amplatzer vascular plug, Laura L. Michel, Ahmad I. Alomari, Journal of vascular and interventional radiology 2008 Oct;19(10):1509-12.