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„Zfp281: A Key Player in Mouse Embryonic Stem Cell Commitment “

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# ABSTRACT

Embryonic stem cells (ESC) are a self-renewing cell type equivalent to pluripotent cells in the developing pre-implantation embryo. Thus, ESCs have the ability to regenerate themselves, but also to differentiate into all embryonic and adult cell types *in vitro* and *in vivo*. Since their discovery more than thirty years ago, the mechanisms keeping ESCs in their pluripotent state have been well described. This has not only led to fundamental insights into properties of stem cell regulatory networks, but also set up the discovery of induced pluripotency. The ability to convert somatic cells into pluripotent cells holds promise to personalized stem cell based disease therapy and was awarded with the Nobel Prize in 2012.

In contrast to mechanisms regulating the establishment and maintenance of pluripotency, the pathways responsible for exit from pluripotency and, consequently, commitment to differentiation, are poorly understood. It is not known if this is solely due to shutdown of the pluripotent regulatory network or if inductive mechanisms are additionally involved. A large-scale loss of function screen for genes required for exit from pluripotency identified the transcription factor Zfp281 as a putative differentiation-promoting factor. Previous work (Wang *et al.*; 2008; Fidalgo *et al.*, 2011) proposed contrary functions – maintenance and exit of pluripotency - for this factor. This study takes a loss of function approach using ESC differentiations and the reprogramming of epiblast stem cells (EpiSC) into ESC. EpiSC are derived from the post-implantation epiblast and therefore represent a cell identity developmentally closely related to ESCs. Consequently, EpiSCs can be reprogrammed into ESC more easily than somatic cells. The aim of this study is to characterize the role of Zfp281 in differentiation and reprogramming and to identify its molecular function using biochemical approaches.

Using gene expression and functional assays, this study shows impaired differentiation of ESC and enhanced reprogramming of EpiSC upon Zfp281 knockdown. These findings suggest that Zfp281 acts as a pro-differentiation factor, by repressing pluripotency genes and down regulating pluripotency promoting signalling pathways. Thus, Zfp281 may act as a safeguard that ensures irreversible exit from pluripotency during development.

# ZUSAMMENFASSUNG (German abstract)

Embryonale Stammzellen (ESC) sind sich selbst erneuende Zellen, die den pluripotenten Zelltyp im *pre-implantation* Embryo widerspiegeln. ESC haben die Fähigkeit sich selbst zu regenerieren aber auch sich in alle embryonale und adulte Zelltypen zu entwickeln. Seit ihrer Entdeckung vor mehr als dreißig Jahren wurden die Pluripotenz erhaltenden Mechanismen ausführlich beschrieben. Das gab nicht nur Einsicht in die grundlegenden Vorgänge der Pluripotenz, sondern führte auch zur Entdeckung der induzierten Pluripotenz. Die Fähigkeit somatische Zellen in pluripotente Zellen zu konvertieren eröffnet die Möglichkeit einer personalisierten Stammzell-basierten Krankheitstherapie. Diese Entdeckung wurde 2012 mit dem Nobelpreis ausgezeichnet.

Im Vergleich zu den Pluripotenz etablierenden und erhaltenden Mechanismen, sind Mechanismen die für den Verlust der Pluripotenz und der daraus resultierenden Differenzierung verantwortlich sind nur schlecht verstanden. Es ist nicht bekannt ob der Verlust der Pluripotenz nur durch Herabregulation des Pluripotenz Netzwerkes bewerkstelligt wird oder ob auch induzierende Signale beteiligt sind. Ein *loss of function screen* für Gene die für den Verlust der Pluripotenz benötigt werden, identifizierte den Transkriptionsfaktor Zfp281 als einen möglichen differenzierungsfördernden Faktor. Frühere Studien (Wang *et al.*; 2008; Fidalgo *et al.*, 2011) schlagen konträre Funktionen – Erhaltung und Verlust der Pluripotenz – für diesen Faktor vor. Diese Studie arbeitet mit einem *loss of function* Ansatz für ESC Differenzierungen und Epiblast Stammzellen (EpiSC) Reprogrammierungen. EpiSC stammen aus dem *post-implantation* Epiblast und entsprechen einem ESC nahen Zelltyp. Daher können diese Zellen leichter reprogrammiert werden als somatische Zellen. Das Ziel dieser Studie ist die Charakterisierung von Zfp281 während der Differenzierung und des Reprogrammierens und der Identifizierung seiner molekularen Funktion mittels biochemischer Experimente.

Genexpressionsanalysen und funktionelle Experimente zeigen beeinträchtige Differenzierung von ESC und verbesserte Reprogrammierung von EpiSC nach einem Zfp281 knockdown. Das lässt darauf schließen, dass Zfp281 durch Unterdrückung von Pluripotenz Genen und Pluripotenz unterstützenden Signalen als differenzierungsfördernder Faktor agiert. Zfp281 könnte als Sicherungsmechanismus fungieren, der den irreversiblen Verlust der Pluripotenz während der Entwicklung garantiert.

# 1 INTRODUCTION

## 1.1 Embryonic Stem Cells

### *1.1.1 Origin of Embryonic Stem Cells*

Early development in mammals is orchestrated by mutual inductive signalling between extra-embryonic and embryonic lineages. The embryo proper is formed by cells of the epiblast, a derivative of the inner cell mass (ICM) in the blastocyst. These cells are capable of forming the three embryonic germ layers and are therefore called pluripotent. Single epiblast cells can be isolated and microinjected into another blastocyst, where they are still able to give rise to all lineages (Gardner, 1998) and integrate into the embryo forming chimeric animals.

Pre-implantation epiblast cells cultured under appropriate conditions give rise to embryonic stem cells (ESC) (Evans and Kaufman, 1981; Martin, 1981). ESC are able to indefinite self-renew and proliferate. They maintain their pluripotent state, which means they are capable of differentiating into derivatives of all three embryonic germ layers including germ cells. When injected into a blastocyst, ESC are able to incorporate into the epiblast and participate in normal embryonic development to produce functional soma and germ cells (Bradley *et al.*, 1984). Another characteristic shared by female ICM cells and ESC are two active X-chromosomes. During cleavage the paternal inherited X-chromosome is silenced, but prior to implantation it is reactivated in pluripotent cells (Heard, 2004). ESC and epiblast cells also express a common set of transcription factors, which maintain pluripotency. The three most important factors are Oct4, Sox2 and Nanog, which form a self-propagating circuit by transcriptionally activating each other (Chambers and Smith, 2004). One important role seems to be suppression of extra-embryonic lineage decisions by repression of the respective lineage specifying genes (Niwa, 2007). Apart from maintaining pluripotency, Oct4 and Sox2 are also responsible for FGF4 (fibroblast growth factor 4) expression which poises ESC for entry into differentiation (Kunath *et al.*, 2007). This pro differentiating role of FGF4 expression can be blocked by constitutive expression of Nanog. Compared to the two other factors Nanog overexpression does not lead to differentiation but is capable of maintaining the pluripotent state even in the absence of signalling molecules required for culture of wild type ESC (Chambers *et al*., 2003).

Derivation of ESC was historically only possible from certain permissive mice strains with varying efficiencies. The recent development of refined, so-called naïve,

culture conditions now allows derivation of ESC not only from recalcitrant mice strains (Ying *et al.*, 2008) but rats as well (Buehr *et al.*, 2008). The similarity of pre-implantation epiblast cells and these, so called naïve pluripotent, ESC has suggested the concept of a developmental ground state (Nichols and Smith, 2009). A reason that naïve ESC can only be derived from rodents may be due to their unusual early development. Rodents, unlike other mammals, do not form an embryonic disc but a structure called egg cylinder after blastocyst formation. Events, which are necessary for the reorganisation of the cells may delay the exit of the pluripotent ground state which facilitates derivation of ESC compared to other species where no ground state ESC have been reported yet (Nichols and Smith, 2009).

### *1.1.2 Maintaining Ground State ESC*

As for all other cell lines the appropriate culture conditions are from utmost importance to derive and culture ESC. Initially, ESC were co-cultured in the presence of foetal bovine serum and embryonic fibroblasts (Evans and Kaufman, 1981; Martin, 1981). The main function of these feeder cells is secretion of the cytokine LIF (leukaemia inhibitory factor) (Smith *et al.*, 1988) and, consequently, LIF can replace the requirement for feeder cells. LIF activates, via binding to the GP130 LIF receptor, the JAK/STAT3 (janus kinase / signal transducer and activator of transcription 3) signalling pathway which hereupon drives self-renewal of ESC (Niwa *et al.*, 1998) by expression of c-Myc (Cartwright *et al.*, 2005) which promotes DNA replication and subsequently facilitates Oct4 binding to its target genes (Niwa, 2007). Furthermore, STAT3 signalling activates Sox2 via Klf4 expression. Nanog expression is also regulated via LIF signalling but in a STAT3 independent way, since LIF also activates the PI(3)K-Akt (phosphatidylinositol 3-kinase / protein kinase B) pathway, which leads to Tbx3 dependent Nanog expression. Together with Sox2 (Masui *et al.*, 2007), Nanog regulates Oct4 expression, completing the core circuitry of pluripotency genes (Niwa *et al.*, 2009) and demonstrating the importance of LIF on maintaining pluripotency. LIF is not only responsible for STAT3 and PI(3)K-Akt mediated self-renewal but also activates the MAPK (mitogen-activated protein kinase) pathway, which blocks Tbx3 and subsequently Nanog expression (Niwa *et al.*, 2009). STAT3 signalling is however not sufficient to support self-renewal on its own, since in the presence of LIF but absence of serum self-renewal is attenuated and ESC enter neural differentiation instead. To block differentiation and support self-renewal in

serum free conditions induction of inhibitor of differentiation genes via bone morphogenic proteins (BMP) is necessary (Ying *et al.*, 2003).

Gene expression profiling of ESC cultured in Serum+LIF revealed heterogeneous expression of pluripotency factors, such as Nanog, as well as of genes responsible for differentiation (Silva and Smith, 2008). This heterogeneity has been suggested to be a requirement for stem cell lineage priming during differentiation, but may also be a consequence of on-going differentiation *in vitro* due to imperfect culture conditions. One reason for heterogeneity is autocrine Fgf4/Erk signalling (Kunath *et al.*, 2007), which acts as a pro-differentiating signal (Burdon *et al.*, 1999). LIF mediated (Niwa *et al.*, 2009) or Fgf4 dependent (Kunath *et al.*, 2007) Erk1/2 phosphorylation seems to be the major repressor of Tbx3 and Nanog expression and therefore induces differentiation. A comparison of Fgf4 null ESC and ESC cultured in the presence of PD184352, a small molecule inhibiting the Erk phosphorylating enzyme MEK, by Kunath *et al.* showed massively decreased phosphorylation of Erk1/2, suggesting that Fgf4 is the main Erk1/2 activator (Lanner *et al.*, 2010). Apart from activating Erk1/2, which leads to lineage priming, Fgf4 additionally induces a negative feedback loop, which facilitates the return to the ground state. This dual function of Fgf4 partially explains the heterogeneity observed under classic culture conditions (Lanner and Rossant, 2010). Studies additionally identified that activation of the canonical WNT signalling pathway, using a glycogen synthase kinase 3 (GSK3) inhibitor - 6-bromoindirubin-3′-oxime (BIO) - (Sato *et al.*, 2003), blocks non neural differentiation and enhances self-renewal of ESC. Activation of the canonical WNT pathway leads to accumulation of β-catenin, which subsequently directly interacts with the transcription factor Tcf3 and interferes with its repressing function, leading to transcription of Tcf3 target genes (Wray *et al.*, 2011). Pereira and colleagues identified the key pluripotency gene *Nanog*, whose overexpression liberates ESC self-renewal from LIF dependence (Chambers *et al.*, 2003), as one of the target genes repressed by Tcf3 (Pereira *et al.*, 2006). Martello and colleagues recently identified another essential gene, the estrogen related receptor beta (*Esrrb*), which is repressed by Tcf3 (Martello *et al.*, 2012). It has been shown that Esrrb together with Oct4 activates transcription of Nanog (van den Berg *et al.*, 2008) and that Esrrb and Nanog are positively regulating Oct4 transcription (Zhang *et al.*, 2008). Lately Festuccia *et al.* have demonstrated that *Esrrb* is one of the main target genes of Nanog (Festuccia *et al.*, 2012). Festuccia *et al.* and Martello *et al.* independently

have shown that overexpression of Esrrb is sufficient to supress differentiation and sustain self-renewal and to substitute for the self-renewal and reprogramming function of Nanog (Festuccia *et al.*, 2012; Martello *et al.*, 2012). These findings put Esrrb in a central role of the pluripotency network and, together with the findings from Pereira and colleagues, clarify the importance of abrogating the Tcf3 function via WNT signalling to maintain pluripotency. Combining and refining these discoveries, especially substitution of PD184352 and BIO with the more specific inhibitors PD0325901 (PD03) and CHIR99021 (Chiron) (Bain *et al.*, 2007) in a defined serum free medium - N2B27 - (Ying *et al.*, 2003) established a culture regime, called 2i, for efficient derivation of ES cells and their maintenance in a highly homogenous naïve ground state in the absence of additional inductive signals (Ying *et al.*, 2008).

## 1.2 Epiblast Stem Cells

Recently pluripotent cells have been derived from the mouse post-implantation epiblast that are called epiblast stem cells (EpiSC) (Brons *et al.*, 2007; Tesar *et al.*, 2007). Compared to ESC, EpiSC derivation and culture requires addition of FGF2 (fibroblast growth factor 2), which activates the MAPK/Erk signalling, and ActivinA which activates Nanog via SMAD2/3 signalling (Greber *et al.*, 2010). EpiSC transferred into ESC culture conditions start to differentiate or die. EpiSC express the core pluripotency factors Oct4, Sox2 and Nanog but differ in expression of other naïve pluripotent transcription factors. For example EpiSC do not express Klf2, Klf4 or Zfp42/Rex1, which is one of the earliest markers down regulated during ESC differentiation, but dispensable for maintaining pluripotency (Masui *et al.*, 2008). However, EpiSC express post-implantation specific markers like FGF5 (fibroblast growth factor 5) or T (brachyury) (Nichols and Smith, 2009). EpiSC, like ESC are able to form teratomas, which are tumours consisting of cell types from all three embryonic germ layers, when grafted into immunodeficient mice and are able to differentiate into various cell lines *in vitro* and *in vivo*. However, EpiSC have not been demonstrated to contribute to adult chimeras when injected into the blastocyst (Tesar *et al.*, 2007; Guo *et al.*, 2009). Consistent with their post-implantation identity, and difference to ESC, female EpiSC feature X-chromosome inactivation. ESC can be differentiated to EpiSC *in vitro* (Guo *et al.*, 2009) but not vice versa without genetic manipulations, consistent with a developmentally more advanced cell type. These observations lead to the suggestion that the ESC state represent a developmental

ground state whereas EpiSC represent a primed pluripotent state (Nichols and Smith, 2009).

Cells derived from human blastocysts (Thomson *et al.*, 1998) share characteristics with mouse EpiSC, both in terms of cell culture requirements and transcription factor expression patterns. These findings suggest that human ESC are more similar to mouse EpiSC than to ground state mouse ESC (Nichols and Smith, 2009). The ultimate test for full pluripotency, formation of chimeras, is obviously not possible with human “ESC”. Reports of genetically non-manipulated rodent EpiSC and “hESC” to differentiate into extra embryonic trophoblasts (Rossant, 2008) raise the question if these cell types really are descendants from the post-implantation embryo or if they rather resemble *in vitro* artefacts (Silva and Smith, 2008).

## 1.3 Exit from Pluripotency

In order to commit for differentiation, ESC have to exit pluripotency irreversibly. Understanding this process has implications for potential cell replacement applications, since cells destined for transplantation need to be devoid of ESC that have the potential to cause teratomas. Inefficient exit from pluripotency and persistence of ESC is frequently observed in differentiations, demanding better control over exit of pluripotency. Pluripotency factors are down regulated during differentiation but it is unclear if this is the only reason, which triggers exit of pluripotency. In fact, knockdown of Oct4 or Nanog in ESC leads to the emergence of extra-embryonic cell identities, suggesting that differentiation into embryonic cell types needs to be coordinated with breakdown of the pluripotency network. The only factor known to be crucial for accurate differentiation is the transcription factor Tcf3 whose knockdown or knockout impairs commitment of ESC by derepression of Nanog (Pereira *et al.*, 2006) and Esrrb (Martello *et al.*, 2012; Festuccia *et al.*, 2012). Understanding the mechanisms, which are responsible for exit from pluripotency, will lead to better insight in early mammalian development and future stem cell based therapies.

## 1.4 Reprogramming

The potency of ESC to produce every cell type in the adult has raised high hopes for disease treatment. To allow for patient-specific cell replacement therapies it has been a long-term aim to generate pluripotent cells from somatic cells. The first

method to produce pluripotent cells from somatic cells was somatic cell nuclear transfer (SCNT) where the nucleus from a somatic cell is injected into an enucleated oocyte. Gurdon and colleagues used this technique to create adult frogs from tadpole intestine cell nuclei transferred into enucleated oocytes (Gurdon *et al.*, 1962). However, the attempt of creating an adult frog with SCNT using adult somatic cell nuclei failed, as only tadpoles could be created (Laskey and Gurdon, 1970). This had raised the question if terminally differentiated cells lose the ability to reactivate all genes required for full development. Not until Wilmut and colleagues showed that SCNT with adult somatic cells leads to full development, by creating Dolly, the first cloned mammal (Wilmut *et al.*, 1997). However, these approached are not applicable to disease therapy, since derivation of human ES cells either directly from blastocysts or through SCNT into oocytes is prohibited by law in most countries. Cell fusion is another technique to reprogram somatic cells, where somatic cells are fused with ES, embryonic germ or embryonic carcinoma cells. Tada and colleagues reported generation of pluripotent thymocyte–ES hybrid cells, displaying methylation patterns of thymocytes and gene expression and X-chromosome activation of ES cells (Tada *et al.*, 2001). Spontaneous cell fusion has also been observed by co-culturing neural cells and ESC. This co-culturing led to pluripotent cells expressing transgenic reporters of both cell types, indicating cell fusion rather than conversion of neural cells back to an ESC-like state (Ying *et al.*, 2002). This method of reprogramming circumvents the judicial problems of SCNT by using commercially available human ES cell lines but yields polyploid cells with abnormal sets of chromosomes. SCNT and cell fusion showed that the oocyte and ES cell cytoplasm contains all signals necessary for re-establishing a developmental ground state from fully differentiated cells. This knowledge, together with the assumption that these reprogramming factors probably overlap with the factors responsible for maintaining pluripotency led to a third technique to produce pluripotent cells from somatic cells: Reprogramming with defined factors (Yamanaka, 2008). Takahashi and colleagues designed a system to identify four genes – c-Myc, Klf4, Oct4 and Sox2 -, which upon viral transduction induce reprogramming of mouse embryonic and adult fibroblasts into pluripotent cells (Takahashi and Yamanaka, 2006). The obtained induced pluripotent stem (iPS) cells show morphology and proliferation rates similar to ESC cells and when transplanted into nude mice are capable of forming teratomas and can also contribute to adult and germ line chimeras, indicative of full pluripotency (Maherali *et*

*al.*, 2007; Okita *et al.*, 2007; Wernig *et al.*, 2007). The initial methods for generation of iPS cells have been significantly improved ever since. For example, c-Myc can be omitted from the reprogramming cocktail (Nakagawa *et al.*, 2008), as it is a known proto-oncogene that led to formation of tumours in 20% of the mice derived from iPS cells. Furthermore, Nanog, although not included in the original reprogramming factor combination, was later shown to enhance reprogramming after cell fusion (Silva *et al.*, 2006) and to be sufficient and required for induced pluripotency (Silva *et al.*, 2009). Apart from Nanog, many other genes, as well as micro RNAs and epigenetic modifiers have been identified as prospective reprogramming factors. Moreover, various differentiated cell types have been reprogrammed successfully. Refinements have also been made concerning the factor delivering method. Takahashi and colleagues used retroviral transduction to insert the reprogramming genes into the cells. This technique is not applicable to potential human applications, as the DNA will stably integrate into the host genome. Other virus-based methods have been tested, but all of them carry the risk of unpredictable DNA replication. A safer method of inserting DNA is the *piggyBac* system, as these transposon-originated plasmids can be completely excised after reprogramming is attained (Woltjen *et al.*, 2009). DNA free reprogramming assays have also been established, like Sendai RNA virus transduction (Fusaki *et al.*, 2009) or direct introduction of proteins (Kim *et al.*, 2009) or RNA (Warren *et al*., 2010) into the cells. Despite all these achievements, reprogramming efficiencies are very low and molecular mechanisms are ill defined. For example, reprogramming is frequently obscured by the appearance of partially reprogrammed cells that fail to acquire naïve pluripotency (Silva and Smith, 2008).

As mentioned above, ESC can be differentiated into EpiSC but not *vice versa*. Therefore, EpiSC also have been subject to reprogramming and because of their early embryonic identity, they convert more easily to naïve pluripotent ES cells as compared to fully differentiated cells. In fact, expression of single factors such as Klf4 or Nanog is sufficient to reprogram EpiSC (Guo *et al.*, 2009; Silva *et al.*, 2009). Additionally, STAT3 signalling is capable to reprogram EpiSC as well. Due to reduced expression of LIF receptors in EpiSC, the LIF signalling pathway strength has to be increased. Therefore, a chimeric LIF receptor, containing the extracellular domain of the G-CSF (granulocyte colony stimulating factor) receptor and the trans- and intracellular domain of the gp130 LIF receptor needs to be expressed in EpiSC (Yang *et al.*, 2010) to achieve sufficient G-CSF-dependent STAT3 activation in order

to allow EpiSC to enter naïve pluripotency. A complete description of this receptor can be found on page 50.

## 1.5 Zfp281

Zfp281 and its orthologs belong to a class of transcription factors conserved in mammals containing an array of four Krueppel type zinc fingers, originally discovered in humans (Lisowsky *et al.*, 1999). Zfp281 was first associated with regulatory functions in mouse ESC pluripotency as it is expressed in pluripotent cells but its expression is reduced during differentiation (Brandenberger *et al.*, 2004; Wei *et al.*, 2005). Due to the highest expression of Zfp281 in the pluripotent cell state its importance in maintaining pluripotency has been investigated and it has been shown that Zfp281 is dispensable for self-renewal and proliferation of ESC, as no differences were observed between normal ESC and Zfp281 knockout ESC (Fidalgo *et al.*, 2011). Despite decreased expression during differentiation, Zfp281 is still expressed in adult tissues, for example brain, heart, kidney and liver (Wang *et al.*, 2008). Zfp281 directly interacts with the pluripotency factor Nanog, both on the chromatin as well as the protein level, and regulates its expression (Wang *et al.*, 2006; Wang *et al.*, 2008; Fidalgo *et al.*, 2011; Fidalgo *et al.*, 2012). It has been shown that Zfp281 knockout abolishes binding of Nanog to its own promoter, which could be a fine tune mechanism to prevent overexpression of Nanog during normal development, as overexpression of Nanog sustains pluripotency in ESC (Cambers *et al.*, 2007). Heterologous expression of Nanog in an ESC population is not affected by Zfp281 knockout, which reinforce its supposed function of fine tuning (Fidalgo *et al.*, 2011). However, the influence of Zfp281 on Nanog as well as on other pluripotency factors is contradictory. One study identified Zfp281 as an activator of Nanog and other pluripotency factors, as their expression is decreased after Zfp281 knockdown (Wang *et al.*, 2008) whereas another study showed a contrary result, with increased Nanog and Oct4 expression after Zfp281 knockout (Fidalgo *et al.*, 2011). Fidalgo and colleagues recently discovered that Zfp281 mediates Nanog auto repression via recruitment of the chromatin-remodelling complex NuRD (nucleosome remodelling and histone deacetylase) and that its repressing function blocks re-expression of Nanog during somatic reprogramming. Furthermore Zfp281 is important to ensure the physical integrity of the core NuRD proteins (Fidalgo *et al.*, 2012*)*. The discrepancy between the published studies is also observable in differentiation assays. Wang and

colleagues claim that knockdown of Zfp281, with shRNA mediated RNAi, prevents the colony forming ability of single cells in Serum+LIF conditions and overexpression of Zfp281 sustains proliferation of ESC even in the absence of LIF (Wang *et al.*, 2008). On the other hand, it was published that Zfp281 knockout leads to delayed differentiation in embryonic body assays, which is accompanied with insufficient down regulation of pluripotency markers Nanog and Oct4 and simultaneous up regulation of differentiation marker for the endoderm lineage, like Cxcr4 or Sox17. Furthermore, Zfp281 knockout enhances self-renewal ability of ESC even in the absence of LIF (Fidalgo *et al.*, 2011). However, both studies show upregulation of the extra embryonic endoderm gene Gata6 upon loss of Zfp281 (Wang *et al.*, 2008; Fidalgo *et al.*, 2011). Gata6 expression is repressed by Nanog (Loh *et al.*, 2006; Kim *et al.*, 2008), suggesting that Gata6 induction in Zfp281 knockout ES cells is an indirect consequence of deregulated Nanog. A study revealed Znf281, the human ortholog to mouse Zfp281, as a direct interaction partner of c-Myc in colorectal cancer cells and embryonic kidneys (Koch *et al.*, 2007), which has been shown to be an important factor in maintaining pluripotency (Cartwright *et al.*, 2005). Another study identified Zfp281 as a target of miRNA-203, which represses stemness in epidermis (Yi *et al.*, 2008), indicating a role in adult stem cell maintenance and skin development (Fidalgo *et al.*, 2011). Summing up these findings it is most likely that Zfp281 plays an important role in early development, based on its binding to core pluripotency factors Nanog and Oct4 and their changed expression after Zfp281 knockdown or knockout. This assumption is supported by the fact that Zfp281 null embryos die around E 7.5 and E8.5 (Fidalgo *et al.*, 2011). The exact role of Zfp281 however is not defined as its effect on core pluripotency factors differs in different studies. It is possible that it plays an activating role on pluripotency factors, indicative for the importance of Zfp281 on maintaining pluripotency, as well as a repressing role to prevent overexpression of these factors, for example the described fine-tuning of Nanog. Additionally, Zfp281 seems to prevent differentiation by repression of lineage specifier genes, such as Gata6, Cxcr4 or Sox17.

## 1.6 Project

### *1.6.1 Background*

The little knowledge about the exit of the ground state pluripotent cell state was the reason for a large-scale loss of function screen to identify genes, required for the

exit from pluripotency (Betschinger J., unpublished data). The screen was performed in *O4GIP* ESC where a transgenic GFP reporter gene and a Puromycin resistance are under the control of the *Oct4* promoter (Ying *et al.*, 2002). Loss of function of each tested gene was achieved using siRNA mediated RNA interference (Fire *et al.*, 1998). RNAi is a cell intrinsic mechanism whose basis is dsRNA. The origin of the dsRNA can be endogenous, regulating gene expression by post-transcriptional-gene-silencing, which can be achieved through mRNA degradation or translational repression (miRNA and esiRNA) – depending on the complementarity of the dsRNA guide strand and target mRNA - or controls the activity of transposons during spermatogenesis and development (piRNA). dsRNA can also be added exogenously (siRNA or shRNA), leading to target mRNA degradation. dsRNA is recognised by an enzyme complex with the appropriate name Dicer, which cuts the double strand RNA in single strands. The guide strand is incorporated into a complex called RISC (RNA induced silencing complex), which then can bind to, and degrade or block translation of mRNA complementary to the guide strand. This mechanism can be induced artificially by transfection of siRNAs targeting the transcript of genes of interest, which then are silenced transiently. Depending upon transcript and siRNA stability as well as dilution through cell proliferation, transcript knockdown persists for only three to four days.

ESC cultured in 2i conditions are transfected with siRNAs targeting each of the tested genes. 24 hours after transfection 2i conditions are withdrawn to induce differentiation. Cells differentiate for 72 hours before 2i conditions are re-established. During this time course, differentiating cells pass a “point of no return” and are not able to respond to 2i conditions any more. After re-adding 2i, only cells that have not exited the EC state, due to siRNA mediated gene knockdown, are able to proliferate. Puromycin is added to select for undifferentiated, therefore still Oct4 positive, cells. To quantify exit from pluripotency, surviving and proliferating cells are quantified with a live/dead stain. The screen revealed genes, which are important for exit from ground state pluripotency as their knockdown impairs differentiation. The strongest hit in this screen was the above-mentioned Tcf3 transcription factor, which is known to impair differentiation strongly (Pereira *et al.*, 2006; Martello *et al.*, 2012). Further hits confirm the accuracy of the screen, as knockdown of genes involved in pathways – GSK3 and MAPK/Erk signalling- normally blocked with 2i, also lead to defects in exit from the ESC state. Apart from these obvious hits, one gene knockdown strongly

impaired differentiation and could therefore be a plausible factor for regulating exit from pluripotency. This gene is the transcription factor Zfp281.

### *1.6.2 Outline*

In the present study, the effect of Zfp281 on exit of pluripotency is investigated in greater detail, as it is one of the genes with the strongest phenotype in the primary siRNA screen. Its influence on differentiation of different mouse ES cells is assessed by transient and stable gene knockdown followed by multiple phenotype characterisation assays, including gene expression profiling and phenotypic quantification. Furthermore, genetic interaction of Zfp281 with known pluripotency enhancing (JAK/STAT) or repressing (WNT pathway and MAPK/Erk) signals is carried out.

A possible role of Zfp281 in reprogramming into naïve pluripotency is also examined. This is performed in EpiSC that are closely related to ESC. To quantify the influence of Zfp281, genetically manipulated EpiSC are used that provide a sensitized reprogramming background. These cell lines either carry a chimeric LIF receptor or ectopically express the reprogramming factors Klf4 or Nanog. Subsequently non-manipulated EpiSC lines are used to test the effect of Zfp281 knockdown on reprogramming. To extend the EpiSC framework the effect of Zfp281 knockdown on somatic reprogramming of NSC using the retroviral reprogramming protocol established by Takahashi and Yamanaka is investigated.

To shed light on how Zfp281 functions on a molecular basis Co-Immunoprecipitation experiments are carried out to identify interaction partners. To facilitate protein complex purifications, a 3xFlag version of Zfp281 is cloned. This is also used in Chromatin Immunoprecipitation experiments to identify chromatin regions bound by Zfp281 that may be functionally relevant targets during exit of pluripotency and reprogramming. Another approach to identify the *in vivo* function of Zfp281 is the identification of genes with a similar phenotype. These may genetically interact with Zfp281 and could therefore be indicative of its function. Genes used for this approach were hits in the primary loss of function screen: Ewsr1/Ews, an already identified direct interaction partner of Zfp281 (Wang *et al.*, 2008), and its gene family member Fus (Law *et al.*, 2006), as well as members of the SWI/SNF complex. The first gene tested is Arid1a since knockout of this gene leads to rapid reduction of predicted Zfp281 target genes (Gao *et al.*, 2008), which is suggestive of a genetic

interaction. Furthermore two other members of the SWI/SNF complex are tested, namely Smarca4 and Smarcb1. The reason to choose those genes are that they interact with Arid1a in the chromatin-remodelling complex and both of them were recovered in the screen, although below the threshold for high confidence hits (Betschinger J., unpublished data). siRNA-mediated knockdown of all these genes is tested in differentiation and reprogramming to identify a Zfp281 pathway candidate.

The study revealed that transient and stable Zfp281 knockdown impairs differentiation of ESC. Furthermore, transient knockdown of Zfp281 enhances EpiSC reprogramming, at least in sensitised genetic backgrounds. Reprogramming of NSC into naïve pluripotent ESC seems to be impaired upon Zfp281 knockdown, but these results will need further investigation. None of the tested putative interaction partners showed striking results, neither in terms of phenotypes during differentiation or reprogramming nor in terms of physical interactions. The characterisation of Zfp281 revealed its strong involvement in differentiation of ESC and an inhibitory role in reprogramming of EpiSC.

# 2 MATERIAL AND METHODS

## 2.1 Cell Culturing

ESC are cultured on 0.1% gelatine/PBS coated plates in N2B27 (Ying and Smith, 2003) medium (StemCells, Inc.) supplemented with a MEK inhibitor [PD0305901 (1 µM)], a GSK3 inhibitor [CHIR99021 (3 µM)], together known as 2i (Ying *et al.*, 2008), and Penicillin/Streptomycin (100U/ml). Cells are detached by aspirating the old medium and adding pre-warmed Accutase (PAA Laboratories) with occasional rocking for about five minutes. This cell suspension is collected by adding N2B27 medium and transferred to a sterile tube. After spinning the cell suspension for 3.5 minutes at 1300 rpm, the supernatant has to be aspirated carefully. The cell pellet is resuspended in new 2i medium and pipetted up and down several times to get a single cell suspension. Depending on the following experiments, cells are counted and seeded in an appropriate number. For passaging, cells are split 1/10 in a new plate every second or third day. If cells are cultured in Serum+LIF conditions, these cells have to be washed with PBS after aspirating the old medium and have to be detached with Trypsin with Chick serum. Serum consists of GMEM (Sigma Aldrich) supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 1mM Pyruvate, 1mM L-Glutamine, $10^{-4}$ M 2-Mercaptoethanol and Penicillin/Streptomycin (100U/ml). LIF (Smith, 1991) is added at a concentration of 10ng/ml. EpiSC are cultured on Fibronectin coated plates in N2B27 Medium supplemented with FGF2 (12 ng/ml), ActivinA (20 ng/ml) and Penicillin/Streptomycin (100U/ml). Collecting EpiSC is performed with Accutase as described above. Culturing NSC is performed on 0.1% gelatine/PBS coated plates in RHB-A Medium (StemCells, Inc.) supplemented with FGF2 (10ng/ml), EGF (0.1ng/ml) (Roche) and Penicillin/Streptomycin (100U/ml). Collecting is performed as described for EpiSC. *Plat*E cells are cultured on non-coated plates with DMEM (PAA Laboratories) supplemented with 10% foetal bovine serum, 1mM L-Glutamine and Penicillin/Streptomycin (100U/ml) and collected as described above.

## 2.2 Cell Lines

*Rex1-d2EGFP* ESC (provided by Kalkan T.) are male ES cells on a *129* mouse strain background with a destabilized GFP (Li *et al.*, 1998), which means that the GFP protein – used as reporter – will be degraded shortly after the gene expression is turned off, and a Blasticidin resistance for selection, knock-in at the *Rex1* locus.

*Oct4GIP* ESC are also on a *129* mouse strain background and carry a transgene with enhanced GFP and a Puromycin resistance (*eGFP-IRES-Puro*) under the control of the *Oct4* promoter (Ying *et al.*, 2002). OE EpiSC lines, these are *OEC-2*, *OEC-4* and *O4G-7*, are derived from embryos carrying the *Oct4GIP* transgene (Guo *et al.*, 2009) and have therefore also the GFP reporter and the Puromycin resistance. T*NGA* EpiSC are derived from *TNGA* ES cells, which are on a mixed *129/ C57BL/6* background and carry an *eGFP* knock-in at the *Nanog* gene (Chambers *et al.*, 2007). *NSα-O4G* NSC (provided by van Oosten A.L.) are also derived from an embryo with the *Oct4GIP* transgene and have therefore the same properties as mentioned for *Oct4GIP* ESC above. *PlatE* cells (provided by van Oosten A.L.) are a packaging cell line for producing viral structure proteins, whereat only the protein coding sequence of viral genes is used to avoid the possible production of a replication competent retrovirus. To maintain high expression of viral proteins, a Blasticidin and a Puromycin resistance are inserted with an anterior *IRES* sequence for selection of virus protein producing cells (Morita *et al.*, 2000). This cell line is used to generate retroviral particles containing the gene of interest with which another cell line can be transfected.

## 2.3 Plasmids and siRNAs

Lentiviral *pLKO.1* plasmids containing Zfp281 targeting shRNAs and a Puromycin resistance for selection (Open Biosystems, see table 1A) are used for creating stable Zfp281 knockdown embryonic stem cell lines. These plasmids contain a shRNA cassette consisting of 21 bps of the Zfp281 mRNA sequence - sense and antisense strand - and a sequence (5'*CTCGAG*3') which forms the hairpin loop. To produce this hairpin single strand DNA is required and therefore an *f1* origin of replication, which triggers single strand DNA replication, is implied on the plasmid. The human *u6* promoter is used for expression of shRNA. Furthermore the plasmid contains a self-inactivating 3' long terminal repeat (SIN 3'LTR) which lacks parts of the enhancer and promoter regions of the provirus and therefore blocks its transcription (Figure 1A).

Stable transfection with transgenes is performed with *pPB-CAG-DEST-pgk-hph* plasmids (provided by Betschinger J., Figure 1B). These plasmids use the *piggyBac* transposition system. They consist of a transgene of interest, a Hygromycin resistance for selection and corresponding promoters flanked by inverted terminal repeats (ITR) of the *piggyBac* element, which belongs to class II transposable

elements. These ITR are recognised by *CMV* (*cytomegalovirus* promoter)-*pBase* transposase (provided by Betschinger J.), located on a helper plasmid with which the target cells are cotransfected, which triggers transposition via a cut and paste mechanism at specific *TTAA* sites in the host DNA (modified from http://piggybac.bio.nd.edu/, 12.May 2012). To create a shRNA immune version of Zfp281 its coding sequence is amplified using mutagenic primers (Sigma-Aldrich, Table 2) to insert point mutations at the shRNA recognition sequence and cloned into a *pcR8/GW/TOPO* vector (Invitrogen). These constructs are sequenced using *M13* forward and reverse primers (Sigma-Aldrich, see table 2) for verification and recombined into the *pPB-CAG-DEST-pgk-hph* vector or for biochemical experiments into a *pPB-CAG-3xFlag-DEST-pgk-hph vector* using LR clonase II (Invitrogen).

Retroviral particles for stable NSC knockdown cell lines and reprogramming of NSC are established by using the *pMXs* retroviral vector containing the *pLKO.1* shRNA cassette, *pMXs-Oct3/4*, *pMXs-Klf4*, *pMXs-c-Myc, pMXs-Sox2* (Addgene; Takahashi K. *et al.*, 2006) and *pMXc-GFP* (provided by Takashima Y.), as control for transfection efficiency, for transfection of *PlatE* packaging cells (table 1B). To create *pMXs*-Zfp281 knockdown constructs the *pLKO.1* shRNA cassette including u6 promoter and Puromycin resistance is amplified using primers listed in table 2 and KOD Hot Start DNA Polymerase (Novagen). These primers insert a *BglII* and a *NotI* endonuclease recognition site. After excision of the shRNA cassette using the restriction enzymes mentioned above, it is cloned into a *pMXs-Oct3/4* vector, using T4 Ligase (Invitrogen), which was cut with *BamHI* and *NotI* endonucleases to excise the *Oct3/4* sequence and replace it with the *shRNA* cassette. Plasmids are sequenced for verification using sequencing primers listed in table 2. Used primers are from Sigma-Aldrich.

siRNAs (Qiagen GeneSolution; Dharmacon) used for transient knockdown through RNAi are listed in Table 3.

| **A** | **shRNA target** | **Catalogue number** | **Supplier** |
|---|---|---|---|
| | Zfp281 #2 | RMM3981-99015280 | |
| | Zfp281 #4 | RMM3981-99015292 | Open Biosystems |
| | Empty vector | RHS4080 | |

| B | pMXs Plasmid | Plasmid ID | Supplier |
|---|---|---|---|
| | Oct3/4 | 13366 | Addgene |
| | Sox2 | 13367 | |
| | Klf4 | 13370 | |
| | c-Myc | 13375 | |

**Table 1. Plasmids used for stable transfected cell lines.**
**(A) *pLKO.1* plasmids for stable Zfp281 knockdown cell lines (https://www.openbiosystems.com/Query/?i=0&q=ZFP281). (B) *pMXs* plasmids used for *PlatE* packaging cell line transfection to create retroviral particles for NSC transfection (http://www.addgene.org/browse/article/1043/).**

| Primer | Sequence (5'→ 3') |
|---|---|
| Zfp281 Rescue forward* | caatataaacatgcaATCAtaCAGTgtTgaGatgcctactgtgtct |
| Zfp281 Rescue reverse* | agacacagtaggcatCtcAacACTGtaTGATtgcatgtttatattg |
| Rescue sequencing (M13) forward | gtaaaacgacggccagt |
| Rescue sequencing (M13) reverse | aacagctatgaccatg |
| shRNA cassette forward** | cggAGATCTgagggcctatttcccatgatt |
| shRNA cassette reverse*** | caagCGGCCGctcaggcaccgggcttgcgggt |
| shRNA cassette sequencing forward | tggactatcatatgcttaccgtaac |
| shRNA cassette sequencing reverse | aaacccagggctgccttggaaaag |

**Table 2. Primers used for cloning of plasmids (Sigma Aldrich)**
***capital letters indicate exchanged bases for site directed mutagenesis; **AGATCT *BglII* recognition site; *** *CGGCCG NotI* recognition site**

| siRNA target | Catalogue number | Source |
|---|---|---|
| Zfp281 #1 - #4 | SI01477959, SI01477966, SI01477973, SI01477980 | Qiagen GeneSolution |
| Ewsr1 #1 - #4 | SI00997115, SI00997122, SI00997129, SI00997136 | |
| Fus #1 - #4 | SI01006803, SI01006810, SI01006817, SI01006824 | |
| Arid1a #1, #5-#7 | SI00230404, SI02676058, SI02696771, SI02745379 | |
| Smarc4a #1, #2, #5, #6 | SI00200690, SI00200697, SI02671424, SI02691626 | |
| Smarcb1 #1, #3 - #5 | SI01426411, SI01426425, SI01426432, SI02738792 | |
| GFP | 1022064 | |
| Negative control | 1027281 | |
| TCF3 | 21415 | Dharmacon |

**Table 3. siRNAs used for transient gene knockdown**

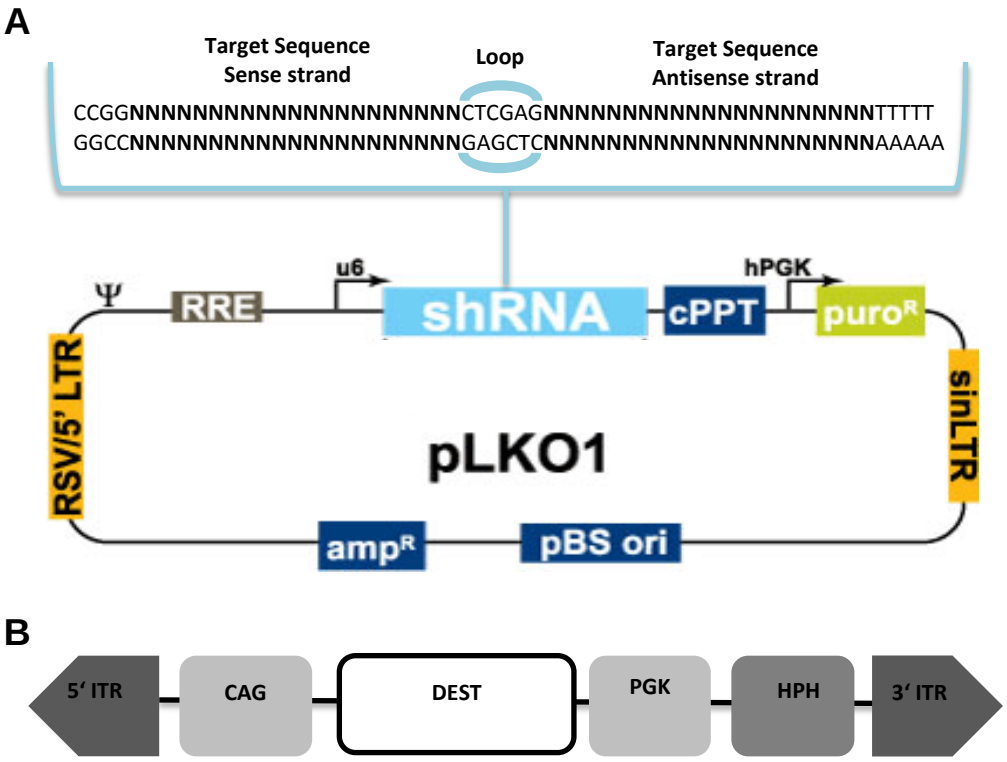


**Figure 1. Plasmids used for stable transfections.**
**(A) *pLKO.1* plasmid used for stable Zfp281 knockdown cell lines ( modified from https://www.openbiosystems.com/RNAi/shRNALibraries/TRCLibraryDetails/); u6, human u6 promoter for stable shRNA expression; cppt, central polypurine tract, primer for +strand DNA synthesis during reverse transcription; hPGK, human *phospho glycerate kinase* promoter; puro$^R$, Puromycin resistance for mammalian selection; sin/3'LTR, self-inactivating long terminal repeat with deletions in enhancer and promoter regions for a transcriptional inactive provirus; pBS ori, origin of replication from *pBluescript* phagemid, contains two individual oris, f1 ori for ssDNA replication and pUC ori for replication in bacteria; amp$^R$, Ampicillin resistance for selection in bacteria; RSV/5'LTR, 5' long terminal repeat; ψ, RNA packaging signal; RRE, Rev responsive element, export signal for mRNA from nucleus to cytoplasm for processing. (B) Schematic illustration of the *pPB-CAG-DEST-pgk-hph* plasmid. ITR, inverted terminal repeat of the *piggyBac* element which are identified by *pBase* transposase; CAG, promoter consisting of *Cytomegalovirus* early enhancer and *β-actin* promoter, high expression levels and no silencing through cell intrinsic mechanisms; DEST, placeholder for transgene; PGK, *phospho glycerate kinase* promoter; HPH, *Hygromycin B phosphotransferase*, Hygromycin B resistance.**

## 2.4 siRNA Transfection

Important for all transfections, transient or stable, is the absence of any antibiotics. Transient siRNA transfections are performed using cationic lipid transfection. For EpiSC Dharmafect1 transfection reagent (Thermo Scientific) is used whereas Lipofectamine RNAiMAX transfection reagent (Invitrogen) is used for ESC. The transfection reagent is diluted in Opti-MEM medium (Invitrogen) and incubated for five minutes at room temperature. siRNA is added and incubated for another twenty minutes. The transfection mix is put in the plates then the cells, at a concentration of $5x10^4$/ml, are added. Cells are plated in their appropriate medium

(see 2.1 Cell Culturing page 15) but 1.5 times as concentrated as for culturing to compensate the extra amount of medium used for the transfection mix. Reaction volumes are listed in table 4.

| | 96 well plate | 24 well plate | 12 well plate |
|---|---|---|---|
| Opti-MEM | 50µl | 300µl | 750µl |
| Transfection reagent | 0,25µl | 1,5µl | 3,75µl |
| siRNA [20µM] | 0,125µl | 0,750µl | 1,875µl |
| Cell number/well | $5x10^3$ | $1,5x10^4$ | $7,5x10^4$ |

**Table 4. Reagent volumes for transient siRNA transfection**

## 2.5 Stable Plasmid Transfection of ESC and EpiSC

Stable transfection uses cationic lipid transfection with Lipfectamine2000 (Invitrogen) as transfection reagent. The transfection reagent is diluted in Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories). 1µg of plasmid DNA is diluted in DMEM in a separate reaction. Both reactions are incubated for five minutes at room temperature then mixed and drop wise added to the plated cells, at a concentration of $10^5$ cells/ml, with occasional rocking. Cells are cultured in normal concentration of their appropriate medium. Table 5 lists reagent volumes.

| | 6 well plate | 12 well plate | 24 well plate |
|---|---|---|---|
| DMEM + Lipofectamine 2000 | 250µl + 3µl | 125µl + 1,5µl | 65µl + 0,75µl |
| DMEM + plasmid DNA | 250µl + 1µg DNA | 125µl + 1µg DNA | 65µl + 1µg DNA |
| Cell number/well | $10^5$ | $5x10^4$ | $10^4$ |

**Table 5. Reagent volumes for stable plasmid transfections of ESC and EpiSC**

Transfection with *pPB-CAG-DEST-pgk-hph* plasmids needs co-transfection with the *piggyBac* transposase *CMV-pBase*, located on a helper plasmid, to enable a cut and paste transposition of the transgene of interest into the host genome. This helper plasmid is additionally added to the DMEM + plasmid DNA reaction at an amount of 1µg. To select for stable transfected cells 24 hours after transfection Hygromycin (150µg/ml) is added to the cells.

To create stable shRNA Zfp281 knockdown cell lines the *pLKO.1* plasmid is linearized - using *ScaI* - prior to transfection to avoid a random linearization after

transfection, which could happen in the shRNA cassette and therefore destroy it. Selection for cells stable transfected with *pLKO.1* is performed with Puromycin at a concentration of 0.5µg/ml after 24 hours.

## 2.6 Transfection of NSC

To obtain retroviral particles for transduction of NSC, *PlatE* cells are individually transduced with one of the *pMXs* vectors (see 2.3 Plasmids and siRNAs page 16 *et seq.*) using FuGENE6 transfection reagent (Roche). For transfection of $2x10^6$ cells in a 10cm dish 27µl transfection reagent are mixed with 570µl DMEM and incubated at room temperature for five minutes. 9µg plasmid DNA are added and at least incubated for 15 minutes at room temperature. Transfection mix is added drop wise to the cells, which are seeded just before transfection to enhance transfection efficiency. After 24 hours, the plasmid-containing medium is replaced with standard *PlatE* cell medium. 48 hours after transfection the medium, which contains the *PlatE* retroviral particles is collected and new *PlatE* medium is added to the cells, which is then collected 72h after transfection as well. Supernatants are filtered through a 0.22µm filter to get rid of any cell debris and enriched with 4µg/ml Polybrene (Sigma Aldrich), which increases retroviral infection efficiency by reducing the charge repulsion between retroviral particles and cell wall. Depending on the volitional combination of the different retroviral particles, the supernatants are combined to get a total volume of 2ml per 6 well. $1.2x10^6$ NSCs are added drop wise to the retroviral supernatants. 24 hours after transfection the medium is switched to NS medium (modified from Takahashi K. *et al*., 2007).

## 2.7 Flow Cytometry

CyAn ADP analyser (Dako) is used for flow cytometry. Dead cells are counterstained with TO-PRO-3 iodide. For analysis Flow Jo software (Tree Star) is used.

## 2.8 Alkaline Phosphatase Staining

Alkaline phosphatase (AP) staining is specific for undifferentiated pluripotent ESC. For detection of AP positive cells Leukocyte Alkaline Phosphatase kit (Sigma-Aldrich) is used. Cells are washed with PBS and fixed for about 45 seconds with a solution consisting of 65% acetone, 10% formaldehyde (37% in water) and 25% 25mM

citrate. After a washing step with water, the substrate for AP activity, consisting of 3 components (FRV Alkaline Solution, Naphtol AS-BI Alkaline Solution and Sodium Nitrite Solution in a composition of 1:1:1 in 15-times water) is added to the cells and incubated for at least 15 minutes, at room temperature in darkness, until red stained colonies are visible. Cells are washed with water one more time, dried and quantified under the microscope.

## 2.9 qRT-PCR/qPCR

For qRT-PCR from cells, they are solubilised using QiaShredder (Qiagen) followed by total RNA extraction using RNeasy Mini kit (Qiagen) with *DNaseI* digest. SuperScript III First-Strand Synthesis system (Invitrogen) is used for first strand cDNA synthesis. qPCR is performed using TaqMan Gene Expression Assays (Applied Biosystems) including TaqMan probes (Table 6A) and TaqMan FastUniv PCR Mastermix 2x (both Applied Biosystems) using the StepOne Real-Time PCR System (Applied Biosystems). Gene expression is determined relative to GAPDH expression using comparative $C_T$. For qPCR following Chromatin-Immunoprecipitation (see page 26 *et. seq*) Fast SYBR Green protocol (Applied Biosystems) is used. For this protocol, no cDNA synthesis is needed. Template DNA, Fast SYBR Green Master Mix (Applied Biosystems) and, for every gene region individually designed forward and reverse primers (Sigma-Aldrich), are mixed. qPCR is performed on a StepOne Real-Time PCR System with the SYBR Green program. This does not involve a relative expression compared to a control but absolute reads. Used primers are listed in Table 6B.

## 2.10 Co-Immunoprecipitation

Cells are collected and once washed in PBS. The cell pellet is resuspended in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP40, 1mM EDTA, 10% glycerol and 5mM MgCl) containing PhosSTOP phosphatase inhibitor (Roche), cOmplete Mini EDTA Free protease inhibitor (Roche) and 1mM DTT. After incubating for thirty minutes on ice the cell suspension is pressed ten times through a - as small as possible - needle to break the cell walls through shearing force.

| A | Target | | Dye | Catalogue number |
|---|---|---|---|---|
| | Zfp281 | | FAM | Mm01296016_s1 |
| | Klf2 | | FAM | Mm01244979_g1 |
| | Klf4 | | FAM | Mm00516104_m1 |
| | Nanog | | FAM | Mm02019550_s1 |
| | Oct4 | | FAM | Mm00658129_gH |
| | GAPDH | | VIC | 4352339E |
| **B** | **Gene** | | **Primer** | **Sequence** |
| | Rex1 | Region 1 | fwd | ctggaatgcccaatgagaag |
| | | | bwd | ggcatttgcataactgagca |
| | | Region 2 | fwd | acaaaagatgcttgggtgaaa |
| | | | bwd | gcctgctcaaagccacac |
| | Klf2 | Region 1 | fwd | gccacgtgaggtagtcacc |
| | | | bwd | acccatgaaggagggactg |
| | | Region 2 | fwd | ggatgtgcacctgatgaaaa |
| | | | bwd | gagtctagcagtgccaagca |
| | Klf4 | Region 1 | fwd | aagaggcctgggaatcaagt |
| | | | bwd | cgggatttctcattttgactct |
| | | Region 2 | fwd | cctgaaacccaaacaactcc |
| | | | bwd | tggtctgtgtgaaatgaaagga |
| | | Region 3 | fwd | tggaatcactgcaaacactttt |
| | | | bwd | cttccaggtttggctctcttt |
| | Oct4 | Region 1 | fwd | ggctgcaggcatacttgaac |
| | | | bwd | ccagggccagcctctaag |
| | | Region 2 | fwd | gccccttttgaacctgaagtc |
| | | | bwd | cctagttcctgggtggagaa |
| | Nanog | Region 1 | fwd | ccaaccaggtctaaggcatc |
| | | | bwd | ttatccagggaagcggttt |
| | | Region 2 | fwd | gggtagggtaggaggcttga |
| | | | bwd | cggctcaaggcgatagatt |

| B | Gene | (continuation) | Primer | Sequence |
|---|---|---|---|---|
| | Tbx3 | Region 1 | fwd | caccattatcatcgtcccatt |
| | | | bwd | gactgcaccactcccagact |
| | | Region 2 | fwd | gacctcctggcgctaattg |
| | | | bwd | ctgactgcagggtcacga |
| | | Region 3 | fwd | tgttcccactcccacttctc |
| | | | bwd | aaaacaaacaggggctgtca |

**Table6. Probes and Primers used for qPCR.**
**(A)TaqMan probes (Applied Biosystems) for comparative CT; (B) Primers (Sigma-Aldrich) used for SYBR Green qPCR.**

In a precooled (4°C) centrifuge, the cells are spun for thirty minutes at 14000rpm. The supernatant is taken off and the cell pellet is discarded. Protein G Sepharose 4 Fast Flow beads (GE Healthcare LifeSciences) are washed three times, five minutes each on a rotating wheel in the cold room, with lysis buffer. Between the washing steps, the beads are spun thirty seconds at 1000rpm. Beads, supernatant and antibody, concentration according to the manufacturer, are combined and incubated overnight on a rotating wheel in the coldroom. Used antibodies are listed in Table 7. Beads are washed six times carefully, as described above. The following step is optional and depending on the used antibody. If a 3xFlag antibody against a protein with 3xFlag tag is used, protein/antibody complexes are eluated from the beads. For this step, 3xFlag peptide (Sigma Aldrich) in lysis buffer (0.2mg/ml) is added to the washed beads and put on the rotating wheel in the coldroom for thirty minutes. Afterwards the beads are spun and the supernatant is taken off carefully. Beads are eluted a second time, both supernatants are combined and spun again to get rid of any beads which are possibly taken off together with the supernatant. Laemmli buffer (Laemmli U. K., 1970) is added to the samples and heated at 94°C for five minutes. After cooling down to room temperature samples are spun for one minute at 14000rpm and loaded on a 10% polyacrylamide gel (homemade or GE Healthcare LifeSciences) (Schaegger H. and von Jagow G, 1987). Gels are run with 120V for ninety minutes using a running buffer consisting of 0.1% SDS, 25mM Tris Base and 192mM Glycin in water.

## 2.11 Western Blot

Proteins from Co-Immunoprecipitation separated on a gel are transferred on a nitrocellulose membrane by electroblotting at 100V for sixty minutes. The used

transfer buffer is similar to the running buffer but without SDS. To test if the transfer was successful the membrane is stained with Ponceau Red (0.1% Ponceau S and 30% acetic acid in water). The membrane is washed with water to get rid of the Ponceau staining and blocked with 5% milk in TBST (200mM Tris-HCl pH 7.6, 1.5M NaCl, 1% Tween 20) for thirty minutes at room temperature on a shaker to prevent unspecific bindings of antibodies later used for detection. Afterwards it is incubated with an antibody against the protein of interest diluted in 5% milk in TBST, concentration according to manufacturer, overnight at the coldroom or for at least three hours at room temperature, depending on the quality of the used antibody, on a shaker. Afterwards the membrane is washed three times for 15 minutes with TBST and incubated with horseradish peroxidase conjugated anti-mouse IgG (GE Healthcare LifeSciences), anti-rat IgG or anti-rabbit IgG (Cell signalling) – depending on the host organism of the primary antibody – antibody, common used dilution is 1:5000, for one hour at room temperature. The membrane is washed again three times with TBST, five minutes each and developed with ECL Western Blotting Detection Reagents (GE Healthcare LifeSciences). Used antibodies are listed in Table 7.

| Target | Antibody | Host | Catalogue number | Source |
|---|---|---|---|---|
| 3xFlag-Zfp281 | Monoclonal ANTI-FLAG M2 | Mouse | F1804 | Sigma-Aldrich |
| Nanog | Monoclonal Anti-mouse Nanog | Rat | 14-5761 | eBioscience |
| Ewsr1 | C-19 Polyclonal Anti-EWS | Goat | Sc-6532 | Santa Cruz |

**Table 7. Primary antibodies used for Co-Immunoprecipitations and western blots.**

## 2.12 Silver Stain

Silver staining is carried out corresponding to Blum *et al.*, 1987. For a silver stain, it is already important to be very exact when running the gel und preparing the running buffer. Only eluted samples from the Co-Immunoprecipitation can be loaded. It is important to use high quality chemicals, just deionized water, to prepare all solutions fresh, to use a really clean chamber for development and carry out all steps on a shaker. Proteins are fixed on the gel by incubating in a solution consisting of 40% ethanol and 10% acetic acid in water for at least one hour. The gel is then washed twice, 20 minutes each, in 30% ethanol in water and once, for at least 20 minutes or even overnight, in water. To increase the sensitivity of the staining, the gel

is incubated for one minute in 0.02% sodium thiosulfate in water and washed three times, twenty seconds each, in water. Afterwards it is incubated for twenty minutes in the cold room in a precooled 0.1% silver nitrate in water solution followed by three water wash steps, each twenty seconds. For developing a solution consisting of 3% sodium carbonate and 0.05% formaldehyde (37%) in water is added for three to five minutes and washed once in water for twenty seconds before adding the stop solution (5% acetic acid in water).

## 2.13 Immunofluorescence

Cells are washed in PBS and fixed with 4% PFA for twenty minutes. To prevent unspecific bindings a block solution [3% donkey serum, 1% BSA in PBST (0.1% Triton X100 in PBS)] is added for thirty minutes. The Primary antibody, diluted 1:1000 in PBST, is added for overnight incubation on the shaker in the coldroom. After incubation cells are washed twice, twenty minutes each, in PBST. Fluorophor labelled secondary antibodies (1:1000 in PBST) are added and incubated for thirty minutes in darkness. If different antigens want to be analysed it is important to use appropriate primary antibodies, from different host species, and appropriate secondary antibodies with different fluorophores. Cells are washed again with PBST and incubated for one minute with DAPI (Wilson W.D. *et al.*, 1990) 1:10000 in PBST. After one final wash in PBST, stained cells can be observed under the fluorescence microscope. Antibodies used for Immunofluorescence are listed in Table 8.

| Target | Primary Antibody | Secondary Antibody |
|---|---|---|
| Oct4 | Oct3/4 (C10) mouse monoclonal $IgG_b$ (Santa Cruz, sc-5279) | Alexa Fluor 555 Donkey anti-mouse IgG (H+L) (Invitrogen, A31570) |
| 3xFlag-Zfp281 | ANTI-FLAG M2 mouse monoclonal IgG (Sigma-Aldrich, F1804) | |

**Table 8. Primary and secondary antibodies used for Immunofluorescence experiments.**

## 2.14 Chromatin Immunoprecipitation

The protocol for Chromatin Immunoprecipitation (ChIP) is according to Brinkman A. B. *et al.*, 2006 with minor modifications from Kalkan T. Cells are resuspended in N2B27 to a concentration of $4x10^6$ cells/ml. 100 ml fixing solution (0.1 M NaCl, 1mM EDTA, 0.5mM EGTA and 50mM HEPES pH 7.5) containing 11% formaldehyde is added to the cell suspension and mixed for ten minutes at room temperature on a rotating wheel. 157µl 1M glycine are added and mixed five minutes at room

temperature. Cell suspension is spun down for five minutes at 1600g in a precooled (four degrees) centrifuge and washed three times with ice cold PBS. The cell pellet is resuspended in 1 ml buffer LB1 (50mM HEPES pH 7.5, 140mM NaCl, 1mM EDTA, 10% Glycerol, 0.5% NP40 and 0.25% Triton X100) with added protease and phosphatase inhibitors (see 2.10 Co-IP, page 22 *et seq.*) and mixed on the wheel for ten minutes at four degrees. After spinning down as mentioned above buffer LB2 (10mM Tris pH 8.0, 200mM NaCl, 1mM EDTA and 0.5mM EGTA) with added protease and phosphatase inhibitors is used for resuspending the cell pellet and incubated for ten minutes on the wheel at four degrees and spun down. 140µl shearing buffer (1% SDS, 10mM EDTA and 50mM Tris pH 8.0) is added at room temperature (SDS would precipitate at four degrees) and sonication, using Bioruptor (Diagenode), is performed. Four cycles, six minutes each with alternating thirty seconds on – thirty seconds off phases, with high settings are used. After every six minutes cycle, fresh ice-cold water has to be added. To test for effective shearing a 14µl aliquot is taken and elution buffer (1% SDS and 0.1M $NaHCO_3$) with 200mM NaCl is added to a final volume of 200µl. The solution is put in a shaker overnight at 65°C at 800rpm, afterwards purified using QIAquick PCR Purification Kit (Qiagen) and analysed on a gel. Sonicated cell suspension is centrifuged for ten minutes with maximal speed at ten degrees, supernatant is taken of and ten times the volume ChIP dilution buffer (50mM Tris pH 8.0, 167mM NaCl, 1.1% Triton X100 and 0.11% Na-Deoxycholate) is added. For the ChIP itself IgG beads for preclearing are prepared by adding 2µg IgGs in PBS to 20µl beads, incubating them overnight and washing them three times, five minutes on the wheel each, with wash buffer 1 (50mM Tris pH 8.8, 0.1% SDS, 0.1% Na-Deoxycholate, 1% Triton X100, 150mM NaCl, 1mM EDTA, 0.5mM EGTA). Beads used for IP are incubated overnight with 1mg/ml BSA (ChIP grade quality) in PBS and also washed three times with wash buffer 1. IgG coated beads are incubated with the supernatant for two hours at four degrees on rotating wheel. The precleared supernatant is preincubated with 2µg antibody [ANTI-FLAG M2 mouse monoclonal (Sigma-Aldrich, F1804); Oct3/4 (N19) goat polyclonal (Santa Cruz, sc-5279)] or IgGs overnight at four degrees. To this preincubated supernatant 20µl BSA coated beads are added for one hour at four degrees. Beads are washed twice with wash buffer 1, once with wash buffer 2 (50mM Tris pH 8.0, 0.1% SDS, 0.1% Na-Deoxycholate, 1% Triton X100, 500mM NaCl, 1mM EDTA and 0.5mM EGTA), once with wash buffer 3 (50mM Tris pH 8.0, 250mM LiCl, 0.5% Na-

Deoxycholate, 0.5% NP40, 1mM EDTA and 0.5mM EGTA) and twice with wash buffer 4 (50mM Tris pH 8.0, 10mM EDTA and 5mM EGTA). Each washing step is performed with 1ml of the buffer for five minutes at four degrees on the rotating wheel. Antibody-Protein-DNA complexes are eluted the first time with 130µl and the second time with 100µl elution buffer for 15 minutes at room temperature in a shaker a maximum speed. Eluted supernatants are combined and spun down to get rid of any beads. NaCl is added to a concentration of 200mM and put in a shaker overnight at 65°C and 800rpm. Samples are purified as described above and can now be used as templates for qPCR using SYBR Green protocol (see 2.9 qRT-PCR, page 22).

# 3 RESULTS

## 3.1 Knockdown of Zfp281 Impairs Commitment of ESC

### *3.1.1 Discovering the Effect of Zfp281 using Transient Gene Knockdown*

To test the influence of Zfp281 on differentiation, an ES cell line (*O4GIP*) is transfected with Zfp281 siRNA to generate a transient gene knockdown using the cell intrinsic RNAi mechanism (Fire *et. al*, 1998). Cells are released into differentiation and, after 72 hours, assayed for exit from the ES cell state, embarking on the expression of a Puromycin resistance gene under the control of the *Oct4* promoter. Since Oct4 is shut down during differentiation, the antibiotic resistance provides an ideal tool analysing changes in cell identity. Transfection of *O4GIP* cells is performed in 96 well plates ($5x10^3$ cells/well) with a pool of four individual Zfp281 siRNAs in 2i medium. Transfections are performed in triplicates. 24 hours after transfection, the two inhibitors are withdrawn to start commitment. Cells are differentiated for 72 hours before medium is switched back to 2i conditions supplemented with Puromycin (1µg/ml) for additional 48 hours to select for proliferating Oct4-expressing and therefore uncommitted cells. As positive control, cells are transfected with siRNA targeting the transcription factor Tcf3, as the absence of Tcf3 is known to delay differentiation in ESC (Pereira *et al.*, 2006). Cells transfected with negative siRNA, which has no targets in ESC and GFP siRNA, are used as negative controls. To exclude an influence of the transfection reagent on commitment cells are also treated with this reagent alone, hereinafter named as no siRNA. To test the transfection efficiency, cells are transfected with GFP siRNA. Cells, which are maintained in 2i for the whole experiment, are also transfected with these siRNAs to investigate a possible influence of Zfp281 on self-renewal of ES cells. Exit from pluripotency is quantified with a life/dead stain [Alamar Blue (Invitrogen)], which results in metabolic activity dependent fluorescence in living cells that is recorded in a plate reader. Fluorescence values are normalised to cells transfected with negative siRNA. Additionally alkaline phosphatase (AP) expressing ES cells are visualised in an enzymatic assay to validate qualitatively the live/dead stain results.

None of the controls has an influence on commitment, as all of them show the same fluorescence intensity as the negative siRNA control (Figure 2A) and hardly any uncommitted cells in the AP stain (Figure 2B). Furthermore, siRNA transfections

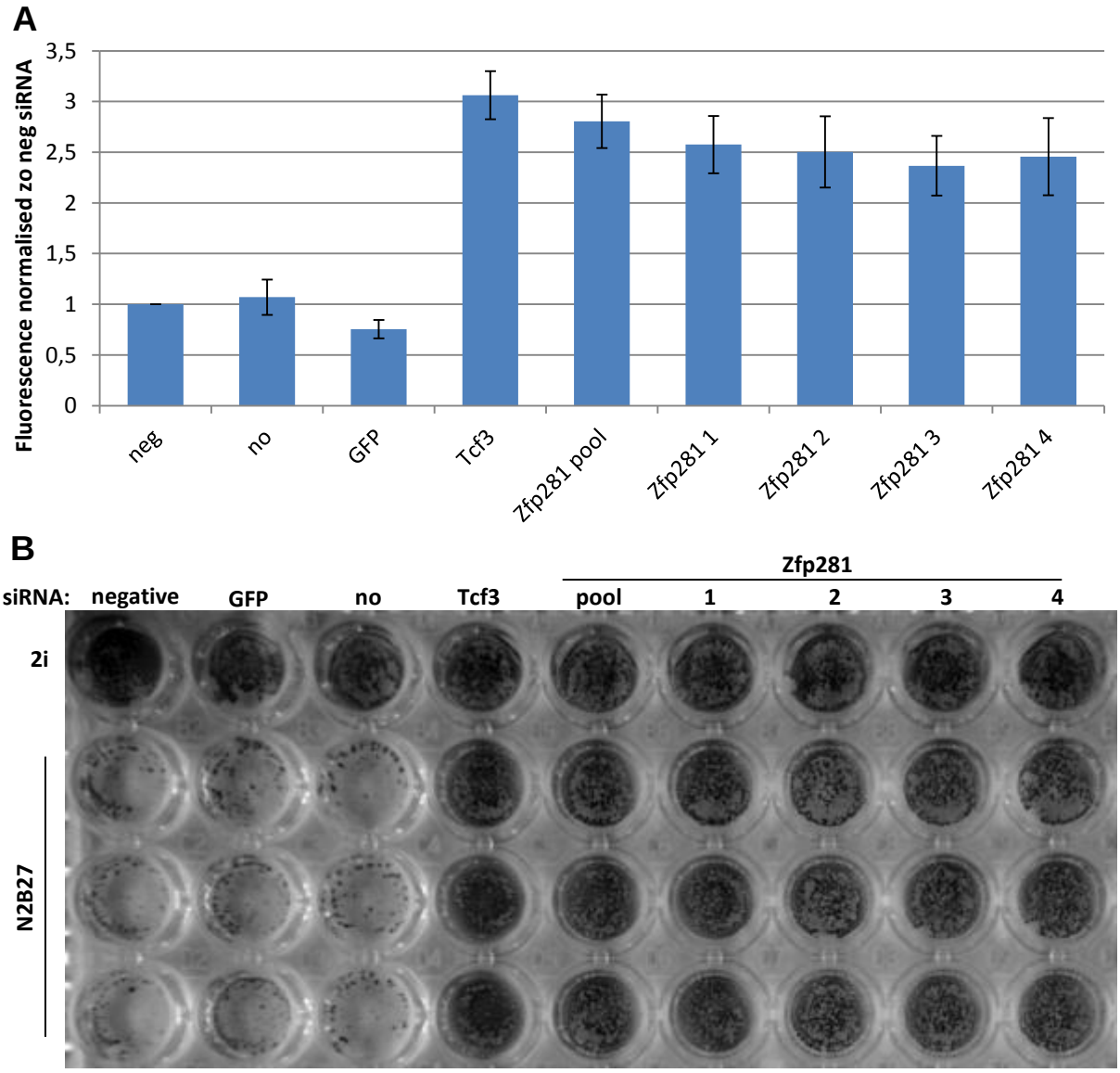


**Figure 2. Zfp281 siRNA gene knockdown mediated delay of commitment in *O4GIP* ESC.**
**(A) Alamar Blue statistics from ESC commitment assay investigating the influence of gene knockdown in commitment by quantification of living and therefore uncommitted cells. Size of bars indicates amount of living cells relative to living cells transfected with negative siRNA. This correlates with the intensity of commitment delay caused by gene knockdown. Experiment is done in triplicates. Results are the mean of three replicates, expressed as mean ± S.E. (B) Photograph of AP stained plate from commitment assay. Intensity of dark spots indicates amount of uncommitted cells. First row shows cells maintained in 2i throughout the experiment as positive control for uncommitted cells. Second till fourth row show experiment done in three replicates. Three individual experiments were carried out with similar results.**

do not affect the proliferation or cell identity of ESC, visible in the control cells maintained in 2i (Figure 2B). In contrast cells transfected with Tcf3 siRNA as positive control show a three times higher fluorescence caused by a higher number of uncommitted and therefore Oct4 expressing and antibiotic resistance expressing ESC (Figure 2A). Zfp281 siRNA transfected cells show a similar result as cells transfected with Tcf3 siRNA (Figure 2A) indicating a similar influence on exit from the ES cell state during differentiation. AP staining affirms these results showing lots of

stained, uncommitted cells compared to controls (Figure 2B). To rule out an off target effect due to target-unspecificity of a particular siRNA sequences, independent Zfp281 siRNAs are tested. This leads to identical results for all four siRNAs (Figure 2A and 2B right side), suggesting that Zfp281 is required for exit from pluripotency.

### *3.1.2 Further Characterisation of Transient Zfp281 Knockdown in ESC*

The phenotype observed in the commitment assay using *O4GIP* ESC is validated in another ES cell line. This transgenic cell line, called *Rex1-d2EGFP*, expresses a destabilised enhanced green fluorescent protein and a Blasticidin resistance under the control of the *Rex1* promoter (Wray *et al.*, 2011). Zfp42/Rex1 is a marker of undifferentiated ES cells and its expression is rapidly reduced after induction of differentiation (Toyooka *et al.*, 2008). The GFP profile of differentiating *Rex1-d2EGFP* ES cells therefore monitors the cell state of individual cells in a heterogeneous cell population. Cells are transfected with siRNA pools as described above using the same controls and subjected to the same differentiation protocol. After 24 hours of differentiation, cells are collected and flow cytometry (Material and Methods page 21) is performed. Cells transfected with negative siRNA show a decrease in peak GFP level (Figure 3A right panel) similar to the profile for the parental, non-transfected, *Rex1-d2EGFP* ESC after 24 hours of differentiation (Figure 3A left panel). The GFP-profile indicates asynchronous differentiation of ES cells where GFP low cells have exited the ESC state while GFP high cells are uncommitted (Kalkan T., unpublished data). Tcf3 siRNA transfection leads to a profile (Figure 3A right panel) nearly identical to cells maintained in 2i (Figure 3A left panel), showing that most cells still maintain *Rex1* promoter activity. Cells with Zfp281 knockdown show a phenotype (Figure 3A right panel) nearly as strong as Tcf3 knockdown, with a little bit more heterogeneity in GFP levels, consistent with Zfp281 being required for exit from the ESC state.

To test the cell state after 72 hours of differentiation, 20% of the cells are replated in 2i conditions on a Laminin coated 24 well plate. After 48 hours, Blasticidin (1ng/ml) is added for at least 48 hours to select for Rex1 expressing, undifferentiated, cells and probed for AP activity. This assay is more stringent than the experiment carried out for *O4GIP* cells, since differentiated cells, which are collected do not attach to the plate under 2i conditions and if nevertheless differentiated cells are able to attach,

the Blasticidin treatment is another step to select for uncommitted cells. The risk of false positive results, for example staining of cell debris, is minimised.

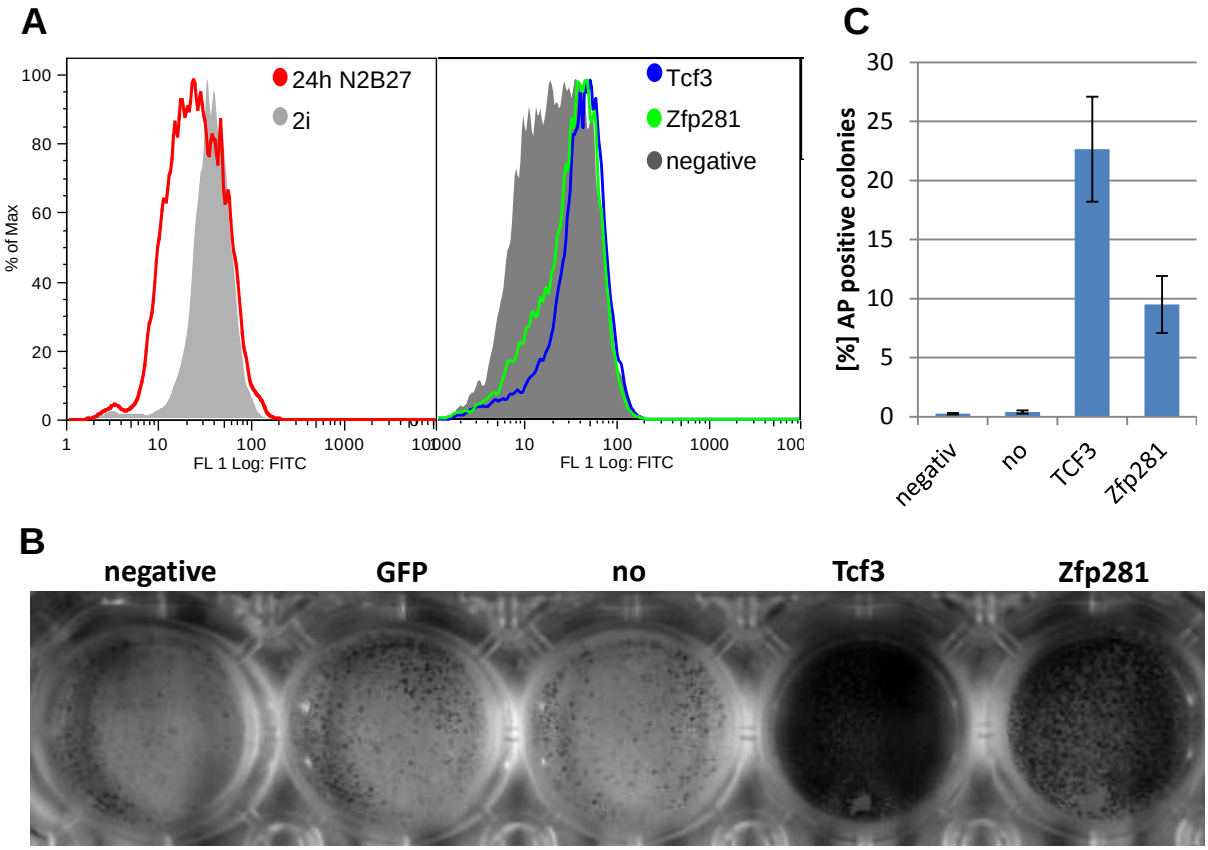


**Figure 3. Impact of Zfp281 knockdown on delayed differentiation in *Rex1-d2EGFP* ESC**
**(A) Flow cytometry histograms showing GFP levels of *Rex1-d2EGFP* ESC; left panel: histogram of parental, untransfected cells cultured in 2i (grey) or after 24 hours of differentiation in N2B27 medium (red); right panel: histogram of siRNA transfected cells after 24 hours of differentiation; grey, negative siRNA; green, Zfp281 siRNA; blue, Tcf3 siRNA. (B) Photograph of AP stained plate from replated cells after 72 hours of differentiation. Black dots represent stained, therefore undifferentiated, colonies. Big white spots in the lower edge of wells with Tcf3 and Zfp281 siRNA transfected cells happened due to too forceful handling of the aspirator. (C) Statistics of clonal density replating experiment. Blue bars indicate the percentage of AP positive colonies formed from $10^3$ seeded cells. Results are the mean of two replicates, expressed as mean ± S.E. Four individual experiments carried out with similar results.**

Similar to the phenotype observed in *O4GIP* ESC, negative controls (negative, no and GFP siRNA) give rise to only few AP positive cells, indicating that most have irreversibly exited the ES cell state. Cells transfected with the positive control Tcf3 and Zfp281 siRNAs give rise to many more AP positive colonies, consistent with defects in differentiation (Figure 3B). To quantitate these phenotypes, single cells are seeded, embarking on the defining property of stem cells to self-renew. From each siRNA transfection, $10^3$ cells are replated on a Laminin coated 12 well plate in 2i conditions and after five days Blasticidin is added. Quantification of resulting AP positive colonies (Figure 3C) revealed that 0.25% of negative siRNA, 0.4% for no

siRNA control but 22.5% of Tcf3 siRNA and 9.5% of Zfp281 siRNA transfected cells self-renewed at the single cell level. Since roughly 50% of ES cells show self-renewal activity in the same assay (Wray *et al.*, 2010), this indicates that approximately one fifth of Zfp281 depleted cells fail to exit from pluripotency during differentiation.

### *3.1.3 Investigation of Stable Zfp281 Knockdown Phenotype in ESC*

Due to the limited knockdown perdurance upon siRNA transfection, *Rex1-d2EGFP* ESC are stable transfected with the lentiviral *pLKO.1* vector carrying Zfp281 shRNA constructs. For detailed information about transfection protocol and used vectors, see Material and Methods pages 16 *et seq.* and *20 et seq*. The purpose of this approach is to assess if a stable Zfp281 knockdown in ESC can be created and if it shows a phenotype similar to the transient knockdown performed with siRNAs. An empty *pLKO.1* vector functions as negative control and two different shRNA constructs are used for the knockdown. Single cell clones from the cells transfected with each of the constructs are picked and expanded in 2i supplemented with Puromycin (0.5 µg/ml) to select for stable transfected cells. The Zfp281 knockdown efficiency is determined by a qRT-PCR according to Material and Methods page 22. Despite a large number of tested clones, only a little fraction shows significant reduction in Zfp281 mRNA levels, indicating Zfp281 knockdown. The transfection is carried out twice to obtain a clone with an as high as possible knockdown efficiency. From those two experiments, six clones are chosen for further experiments. These are three clones transfected with the *pLKO.1* #2 construct, hereinafter called Z2, with a knockdown efficiency of 82% (Z2.2), 60% (Z2.31) and 73% (Z2.36), and one clone from *pLKO.1* #4, hereinafter called Z4, with a knockdown efficiency of 54% (Z4.34) compared to average mRNA level measured in two clones transfected with empty *pLKO.1* vector, e.1 and e.2, (Figure 4A).

The phenotype caused by stable knockdown transfections is determined by seeding the two empty clones, e.1 and e.2, and the knockdown clones Z2.2, Z2.36 and Z4.34 on a gelatine coated 24 well plate at a concentration of $3x10^4$ cells/well in 2i conditions. After 24 hours, the 2i inhibitors are withdrawn to start differentiation, in parallel the clones are maintained in 2i. After 24 hours of differentiation, cells are collected and flow cytometry is performed (Figure 4B). All five clones cultured in 2i show a homogeneous GFP high peak, similar to the parental ES cell line in figure 3A on page 32. After 24 hours of differentiation, the control clones e.1 and e.2 show a

decrease in peak GFP level, indicating differentiation, which is similar to the parental cell line in Figure 3A. Knockdown clones Z2.2 and Z2.36 show GFP levels, similar to cells cultured in 2i conditions, indicating a defect in exit from the ESC state. A little decrease in peak GFP level is visible for the clone Z4.34, consistent with its lower knockdown efficiency, suggesting a dose dependent knockdown phenotype.

Furthermore, clones are differentiated for 72 hours and 20% of those differentiated cells, as well as 5% of the cells from clones maintained in 2i, are replated in 2i conditions on a Laminin coated 24well plate. After 48 hours in 2i and another 48 hours of Blasticidin selection AP staining is carried out to determine the amount of uncommitted cells (Figure 4C). The clones maintained in 2i for the whole experiment are used as a control to show that the difference in stained colonies is not because of a different amount of seeded cells. In contrast to similar amounts of cells obtained from control and knockdown clones kept in 2i conditions, empty clones give rise to only a few number of AP positive colonies after 72 hours of differentiation. However, Zfp281 knockdown clones generate numerous AP positive colonies, indicating defects to exit the ES cell state. A clonogenicity approach is carried out for quantification (figure 4D). $10^3$ cells are replated after 72 hours of differentiation on a Laminin coated 12 well plate in 2i conditions, cultured five days in 2i, selected for undifferentiated cells with 48 hours of Blasticidin (1µg/ml) treatment, stained with the AP kit and counted. 0.7% and 0.25% of differentiating control cells give rise to AP positive colonies while self-renewal ability is retained in 8% and 10% of differentiating cells from the Z2.2 and Z2.36 cell lines. This phenotype is consistent with the phenotypes observed after Zfp281 siRNA transfection and affirms the assumption that stable knockdown using shRNA also leads to delayed differentiation in ESC.

### *3.1.4 Rescue of shRNA Mediated Stable Knockdown Phenotype*

To test for shRNA-specificity, knockdown clones are stably transfected with an shRNA resistant version of Zfp281 using the *piggyBac* transposition system. For protocol and used plasmids see Material and Methods page 20 *et seq* and 16 *et seq.* Compared to the establishment of knockdown clones, where single clones are used, rescue cell lines are cell pools containing cells with different expression levels. The shRNA-resistant Zfp281 transgene is point-mutated in the sequence stretch targeted by the Zfp281 #2 shRNA. Therefore, only Z2 clones, as well as the two control clones e.1 and e.2, are used for rescue transfection.

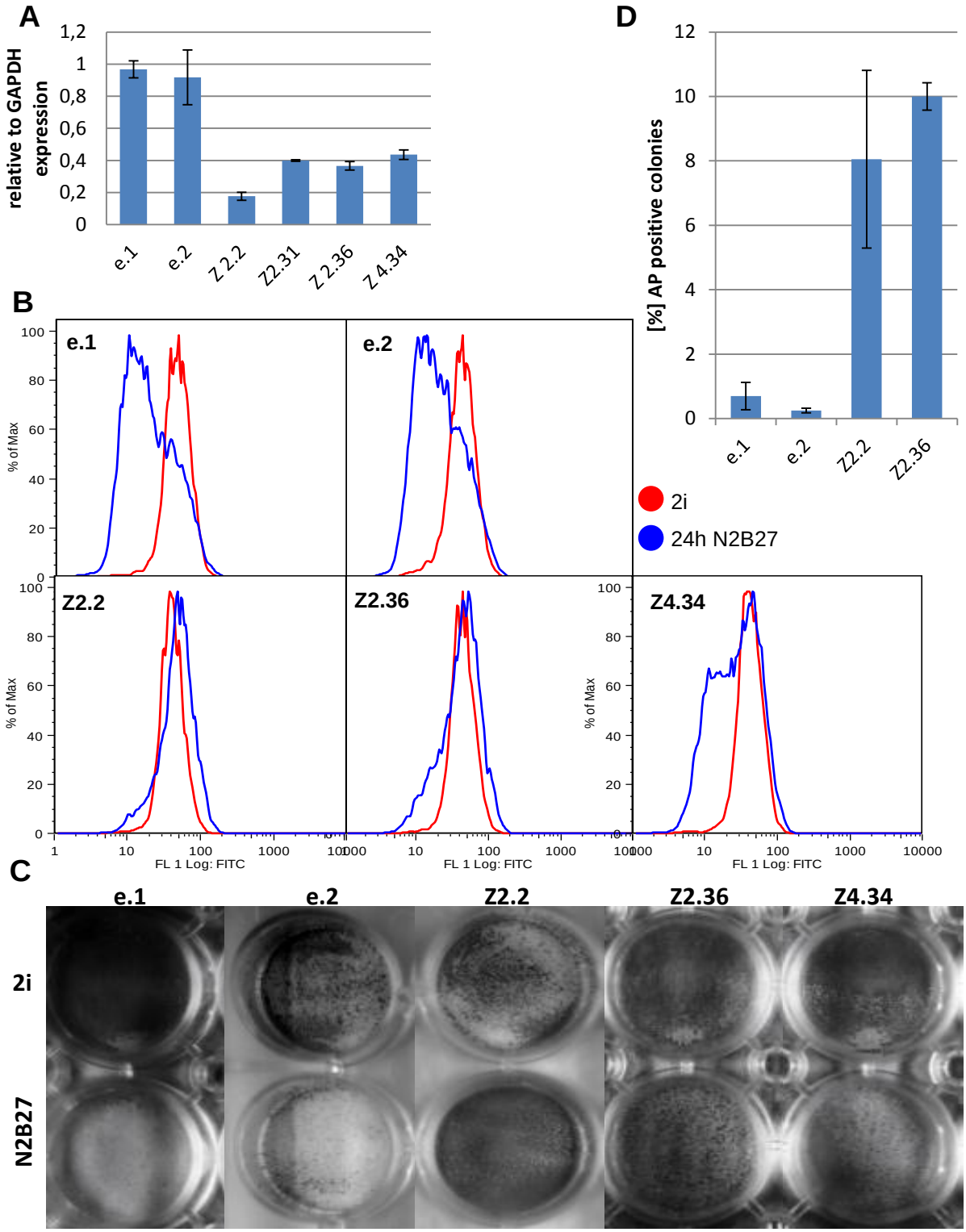


**Figure 4. Stable knockdown of Zfp281 in *Rex1-d2EGFP* ESC**
**(A) qRT-PCR results of Zfp281 mRNA levels in stable knockdown lines. mRNA levels relative to GAPDH expression; results normalised to mean expression level of control clones e.1 and e.2; Results are the mean of two replicates, expressed as mean ± S.E (B) Flow cytometry histograms of GFP expression in knockdown clones; red, cells maintained in 2i; blue, cells collected after 24 hours of differentiation. (C) Photograph of AP stained plate from replated cells after 72 hours of differentiation. First row shows cells maintained in 2i as control for amount of replated cells. Black dots represent stained, therefore undifferentiated, colonies. Pictures are taken from two different plates (e.1/Z2.36/Z4.34 and e.2/Z2.2) and assembled; (D) Statistics of clonal density replating experiment. Blue bars indicating the percentage of AP positive colonies formed from $10^3$ seeded cells. Results are the mean of two replicates, expressed as mean ± S.E. Two individual experiments carried out with similar results.**

An empty rescue construct is used as control and in addition a 3xFlag Zfp281 rescue construct is tested as well, which is needed for later biochemical experiments. After transfection and selection with Hygromycin (150µg/ml) rescue efficiency on mRNA level is determined by carrying out a qRT-PCR (Figure 5A). e.1 and e.2 control clones transfected with the empty rescue construct show no changes in Zfp281 mRNA level whereas both clones have an increased mRNA level by at least 150% when transfected with the Zfp281 and 3xFlag Zfp281 rescue construct. Knockdown clones transfected with empty rescue construct still show decreased Zfp281 mRNA level but knockdown clones transfected with Zfp281 and 3xFlag Zfp281 rescue construct show Zfp281 mRNA levels similar to or even higher than the endogenous transcript. The reason for this moderate increase in Zfp281 mRNA may be based on the fact that no single clones but populations are transfected and tested. Another reason could be that cells with high expression of the rescue constructs die because of unknown reasons. An advantage of this rescue on endogenous levels is that in the following experiments results will not be obscured by overexpression artefacts.

A flow profile after 24 hours of differentiation is carried out to assess if the rescue of Zfp281 mRNA levels rescues the knockdown phenotype (Figure 5B). For this experiment, the 3xFlag Zfp281 rescue construct is not used because it is established for biochemical experiments and not with the primary aim to rescue the knockdown phenotype. e.1 and e.2 transfected with either empty or Zfp281 rescue construct show no difference in GFP profile after 24 hours of differentiation. Most cells start to differentiate indicated by heterogeneous distribution of GFP expression. Due to a clearer differentiation induced GFP profile of e.1, this will be the control clone used for later experiments. The knockdown clones show a partial rescue of the phenotype when transfected with the Zfp281 rescue construct but not with the empty construct. The partial rescue may be due to varying Zfp281 expression levels within the cell pools.

For phenotype rescue quantification, 20% of cells differentiated for 72 hours are replated in 2i conditions on a Laminin coated plate for 48 hours followed by two days of Blasticidin selection and an AP stain (Figure 5C). For this experiment, the 3xFlag Zfp281 rescue cell lines are also used, to determine the rescue cell line, which will be used for later biochemical experiments. Control knockdown cell lines, e.1 and e.2, show no difference in the amount of AP positive colonies comparing empty rescue and Zfp281 / 3xFlag Zfp281 rescue construct transfection. Very few colonies are

stained, indicating normal differentiation behaviour even upon Zfp281 overexpression. The knockdown clones transfected with Zfp281 and 3xFlag Zfp281 show a decreased number in AP stained colonies compared to the empty construct, showing a partial rescue of the phenotype. Especially Z2.31 with 3xFlag Zfp281 shows a reduction of AP colonies after rescuing and is therefore used for biochemical experiments but not for any other of the following experiments. None of the rescue constructs in none of the knockdown clones lead to a perfect rescue consistent with partial rescue of GFP-expression after 24 hours of differentiation. Quantification of the rescue has also been performed, with empty and Zfp281 rescue cell lines, after 72 hours of differentiation and replating of $10^3$ cells in 2i followed by AP stain after colonies have been formed (Figure 5D). As expected and seen in Figure 5C only very few cells form AP positive colonies in e.1 and e.2 , between 0.5% and 1.5%, transfected with either empty or Zfp281 rescue construct. Z2.2 and Z2.36 show an increased number of AP positive colonies (7% and 9% of replated cells) when transfected with empty rescue constructs, showing that the transfection does not affect the delayed differentiation phenotype. Transfection with Zfp281 rescue constructs shows reduction of AP positive colonies only in Z2.2 from 7% to 3%, which fits to results from other experiments showing a partial rescue. In contrast to the other results Z2.36 does not show a phenotype rescue in this experiment, which could be based on the stringency of this assay, as even only 50% of non-manipulated ES cells form AP positive colonies (Wray *et al.*, 2010). These experiments prove that the phenotype observed after shRNA transfection of ESC is dependent on reduced Zfp281 expression as insertion of shRNA immune Zfp281 rescues the phenotype partially.

### *3.1.5 Influence of Stable Zfp281 Knockdown on ESC Cultured in Serum+LIF*

Stable knockdown lines with and without rescue construct expression are cultured in classic ESC conditions using Serum supplemented with LIF (10ng/ml) to assess if the knockdown phenotype is linked to the N2B27+2i culture conditions. ESC cultured in these conditions show heterogeneity concerning their differentiation state and transcriptome (Wray et al., 2010) in comparison to ESC cultured in 2i conditions, which are more homogenous. The aim is to determine if Zfp281 knockdown influences this heterogeneity. After culturing the cell lines for three

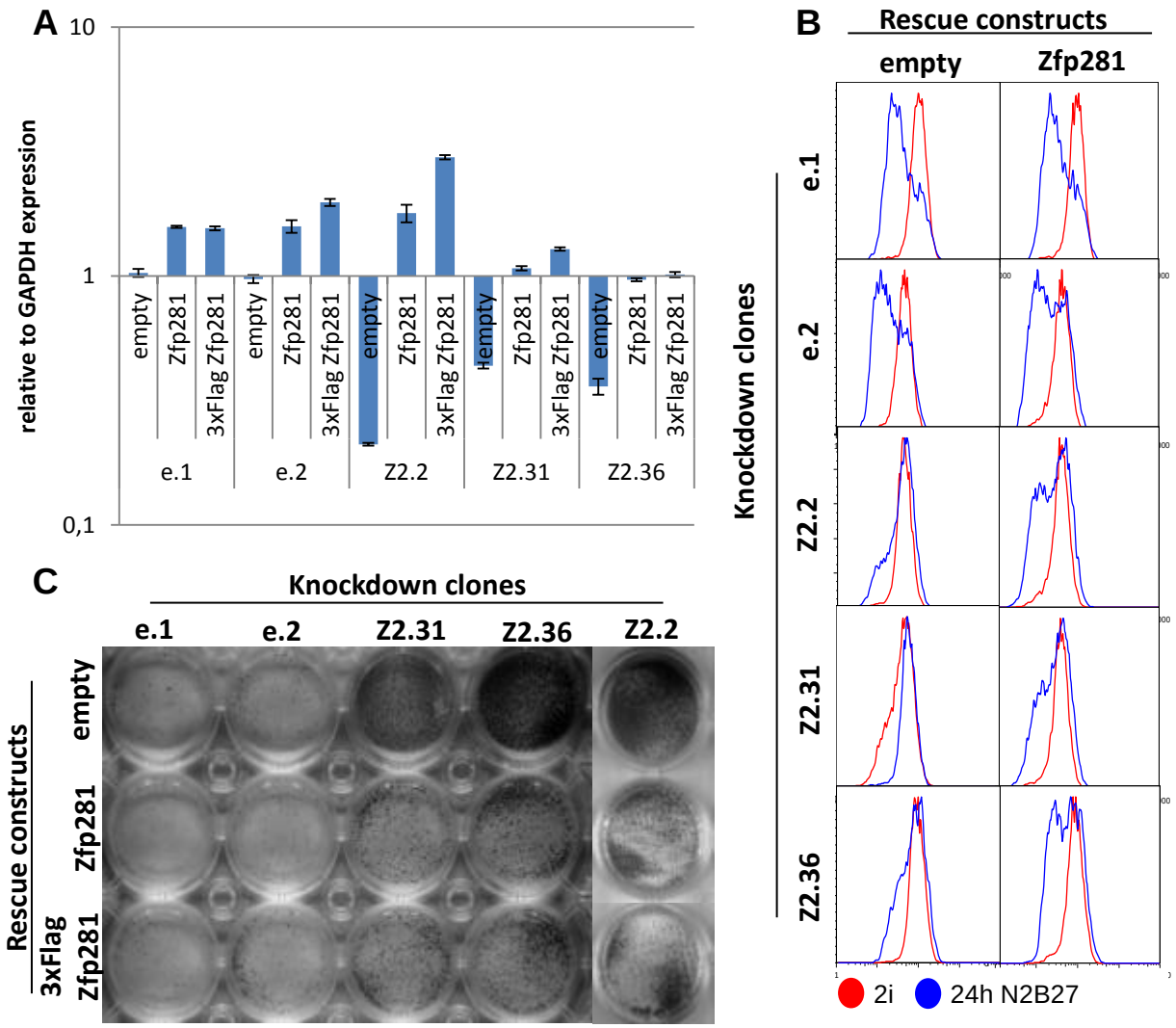


**Figure 5. Rescue of stable Zfp281 knockdown cell lines (A) qRT-PCR results of Zfp281 mRNA levels in rescued knockdown lines. mRNA levels relative to GAPDH expression; results normalised to mean expression level of control clones e.1 and e.2 transfected with empty rescue constructs; Results are the mean of two replicates, expressed as mean ± S.E (B) Flow cytometry histograms of GFP expression in rescue knockdown clones; red, cells maintained in 2i; blue, cells collected after 24 hours of differentiation (C) Photograph of AP stained plate from replated cells after 72 hours of differentiation. First row shows cells transfected with empty rescue construct, second row shows Zfp281 and third row shows 3xFlag Zfp281 rescue construct transfection. Columns show knockdown cell lines as indicated. Black dots represent stained, therefore undifferentiated, colonies. Pictures are taken from two different plates (e.1/e.2/Z2.31/Z2.36 and Z2.2) and assembled (D) Statistics of clonal density replating experiment. Blue bars indicating the percentage of AP positive colonies formed from $10^3$ seeded cells. Results are the mean of two replicates, expressed as mean ± S.E. Two individual experiments carried out with similar results.**

passages in Serum+LIF flow cytometry is performed to determine the level of heterogeneity in knockdown cell lines compared to control cells and rescues (Figure 6A). The empty control clone e.1 shows two GFP level peaks, displaying varying differentiation states indicative for ESC cultured in Serum+LIF - a population of uncommitted cells expressing GFP under the control of active *Rex1* promoter versus a population of differentiated cells where the pluripotency marker is turned off. The knockdown clones Z2.2 and Z2.36 on the other hand show a flow profile very similar to cells cultured in 2i conditions. Only a few differentiating *Rex1-d2EGFP* negative cells distinguish the knockdown cells from cells cultured in 2i. This homogeneity is partially deregulated by introduction of the Zfp281 rescue construct (Figure 6B), indicating that knockdown of Zfp281 is responsible for this phenotype.

$3x10^2$ cells cultured in Serum+LIF are replated on a Gelatine coated 12 well plate and after seven days, an AP stain is performed to quantify the observed reduction in heterogeneity. In contrast to 2i conditions, heterogeneity in Serum+LIF is reflected in heterogeneous colonies consisting of AP positive and negative cells. A colony is defined as AP positive if every cell in this colony is stained, a mixed colony is characterised by a stained, therefore uncommitted, cluster of cells in the centre and unstained, differentiated cells on the outside of the colony. Colonies only consisting of differentiated and therefore unstained cells are AP negative. A figurative explanation of these three colony types can be found in Figure 6C. The e.1 control clone shows 95% of AP mixed colonies, what is expected from ESC cultured in these conditions. Z2.2 and Z2.36 knockdown clones show an approximate 50:50 distribution between AP mixed and AP positive colonies, indicating a reduced heterogeneity to the point of an uncommitted, pluripotent cell state. This phenotype is rescueable for both clones. The Z2.2 rescue shows a stronger rescue verging on colony distribution of the control clone (Figure 6D). Interestingly, the e.1 control rescue shows more fully differentiated colonies due to the rescue mediated Zfp281 overexpression.

The next step is to assess how these cells behave under differentiation favouring conditions. For this assay, the concentration of LIF is reduced, as this is a crucial factor in maintaining pluripotency by activating the STAT3 signalling (Niwa H. *et al.*, 1998). The concentrations used, apart from the normal concentration of 10ng/ml, are 1ng/ml, 0.1ng/ml, 0.01ng/ml and Serum without added LIF. Knockdown cell lines, without the corresponding rescues, cultured under these conditions are analysed by

flow cytometry (Figure 6E) and the heterogeneity is quantified by AP colony ratio determination in a clonogenicity assay (Figure 6F). The e.1 GFP profile (Figure 6E left panel) reveals the importance of LIF for maintaining pluripotency in ESC. The normal concentration (10ng/ml) of LIF leads to the expected heterogeneity consisting of a differentiated and an undifferentiated cell population in an equal ratio. Reduction of LIF concentration to 1ng/ml already favours differentiation over pluripotency visible in the decreased peak for GFP expressing cells. LIF concentrations of 0.1 and 0.01ng/ml lead to a GFP profile of a cell population mainly consisting of differentiated cells described by one low GFP level peak. In the complete absence of LIF, the control cells are not able to survive. The knockdown clones Z2.2 (Figure 6E middle panel) and Z2.36 (Figure 6E right panel) show a reduced dependence on LIF. With normal LIF concentration (10ng/ml) as well as with 10% of the standard concentration (1ng/ml) both clones show a homogeneous GFP distribution representing an uncommitted cell population. The broadened GFP distribution for those LIF concentrations is due to high density of cells in culture plates which favours beginning differentiation. Despite this sub optimal GFP peak the phenotype of Zfp281 knockdown is indicative. A LIF concentration of 0.1ng/ml still leads to a small subpopulation of GFP expressing cells. Cells cultured with 0.01ng/ml LIF are mostly differentiated, however there are still some cells expressing GFP, especially in Z2.36. Z2.36 cells growing in Serum without LIF still show some cells that are GFP positive, compared to Z2.2. Apart from that, it is notably that these cell lines are able to survive without LIF, compared to the control clone, for the whole experiment, which lasts five passages.

The reduced dependence of Zfp281 knockdown clones on LIF under the aspect of homogeneity versus heterogeneity in the classic ESC culture medium Serum+LIF is quantified. A clonogenicity assay with e.1, Z2.2 and Z2.36 is performed by replating $3x10^2$ cells on gelatine-coated plates in Serum with the adjusted LIF concentrations and performing an AP stain after colonies have formed. The distribution between AP positive/mixed/negative colonies sheds light on how these different cell lines behave under differentiation favouring conditions. The ratio of the different AP colonies formed under standard Serum+LIF (10ng/ml) conditions for e.1, Z2.2 and 2.36 are displayed for comparison and already described on page 39 *et seq.* (Figure 6D). Decreasing LIF concentrations lead to an ascent of AP negative colonies in e.1, beginning from 3% at 10ng/ml, 50% at 1ng/ml until 82% at 0,01ng/ml and in culture

conditions without LIF e.1 control cells are not able to survive. The low percentage at the concentration of 0.1ng/ml is because of too few replated cells and therefore not representable. At a concentration of 10ng/ml AP positive colonies (5%) appear in the e.1 cell line. They are also exclusively visible in the knockdown clones Z2.2 (51%) and Z2.36 (59%) at standard LIF concentration. At concentrations beginning with 1ng/ml the majority of knockdown cells are AP mixed, from 100% (Z2.2, Z2.36) at 1ng/ml, 99% (Z2.2) and 94% (Z2.36) at 0,1ng/ml, 92% (Z2.2) and 83% (Z2.36) at 0.01ng/ml up to 83% (Z2.2) and 75% (Z2.36) without LIF. The distribution, between differentiated and partially undifferentiated colonies in Z2.2 and Z2.36 with LIF concentrations equal or lower than 0.1ng/ml and even without LIF, reflects the heterogeneity observed in non-manipulated ES cell lines under standard Serum+LIF conditions. Z2.2 and Z2.36 cultured with LIF concentrations of 10ng/ml and 1ng/ml show reduced heterogeneity.

These findings indicate that a reduction of Zfp281 decreases LIF-STAT3 signalling dependence of ESC. Furthermore, Zfp281 knockdown seems to enable survival and proliferation of ESC in Serum without LIF for at least five passages, whereas control cells die in these conditions.

### *3.1.6 Stable Zfp281 Knockdown Phenotype in the Absence of the MAPK/Erk Inhibitor PD03*

The phenotype of Zfp281 shRNA ESC in N2B27 differentiation medium and classic Serum+LIF conditions shows the role of Zfp281 in exit from pluripotency. Knockdown clones in 2i medium on the other hand behave like normal, non-manipulated ESC. To address if Zfp281 acts in MAPK/Erk signalling, cell lines are cultured in N2B27 supplemented with Chiron (3µM) but without PD03 and after five passages cells are analysed using flow cytometry and AP colony ratio determination in a clonogenicity assay with $3x10^2$ replated cells. Like in Serum+LIF conditions the control clone e.1 (Figure 7A) shows two GFP peaks reflecting two distinct cell populations of differentiated and pluripotent cells. These cells are only partially able to compensate for the absence of the MAKP/Erk inhibitor. By comparison the majority of cells with knocked down Zfp281 (Z2.2 and Z2.36) (Figure 7A) are in the pluripotent state despite the lack of PD03, displayed by the GFP high peak. Only a few cells do not express GFP because of the shutdown of the pluripotency indicating marker Rex1. This may be because Zfp281 mRNA levels are reduced but not absent.

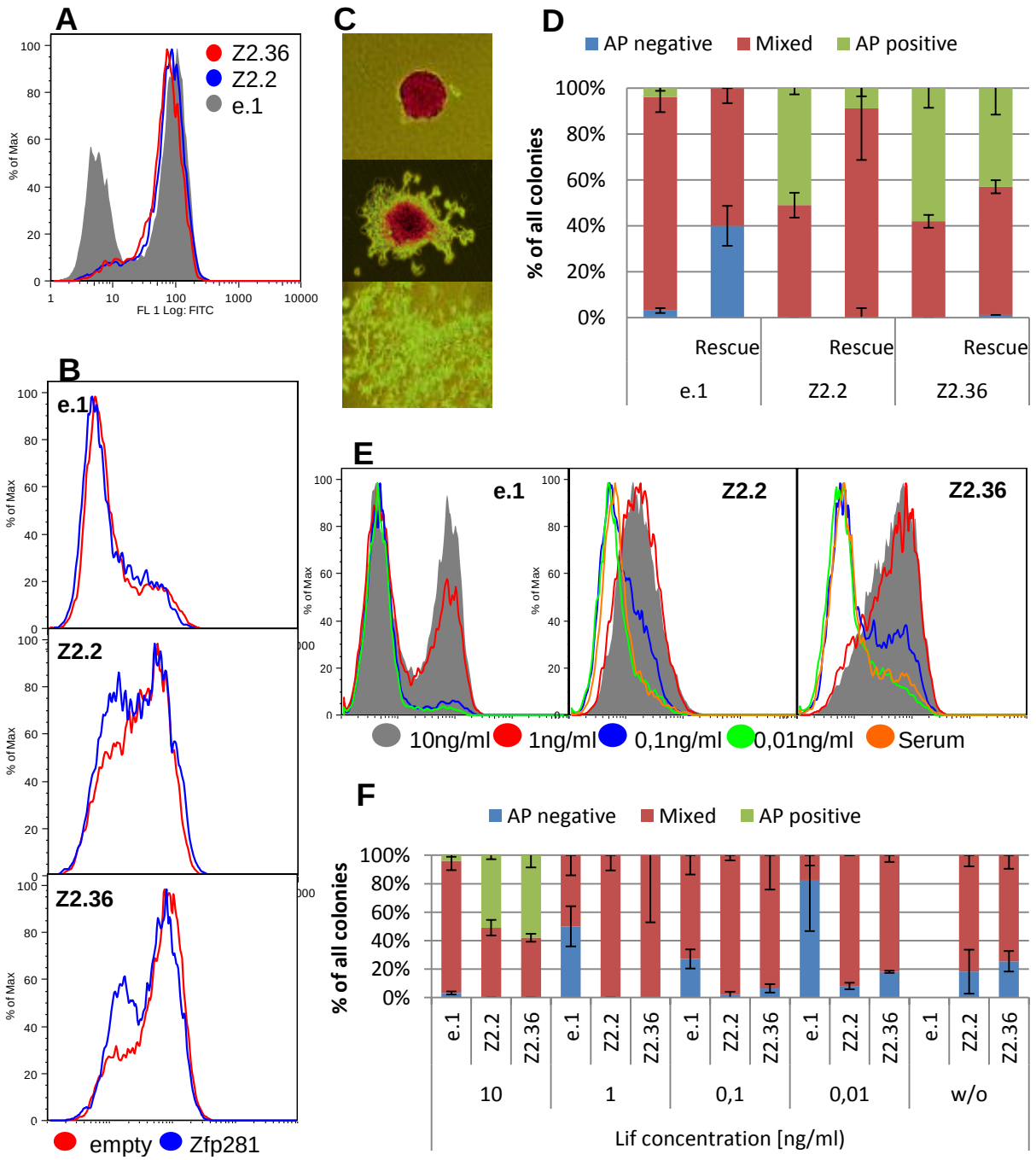


**Figure 6. Stable Zfp281 knockdown phenotype in Serum+LIF culture conditions**

**(A) Flow cytometry histogram of GFP expression levels in knockdown cells; grey, expression profile of e.1; blue, expression profile of Z2.2; red, expression profile of Z2.36 (B) Flow cytometry histogram of rescue cell lines; red, empty rescue construct; blue, *Zfp281* rescue construct; upper panel, e.1 control; middle panel, Z2.2 knockdown clone; lower panel, Z2.36 knockdown clone; (C) Picture of AP stained colonies; upper panel, AP positive colony; middle panel, AP mixed colony; lower panel, AP negative colony (D) Statistics of clonal density replating experiment with knockdown and rescue clones under standard Serum+LIF culture conditions. Each bar represents the amount of all formed colonies from one cell line as total 100%; blue, AP negative cells; red, AP mixed colonies; green, AP positive colonies. Results are the mean of two replicates, expressed as mean ± S.E. Two individual experiments carried out with similar results. (E) Flow cytometry histogram showing GFP expression levels in knockdown cell lines cultured with different concentrations of LIF; left panel, e.1 control; middle panel, Z2.2 knockdown clone; right panel, Z2.36 knockdown clone; grey, 10ng/ml LIF; red, 1ng/ml LIF; blue 0.1ng/ml LIF; green, 0.01ng/ml LIF; orange, no LIF (F) Statistics of clonal density replating experiment of knockdown clones with different LIF concentrations; Each bar represents the amount of all formed colonies from one cell line as total 100%; blue, AP negative cells; red, AP mixed colonies; green, AP positive colonies. Results are the mean of two replicates, expressed as mean ± S.E.**

The phenotype can be rescued by ectopic expression of the shRNA immune Zfp281. The transfected e.1 control clone does not show a change in GFP levels (Figure 7B left panel) between transfection with empty rescue or Zfp281 rescue construct, displaying that the empty rescue vector does not have an influence on the cell state. Rescued knockdown clones Z2.2 (Figure 7B middle panel) and Z2.36 (Figure 7B right panel) show a reduction of the GFP high peak towards the GFP low peak representing differentiated cells. However, rescue of Z2.2 shows a stronger GFP peak shift than in Z2.36. The results of these flow cytometry analyses indicate a reduced dependence of Zfp281 knockdown ESC on MAPK/Erk signalling pathway inhibition for maintaining their naïve pluripotent state.

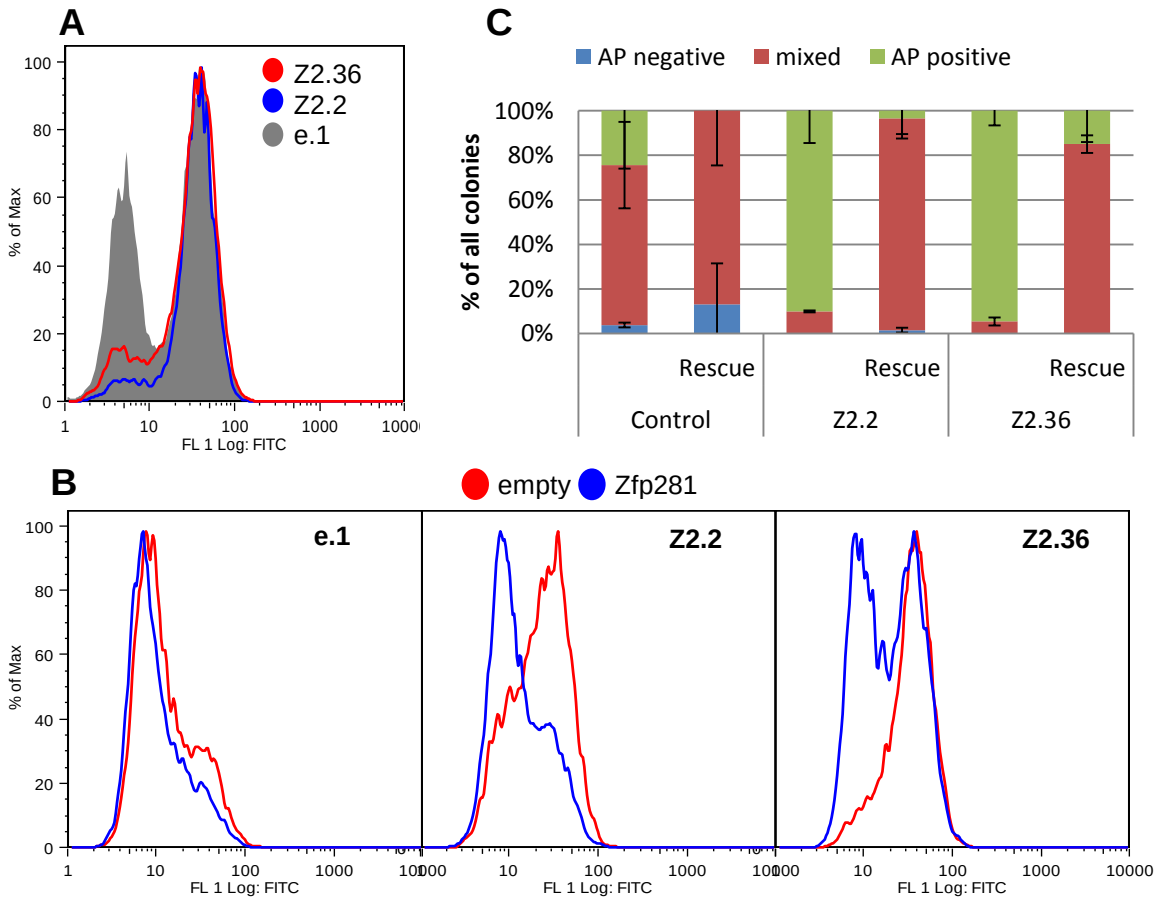


**Figure 7. Phenotype observed in stable Zfp281 knockdown ESC cultured in N2B27+Chiron**
**(A) Flow cytometry histogram of GFP expression levels in knockdown cells; grey, expression profile of e.1; blue, expression profile of Z2.2; red, expression profile of Z2.36 (B) Flow cytometry histogram of rescue cell lines; red, empty rescue construct; blue, Zfp281 rescue construct; left panel, e.1 control; middle panel, Z2.2 knockdown clone; right panel, Z2.36 knockdown clone (C) Statistics of clonal density replating experiment with knockdown and rescue clones cultured in N2B27+Chiron. Each bar represents the amount of all formed colonies from one cell line as total 100%; blue, AP negative cells; red, AP mixed colonies; green, AP positive colonies. Results are the mean of two replicates, expressed as mean ± S.E. Two individual experiments carried out with similar results.**

Validation of the observed phenotype is performed by determination of ESC clone composition using AP staining (Figure 7C). 70% of the colonies formed from e.1 cells are AP mixed and 25% are AP positive, reflecting the results from the flow cytometry analysis showing two cell populations of differentiated and undifferentiated cells. This ratio is changed to 90% AP mixed and no AP positive colonies after transfection with Zfp281 rescue construct, leading to expression of Zfp281 higher than the endogenous level. Knockdown clones cultured in N2B27+Chiron show 90% AP positive colonies for Z2.36 and respectively 95% for Z2.2. After transfection with the rescue construct the ratio changes to 95% AP mixed colonies for Z2.2 and 85% for Z2.36.

These assays show that reduced Zfp281 expression can compensate for MAPK inhibition both in terms of *Rex1-d2EGFP* expression as well as in terms of colony composition in clonogenic assays. On the other hand, expression of Zfp281 at a level higher than the endogenous level favours heterogeneity visible in the clonogenicity assay for the e.1 control clone and its rescue.

### *3.1.7 Phenotype Characterisation of Stable Zfp281 Knockdown Without the GSK3 Inhibitor*

As shown above (Figure 7) Zfp281 knockdown may partially substitute for missing PD03 in 2i culture conditions. To complete this line of thought Zfp281 knockdown clones and their rescues are cultured in N2B27 supplemented with the MAPK/Erk inhibitor PD03 (1µM) but without the GSK3 inhibitor Chiron. For analysis flow cytometry and a clonogenicity assay are performed after two passages. The first result obtained from culturing is that cells cultured in N2B27+PD03 cannot be passaged for more than three or four times before they die. This is independent from the Zfp281 knockdown.

Flow cytometry analysis (Figure 8A) shows two GFP peaks for e.1 representing two cell states in the population, which is exemplarily for the predominant heterogeneity. This heterogeneity in GFP expression, which is equivalent to the pluripotency marker Rex1 expression, is only partially reduced in Zfp281 knockdown clones. Z2.2 shows a stronger increase in the GFP high peak resembling the pluripotent cell population compared to Z2.36. Regardless of the increase of pluripotent cells, there are still many cells, between 30% for Z2.2 and 60% for Z2.36, which do not express GFP because of their differentiated cell state. Flow cytometry of rescue cell lines (Figure 8B) does not give conclusive results either. As expected,

and shown in previous flow cytometry experiments carried out with these rescue cell lines, the e.1 control shows no difference in GFP expression according to the transfected rescue construct. A difference to the untransfected e.1 (Figure 8A) is the missing GFP high peak, which can be caused by too dense growth of cells in the culture plate. Rescue transfection of knockdown clones shows no effect for Z2.36 and only a minimal shift of GFP level from low to high in Z2.2.

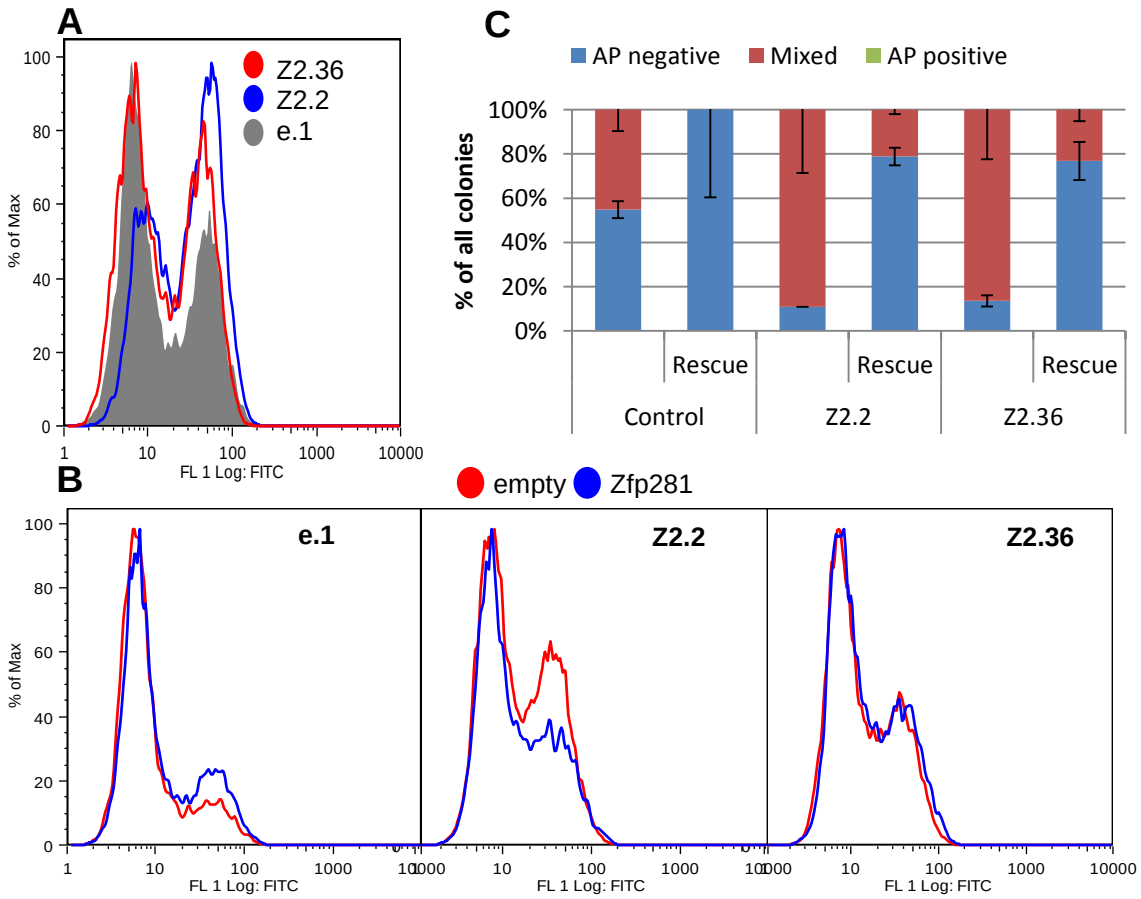


**Figure 8. Phenotype observed in stable Zfp281 knockdown ESC cultured in N2B27+PD03**
**(A) Flow cytometry histogram of GFP expression levels in knockdown cells; grey, expression profile of e.1; blue, expression profile of Z2.2; red, expression profile of Z2.36 (B) Flow cytometry histogram of rescue cell lines; red, empty rescue construct; blue, Zfp281 rescue construct; left panel, e.1 control; middle panel, Z2.2 knockdown clone; right panel, Z2.36 knockdown clone (C) Statistics of clonal density replating experiment with knockdown and rescue clones cultured in N2B27+PD03. Each bar represents the amount of all formed colonies from one cell line as total 100%; blue, AP negative cells; red, AP mixed colonies; Results are the mean of two replicates, expressed as mean ± S.E. Two individual experiments carried out with similar results.**

A clonogenicity assay, as described for knockdown clones cultured in Serum+LIF page 39 *et seq.*, is performed to complete the analysis (Figure 8C). Compared to the e.1 clone with 55% AP negative colonies the knockdown clones, Z2.2 with 10% and Z2.36 with 15%, show reduced percentage of AP negative colonies. None of the cell

lines forms AP positive colonies indicating that the Zfp281 knockdown does not influence the cell state to a point of naïve pluripotency. Transfection of the cell lines with Zfp281 rescue construct increases the number of AP negative colonies, 100% for e.1, 80% for Z2.2 and 75% for Z2.36. This increase of completely differentiated colonies due to slight overexpression of Zfp281, compared to endogenous levels, may be a further indication of its importance in exit of pluripotency.

### *3.1.8 Interaction of Zfp281 with the Pluripotency Network*

The Zfp281 interaction with and influence on transcription factors is examined to figure out how it could possibly be responsible for exit of ground state pluripotency. The first experiment to investigate the influence of Zfp281 on pluripotency factors is gene expression profiling using qRT-PCR in knockdown clones compared to control clones and rescue cell lines in 2i conditions and after 24 hours of differentiation (Figure 9A-9E). The mRNA levels of Nanog, Klf2, Klf4 and Oct4 are determined in e.1 control and Z2.2 and Z2.36 knockdown clones cultured in 2i (Figure 9A). The results are normalized to the mRNA levels of the e.1 control as it represents normal ESC gene expression. Compared to the e.1 control clone, mRNA levels of all four transcription factors in Z2.2 and Z2.36 are decreased whereat the mRNA level of Nanog is down regulated to 55% in Z2.2 and 41% in Z2.36, the mRNA level of Klf2 to 45% in Z2.2 and 30% in Z2.36 and Klf4 mRNA level is down regulated to 60% in Z2.2 and 50% in Z2.36. Down regulation of Oct4 mRNA is stronger than in the previous transcription factors, it is down to 30% in Z2.2 and 5% in Z2.36. This strong down regulation in Z2.36 may be a false result but is observed twice in independent experiments. This result points to an activating role of Zfp281 on transcriptional control of the tested transcription factors in a naïve pluripotent environment. The same experiment is carried out after 24 hours of 2i withdrawal and mRNA levels of the above-mentioned transcription factors are determined (Figure 9B-9E). This assay shows a different result as for cells cultured in 2i conditions. As expected in the e.1 control, the mRNA levels of all four transcription factors decrease massively after 24 hours of 2i withdrawal indicating differentiation. For the Z2.2 and Z2.36 knockdown clones a difference to the gene expression profile carried out in 2i conditions is visible. The mRNA levels of Nanog (Figure 9B), Klf2 (Figure 9C) and Oct4 (Figure 9E) show less reduction compared to the e.1 control. Nanog mRNA level reduction is about 10%, the reduction of Klf2 mRNA level is 50% and for Oct4 it is between 5%

(Z2.2) and 10% (Z2.36) of the mRNA level reduction in the e.1 control clone. The mRNA levels for Klf4 do not show a difference between e.1 control and the knockdown clones (Figure 9D). The mRNA levels of all four transcription factors in the knockdown cell lines can be decreased to the level of the e.1 control, or even more, by transfection with the Zfp281 rescue construct. This effect is also visible for Klf4. Transfection of e.1 with Zfp281 rescue construct leads to further reduction of mRNA levels. This effect and the higher mRNA levels in Z2.2 and Z2.36 compared to e.1 suggest that Zfp281 represses pluripotency transcription factor transcription during differentiation.

A Chromatin-Immunoprecipitation (ChIP), according to Material and Methods page 26 *et seq.*, is carried out to assess whether Zfp281 may regulate these genes directly. ChIP is the technique of choice for this question, as the protein of interest is cross-linked to its DNA binding sites. These can be assigned to cis-regulatory elements of putative target genes using qPCR and specific primers for regions of interest. Additionally to target identification, quantification of the binding strength can be assessed by the amount of qPCR amplification. Published ChIPs for Zfp281 (Kim *et al.*, 2008; Wang *et al.*, 2006; Wang *et al.*, 2008) identified loci like the *Nanog* promoter (Wang *et al, 2008)* or *Gata6* (Wang *et al.*, 2006) and target genes shared with other pluripotency transcription factors (Kim *et al.*, 2008), indicative for the involvement of Zfp281 in the ESC network. However, it is unclear if this binding pattern is regulated during ESC differentiation. For the actual ChIP, the Z2.31 knockdown cell line transfected with the 3xFlag Zfp281 rescue construct is used. The reasons to use the 3xFlag Zfp281 is that no antibodies against Zfp281 are available and the knockdown cell line is used to minimize interference, meaning occupation of DNA binding sites, of endogenous Zfp281 with the 3xFlag construct. The functionality of this cell line is represented on page 38, Figure 5. Cells cultured in 2i and cells cultured for 24 hours in N2B27 only are used to detect possible changes in DNA protein interactions. With both cell populations, three ChIP assays are carried out. Additionally to the 3xFlag Zfp281 ChIP, an Oct4 ChIP is used as positive control and a ChIP with IgGs is used as negative control to normalise the results. Analysis of the experiment is carried out using qPCR by measuring the quantity of reads in promoters and transcriptional start sites recognised by specific primers (see Material and Methods table 6B). The chosen regions are loci, which are co-bound by Nanog, Sox2 and Tcf3 and are therefore likely nodes for the pluripotent transcription factor

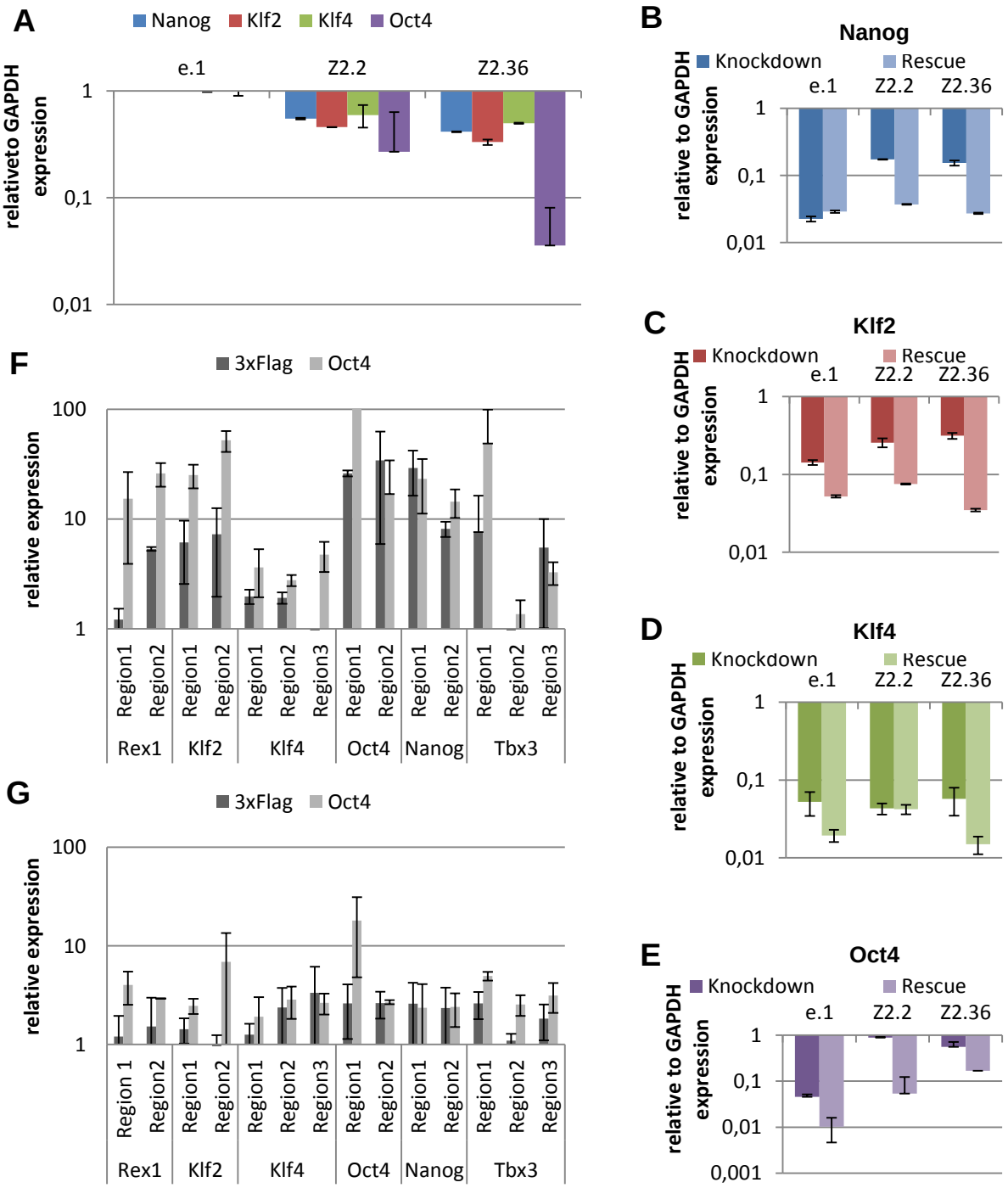


**Figure 9. Interaction of Zfp281 with the pluripotency network**

**(A) qRT-PCR results of pluripotency factor mRNA levels in stable knockdown lines cultured in 2i; blue, Nanog; red, Klf2; green, Klf4; violet, Oct4; mRNA levels relative to GAPDH expression; results normalised to expression level of control clone e.1; Results are the mean of two replicates, expressed as mean ± S.E; (B-E) qRT-PCR results of pluripotency factor mRNA levels in stable knockdown lines and corresponding rescue cell line, represented by adjacent pairs of bars, collected after 24 hours of differentiation; (B) Nanog; (C) Klf2; (D) Klf4; (E) Oct4; mRNA levels relative to GAPDH expression; results normalised to expression level of control clone e.1 in 2i conditions (Figure 9A); Results are the mean of two replicates, expressed as mean ± S.E; (A-E) Experiments carried out in parallel under same conditions; Four individual experiments carried out with similar results; (F,G) ChIP analysis of Z2.31 knockdown cells transfected with 3xFLag Zfp281 rescue construct cultured in 2i (F) or 24 hours after differentiation (G); Results are the mean of two replicates, expressed as mean ± S.E; bars indicating reads of selected gene regions; results normalised to reads in IgG ChIP; light grey, positive control Oct4 ChIP using Oct3/4 (N19) goat polyclonal antibody (Santa Cruz, sc-5279); dark grey, 3xFLag Zfp281 ChIP carried out with ANTI-FLAG M2 mouse monoclonal antibody (Sigma-Aldrich, F1804); experiment carried out once**

circuitry. It has to be said that this experiment has only been done once. The 3xFlag Zfp281 ChIP performed in 2i cells (Figure 9E) shows that Zfp281, similar to Oct4, interacts with most tested regions, but binding is considerably weaker leading to lower enrichment scores. The strongest Zfp281-DNA interaction appears to be with both regions of *Oct4* and *Nanog*. Zfp281 also binds to both regions of *Klf2* and partially to *Rex1*, *Klf4* and *Tbx3*. The ChIPs performed in differentiating cells (Figure 9F) show a general decrease in the amount of reads per region. The decrease of reads of the Oct4 ChIP, which serves as positive control, indicates that the decreases in the 3xFlag Zfp281 are not due to a failure in the experiment but a reaction to the changed culture conditions and onset of differentiation. The regions Zfp281 binds to during differentiation are similar to the ChIP performed in 2i but with lower intensity. A difference in DNA protein interaction is observed for *Klf2*, which does not interact with Zfp281 in differentiating cells. The main statement of this experiment is that Zfp281 seems to interact with the regulatory sequences of pluripotency genes in a direct manner in ground state pluripotency conditions as well as during the exit of pluripotency.

Comparing these results with gene expression profiles carried out for Nanog, Klf2,Klf4 and Oct4 (Figure 9A-9E) in 2i and differentiation conditions suggests a dual function of Zfp281 as an activator in naïve pluripotency (Figure 9A) and repressor during differentiation (Figure 9B-E).

## 3.2 Reprogramming of EpiSC is Enhanced by Zfp281 Knockdown

The impaired differentiation of ESC upon Zfp281 knockdown highlights its involvement in exit from pluripotency. Zfp281 knockdown delays repression of pluripotency genes (Figure 9B-9E) during differentiation, while overexpression of the same induces reprogramming of somatic cells. Thus, knockdown of Zfp281 may promote reprogramming.

### *3.2.1 Effect of Zfp281 siRNA Knockdown on Reprogramming using Chimeric LIF Receptor EpiSC*

A transgenic EpiSC line (*O4G-7*), expressing a GFP reporter and Puromycin resistance under the control of the *Oct4* promoter, is used to investigate the possible influence of Zfp281 on reprogramming. The experiment is a loss of function assay using siRNA mediated transient gene knockdown. Since it is not clear if absence of Zfp281 is sufficient to induce EpiSC reprogramming, a sensitised genetic background

is used. Hyper-activation of the LIF pathway member STAT3 induces reprogramming of EpiSC into naïve ESC at a low frequency (Yang *et al.*, 2010). This is achieved by expression of a chimeric LIF receptor, called GY118F. The receptor consists of the transmembrane and intracellular domain of the LIF receptor with a substitution mutation at residue 118 from tyrosine to phenylalanine to stimulate exclusively JAK/STAT signalling and not the RAS/MAPK and PI3K signalling. It further interferes with the negative feedback regulator SOCS3 to allow continuous STAT3 signalling. The JAK/STAT signalling is driven by G-CSF (Recombinant Murine G-CSF, PeproTech 250-05) which binds to its receptor, the extracellular domain of the chimeric receptor. A schematic description of this receptor can be seen in Figure 10A. The reason for using this receptor is that LIF stimulates reprogramming as well as self-renewal but in cell lines other than ESC the expression of the LIF receptor is too low for signal activation. Furthermore, the use of G-CSF as stimulus is controllable, as it is not expressed and secreted in EpiSC or ESC. Used EpiSC are transfected with the transgene or the empty plasmid as negative control, hereinafter called as 796.4 respectively 131.4 (Yang *et al.*, 2011). Transfection is performed according to Material and Methods page 20 *et seq*. As negative control for influence on reprogramming efficiency 796.4 and 131.4 cell lines are transfected with negative siRNA, no siRNA and Tcf3 siRNA, as Tcf3 has no effect on reprogramming although it strongly inhibits ES cell differentiation. Zfp281 knockdown is carried out with pooled Zfp281 siRNA as well as with the four individual siRNAs targeting Zfp281 for deconvolution and ruling out an off target effect. Transfection is performed in EpiSC medium, consisting of N2B27 supplemented with FGF2 (12 ng/ml) and ActivinA (20 ng/ml). 24 hours after transfection medium is switched to 2i conditions supplemented with G-CSF (30ng/ml). Additionally 796.4 cells are cultured in 2i alone, to control for JAK/STAT independent reprogramming. After four days medium is switched to 2i+LIF, leading to proliferation of reprogrammed cells, and finally a Puromycin (1ng/ml) treatment is performed to select for reprogrammed, Oct4 expressing, cells. The efficiency of reprogramming and the effect of Zfp281 knockdown on it is analysed by an AP stain (Figure 10B). All siRNA transfected 796.4 cells show reprogrammed cells, due to the G-CSF stimulated hyperactive JAK/STAT signalling. Transgenic chimeric LIF receptor cells transfected with the Zfp281 siRNA pool show an 8.5 fold increase in the number of AP positive colonies compared to cells transfected with negative siRNA. As all the single Zfp281 siRNAs show a similar

increase in AP positive colonies an off target effect can be ruled out. As expected Tcf3 knockdown does not have in influence on reprogramming of EpiSC compared to the negative controls. 796.4 cells cultured in 2i without added G-CSF do not show any reprogrammed cells indicating the dependence of reprogramming on activated JAK/STAT signalling. EpiSC with the empty plasmid do not show reprogrammed cells either except for Zfp281 #4 single siRNA, which leads to one AP positive colony. This reprogramming effect might be a false positive stained colony, as no other Zfp281 siRNA knockdown leads to reprogramming without enhanced STAT3 signalling. This result suggests that Zfp281 not only drives commitment but also blocks the opposite effect namely reprogramming.

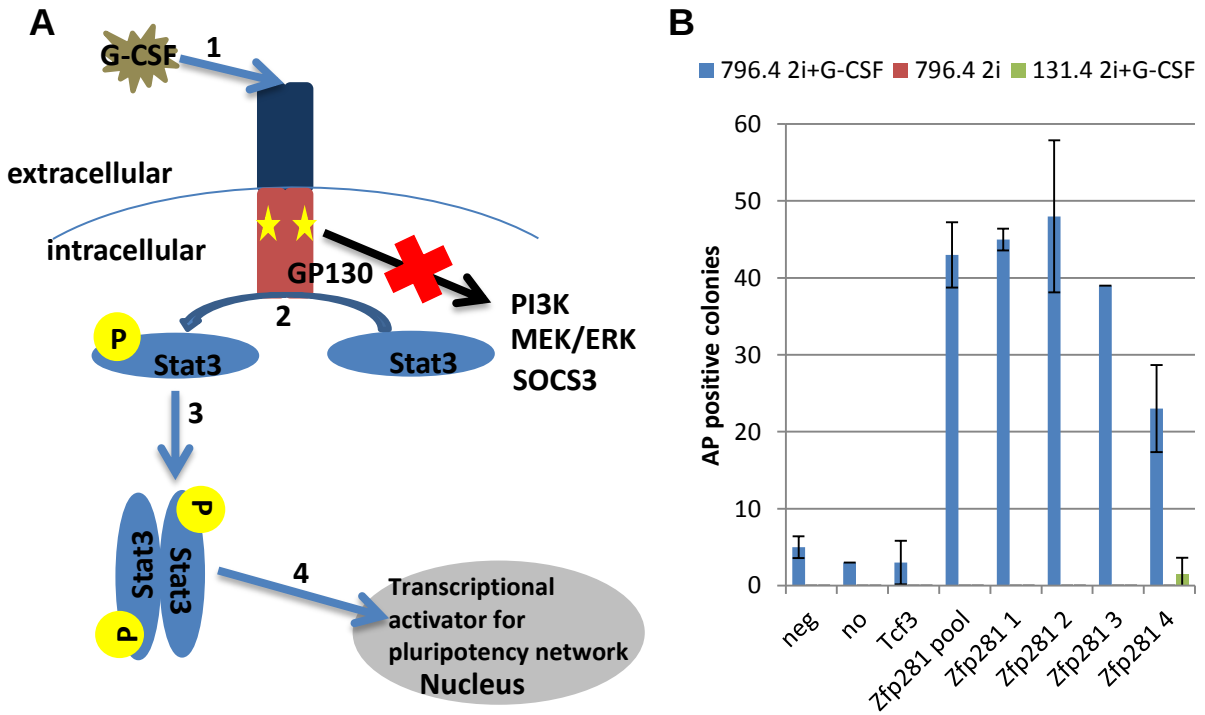


**Figure 10. Effect of Zfp281 siRNA knockdown on chimeric LIF receptor EpiSC reprogramming**
**(A) Schematic illustration of chimeric LIF receptor GY118F; dark blue, dimeric extracellular domain of G-CSF receptor; red, dimeric transmembrane and intracellular domain of GP130 LIF receptor; yellow asterisks, mutation of residue 118 from tyrosine to phenylalanine; 1, extracellular binding of G-CSF at its receptor; 2, phosphorylation of STAT3 through JAK; 3, dimerization of phospho-STAT3; 4, entering the nucleus and acting as transcription actor for pluripotency genes; (B) Statistics of AP stain experiment. Bars indicating the number of AP positive colonies formed from siRNA transfected cells after eight days of reprogramming ; blue , *O4G-7* cells transfected with GY118F reprogrammed in 2i+G-CSF; green, O4G-7 cells transfected with empty plasmid as negative control cultured in parallel to GY118F O4G-7 cells. Results are the mean of two replicates, expressed as mean ± S.E. Five individual experiments carried out with similar results.**

### *3.2.2 Reprogramming of EpiSC Expressing Transgenic Pluripotency Factors with Zfp281 Knockdown*

These results place the reprogramming function of Zfp281 genetically downstream or independent of hyper-activated JAK/STAT signalling. The next

question to address is therefore if this signalling pathway is crucial for enhanced reprogramming or if there are other mechanisms supporting this Zfp281 knockdown phenotype. For this reason two different EpiSC lines, *OEC-2* and *O4G-7*, both with GFP reporter and Puromycin resistance under the control of the *Oct4* promoter, are each stably transfected with *pPB-CAG-Nanog-pgk-hph*, *pPB-CAG-Klf4-pgk-hph*, *pPB-CAG-Esrrb-pgk-hph* or *pPB-CAG-DEST-pgk-hph* empty vector, as control, (provided by Betschinger J.) using the *piggyBac* transposition system ( protocol see Material and Methods page 20 *et seq.*). These cell lines are used in the same reprogramming assay described for the GY118F *O4G-7* EpiSC line (page 49 *et seq.*). In this assay, no G-CSF is used, because of the lack of the receptor. Instead the transfected cells are cultured in 2i ± LIF (10ng/ml) for eight days after siRNA transfection followed by two days of Puromycin selection. Negative siRNA, no siRNA and Tcf3 siRNA are used as controls and Zfp281 siRNA pool is used for knockdown. The results are analysed by counting AP positive colonies, marking reprogrammed pluripotent like cells. Neither *OEC-2* EpiSC (Figure 11A) nor *O4G-7* EpiSC (Figure 11B) are able to reprogram with any of the siRNAs when transfected with the empty plasmid. Overexpression of the reprogramming factors induced formation of AP positive ESC colonies irrespective of siRNA transfection. However, Zfp281 knockdown increased reprogramming effects significantly. In particular, transfection with Zfp281 siRNAs, lead to 14 times and 17 times more AP colonies in the *OEC-2* and *O4G-7* Klf4 overexpressing EpiSC compared to negative siRNA, respectively. Both cell lines reprogram in 2i without addition of LIF indicating the strong reprogramming effect of Klf4 expression (Guo *et al.*, 2009) combined with Zfp281 knockdown. *OEC-2* cells overexpressing Nanog transfected with control siRNAs are able to reprogram in 2i conditions supplemented with LIF (10ng/ml) to a limited degree that is increased twenty fold by Zfp281 knockdown. *O4G-7* EpiSC are not able to reprogram with transgenic Nanog neither with nor without Zfp281 knockdown. This reduced support for reprogramming could be based on too low expression of transgenic Nanog, whose expression as well as the expression levels of the other transgenic pluripotency factors, has not been determined. However, similar EpiSC line differences have been observed by lab members, suggesting that the *O4G-7* and *OEC-2* lines show different disposition for reprogramming. Expression of transgenic Esrrb leads to massive reprogramming in *OEC-2* cells so no potential difference between negative control and Zfp281 knockdown can be quantified. In *O4G-7* EpiSC,

expressing Esrrb knockdown of Zfp281 leads to formation of six AP positive colonies compared to nil in control siRNA transfections. This is consistent with the findings of Festuccia and colleagues that Esrrb can promote reprogramming, even in the absence of Nanog (Festuccia *et al.*, 2012).

This experiment shows that Zfp281 knockdown increases reprogramming efficiency of EpiSC independent of hyperactive JAK/STAT signalling. Without additional signals Zfp281 knockdown does not lead to reprogrammed cells in neither of the two tested EpiSC lines.

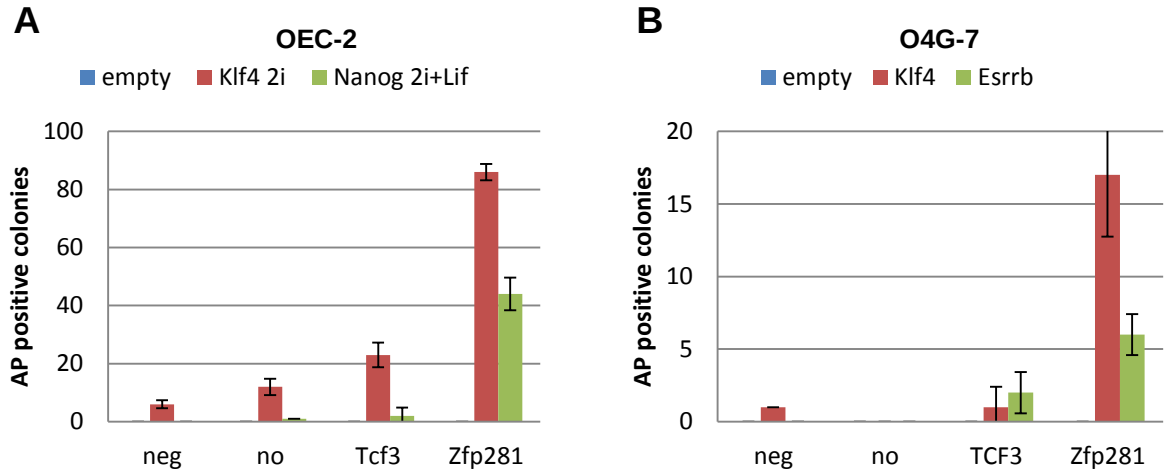


**Figure 11. Zfp281 knockdown effect on reprogramming of transgenic transcription factor expressing EpiSC lines.**
**(A-B) Statistics of AP stain performed after reprogramming of transgenic EpiSC; Bars indicating the number of AP positive colonies formed from siRNA transfected cells after eight days of reprogramming; Results are the mean of two replicates, expressed as mean ± S.E. Three individual experiments carried out with similar results. (A) *OEC-2* EpiSC line; red, transgenic expression of Klf4, reprogramming performed in 2i; green, transgenic expression of Nanog, reprogramming performed in 2i+LIF (10ng/ml); (B) *O4G-7* EpiSC line; reprogramming performed in 2i conditions; red, transgenic expression of Klf4; green, transgenic Esrrb expression.**

### *3.2.3 Reprogramming of EpiSC Lines Using Zfp281 Knockdown Without Additional Signals*

It has been shown that Zfp281 knockdown can increase reprogramming of EpiSC if additional signals, hyperactive JAK/STAT signalling or transgenic expression of pluripotency transcription factors, are provided. Despite the negative results from the previous experiments using empty control plasmids, which do not reprogram in the absence of Zfp281 it has to be tested in more detail if Zfp281 knockdown is sufficient to initiate EpiSC reprogramming even without supporting signals. In this experiment, five different EpiSC lines are used for a reprogramming assay using the same controls as mentioned above and the Zfp281 siRNA pool. After transfection, cells are switched to ground state medium supplemented with LIF (10ng/ml) and cultured for

ten days. To analyse the experiment an AP stain is performed (Figure 12). None of the tested EpiSC lines is able to reprogram when transfected with control siRNAs. Transfection with Zfp281 siRNA leads to a certain degree of reprogramming. *OEC-2* EpiSC form 11 AP positive colonies and *OEC-4* cells six AP positive colonies. *OEC-2-E* and *O4G-7* EpiSC form one AP positive colony whereas *TNGA* EpiSC are not able to reprogram at all. The result for the *OEC-2* line is unexpected because in the experiment using the transgenic transcription factors to facilitate reprogramming the empty control vectors, which are basically the same cell line as used for this experiment, does not give rise to any reprogrammed cells. This difference may be based on different batches of used media, as diverging results have also been obtained in other experiments if the batch of medium differs. The difference between formed colonies from *OEC-2* and *O4G-7* reflects the general tendency of these lines to reprogram. The result is not convincing even if some reprogrammed colonies emerged. The difference between the individual lines and also between the experiments carried out suggest that Zfp281 knockdown might be able to trigger reprogramming of EpiSC on its own but evidentiary and reproducing results are only obtained if additional signals support the reprogramming.

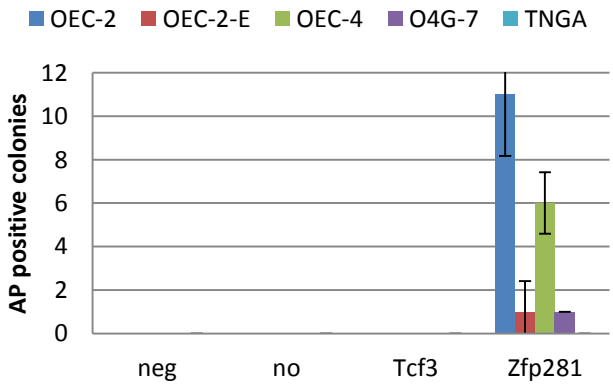


**Figure 12. Influence of Zfp281 knockdown on reprogramming of EpiSC lines without supporting signals**
**Statistics of AP stain performed after reprogramming of different EpiSC lines; Bars indicating the number of AP positive colonies formed from siRNA transfected cells after ten days of reprogramming; Results are the mean of two replicates, expressed as mean ± S.E. Experiment carried out once; blue, *OEC-2* EpiSC; red, *OEC-2-E* EpiSC; green, *OEC-4* EpiSC; violet, *O4G-7* EpiSC; light blue, *TNGA* EpiSC**

## 3.3 Somatic Reprogramming of NSC

The next question to address is if the effect of Zfp281 knockdown on enhanced reprogramming of EpiSC reflects its general reprogramming capacity. For this reason, the influence of Zfp281 knockdown is determined on reprogramming of NSC. The used NSC line expresses transgenic GFP and a Puromycin resistance under the control of the *Oct4* promoter. Different to ESC and EpiSC, NSC are transfected with retroviral particles, as the transient siRNA knockdown will not last long enough to reprogram the cells. The production of retroviral particles and the transfection of NSC

are performed according to Material and Methods page 21. The used particles consist of the *pMXs* vector each carrying one of the pluripotency factor *Sox2*, *Klf4*, *c-Myc* or *Oct4* (Takahashi K. and Yamanaka S., 2006).

Stable Zfp281 knockdown NS cell lines are established for comparable experiments to determine the influence of Zfp281 knockdown on reprogramming. For this reason, the shRNA cassette including the u6 promoter and the Puromycin resistance from the pKLO.1 vector used for stable knockdown cell lines in ESC is cloned into the pMXs vector. As negative control the empty pLKO.1 cassette and for the knockdown the constructs from pLKO.1 #2 and #4, hereinafter called Z2 and Z4, are cloned into the retroviral vector. After transduction of NSC, the knockdown efficiency of the whole transfected cell population is determined by measuring the Zfp281 mRNA level using qRT-PCR (Figure 13A). The empty control does not show a reduction in mRNA level but transduction of NSC with Z2 leads to a reduction of 50% and with the Z4 construct to a reduction of 40%. With these knockdown lines somatic reprogramming is performed.

During establishing the knockdown cell lines, a surprising observation has been made and reproduced with three individual knockdown transductions. Four days after transduction with Z2, but not Z4, retroviruses, few green cells become visible under the fluorescence microscope (Figure 13B). Because the knockdown construct does not consist of a GFP reporter, it has to be the GFP reporter from the NS cell line, which is under control of the *Oct4* promoter. This observation therefore raises the possibility that knockdown of Zfp281 activates Oct4 expression, which may provide the mechanism for the role of Zfp281 in exit and induction of pluripotency. The low frequency of GFP activation in NSC may be due to heterogeneous knockdown efficiencies in the NSC pool and the need for very low Zfp281 levels to allow activation of the Oct4 reporter. To test if GFP activity is correlated with Oct4 expression, an immuno staining (Figure 13B), according to Material and Methods page 26, and a gene expression profiling for Oct4 (Figure 13C) are carried out. The immuno staining for Oct4 does not provide a conclusive result, which may be based on the fact that only very few cells are green and therefore possible Oct4 expressing, so that the staining is not sensitive enough. However a minimal Oct4 staining (Figure 13B, arrow) can be observed. To validate this assumption mRNA levels are determined in the empty control, both knockdown lines and in an ESC line as positive control. Compared to the empty control and the Z4 line, which does not show GFP

positive cells, the Oct4 level in the Z2 line is eight times enriched (Figure 13C). Due to these results, the Z2 knockdown cell line is sorted for GFP positive cells using the MoFlo Sorter (SBeckman Coulter). Only 0.15% of the sorted cells are GFP positive. The sorted cells are immuno stained for Oct4 (Figure 13D) and this time a clear Oct4 staining is observed. An expression profiling is performed again but despite repeated assays, no mRNA level determination could be achieved. This may be based on an incorrect performance of the assay or that eventual mRNA levels are so low that they cannot be detected anymore. A gene expression profile for Zfp281 is also performed in the GFP positive cell population, as well as for the other knockdown cell lines, to see if there is a correlation between GFP expression and level of Zfp281 expression (Figure 13E). Expression levels of empty control, Z2 and Z4 are similar to the initial qRT-PCR results, ruling out a failure in the technique. However, the population sorted for GFP expressing cells shows a 1.7 times higher Zfp281 expression compared to the empty control. This observation is not in line with the assumption that Zfp281 knockdown causes GFP expression, and needs to be investigated further.

The influence of Zfp281 knockdown on somatic reprogramming is tested by individual transduction of the knockdown and the parental NS cell line with pMXs-Oct4 + pMXs-Klf4 + pMXs c-Myc (3 TF) or pMXs-Oct4 + pMXs-Klf4 + pMXs c-Myc + pMXs-Sox2 (4 TF). For transduction particles are used which are produced after 48 hours and after 72 hours of transfection of the packaging cell line *PlatE*. For further information, see Material and Methods page 21. The transduction efficiency is monitored by transduction with pMXc-GFP (Figure 13F). Compared to the GFP negative parental NSC, the pMXc-GFP transduced cells show a strong expression of GFP indicating an effective transduction, especially with particles collected 72 hours after transfection of the packaging cell line. After transduction, the cell lines are cultured in NS medium for three days. Afterwards the medium is switched to Serum+LIF conditions favouring proliferation of cells that start reprogramming. In these conditions cells, which are not able to reprogram stop proliferation and form a dense layer of stretched cells. In comparison, cells starting reprogramming form colonies of proliferative cells. As soon as this phenomenon is observable, the medium is switched to ground state condition medium supplemented with LIF. In a time course of seven to 14 days the proliferative colonies continue to grow, compared to the other cells which are dying, and when reprogramming is successful these colonies begin to express GFP under the control of the *Oct4* promoter (Figure 13G).

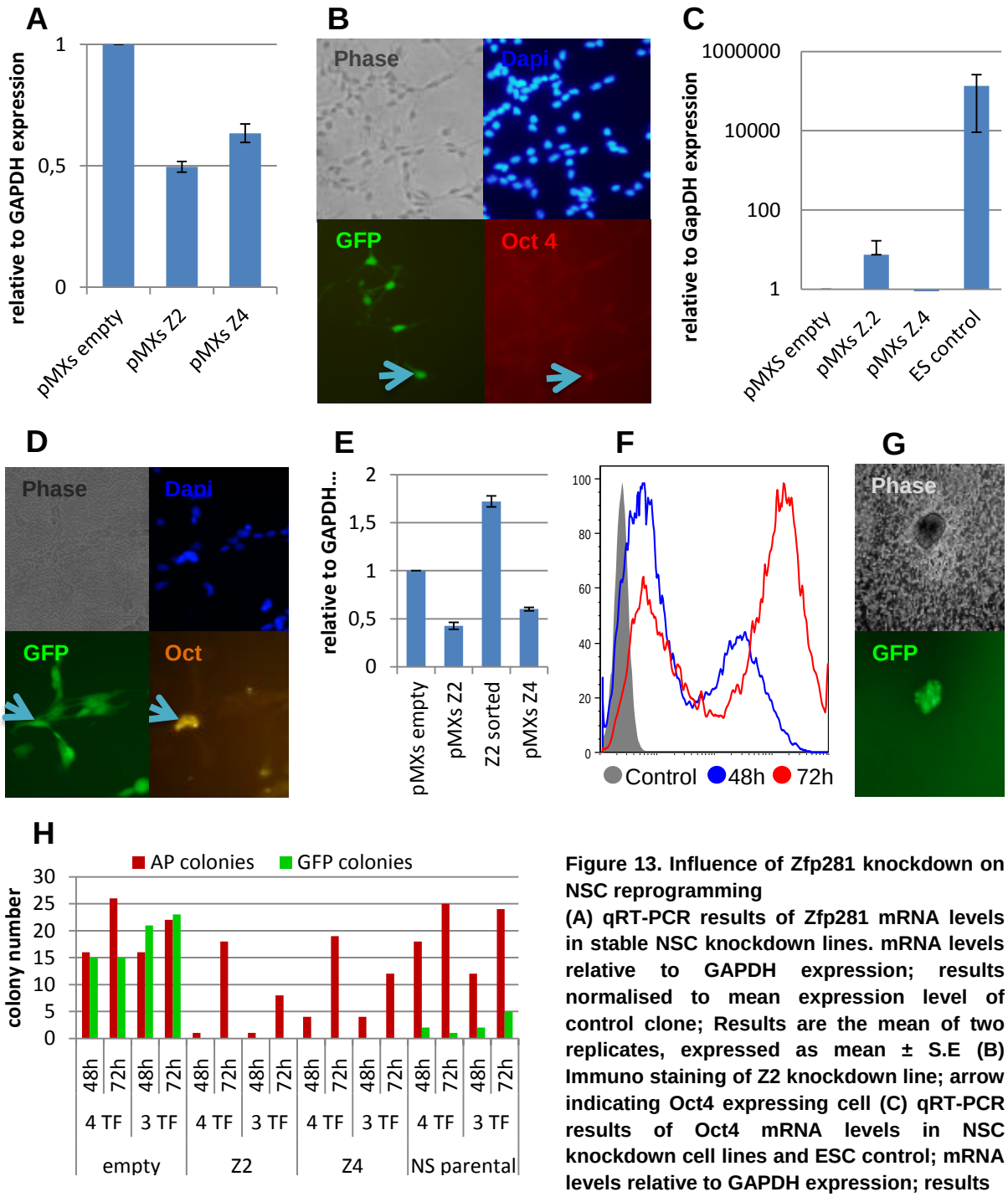


**Figure 13. Influence of Zfp281 knockdown on NSC reprogramming**

**(A) qRT-PCR results of Zfp281 mRNA levels in stable NSC knockdown lines. mRNA levels relative to GAPDH expression; results normalised to mean expression level of control clone; Results are the mean of two replicates, expressed as mean ± S.E (B) Immuno staining of Z2 knockdown line; arrow indicating Oct4 expressing cell (C) qRT-PCR results of Oct4 mRNA levels in NSC knockdown cell lines and ESC control; mRNA levels relative to GAPDH expression; results normalised to expression level of control clone; Results are the mean of two replicates, expressed as mean ± S.E (D) Immuno staining of Z2 line sorted for GFP expression; arrow indicating Oct4 expressing cell (E) qRT-PCR results of Zfp281 mRNA levels in stable NSC knockdown lines. mRNA levels relative to GAPDH expression; results normalised expression level of control clone; Results are the mean of two replicates, expressed as mean ± S.E (F) Flow cytometry histogram of GFP expression levels in NSC transduced with *pMXc-GFP* vector; grey, expression profile of parental NSC; blue, transduction with particles after 48h; red, transduction with particles after 72h (G) Picture of reprogrammed NSC colony (H) Statistics of reprogrammed parental and knockdown NS cell lines; green, GFP positive colonies; red, AP positive colonies; 3 TF, Transduction with Oct4, Klf4, c-Myc; 4 TF, Transduction with Oct4, Klf4, Sox2 and c-Myc**

Reprogramming efficiency is determined by counting GFP and AP positive colonies. At the beginning of reprogramming of the Z2 cell line a strong increase in GFP expressing cells is observable, which is likely because of the phenomenon described above. After 21 days of reprogramming empty vector expressing as well as parental NSC lines give rise to 15 – 25 ESC colonies with both pluripotency factor combinations. However, most colonies in reprogramming of the parental NSC cells failed to express Oct4, indicative of pre-iPS cells, which are partially reprogrammed cells that have not acquired full pluripotency (Silva *et al.*, 2008). The two knockdown cell lines Z2 and Z4 do not give rise to any GFP positive colonies but colonies that show AP activity, which therefore may be pre-iPS cells.

These results suggest Zfp281 is responsible for complete reprogramming of NSC, apparent in the absence of GFP positive colonies in knockdown cell line reprogramming. However, induction of GFP expression in NSC by Zfp281 knockdown as well as a further increase after transduction of the reprogramming factors may indicate that a reduction in Zfp281 mRNA level may favour the initial steps in reprogramming.

## 3.4 Interaction Partner of Zfp281

As little is known about how Zfp281 acts, finding genes with a similar phenotype or directly interacting with Zfp281 could be an indirect way of identifying a mechanism. The tested genes are Ews/Ewsr1, which has been shown to be an interaction partner of Zfp281 *(*Wang *et al.*, 2008) and was a hit in the large-scale screen and its family member Fus, to exclude redundancy. The other tested genes belong to the SWI/SNF complex and have been hits in the large-scale screen, even if only Arid1a was above the threshold defined for selection of high confidence hits (Betschinger J., unpublished data). Furthermore, Arid1a knockout was shown to influence transcription of Zfp281 target genes (Peri Tate, unpublished observations). Further tested SWI/SNF members are Smarca4 and Smarcb1. Members of this complex are promising interaction partners of Zfp281, as recently it has been shown that Zfp281 interacts with another chromatin-remodelling complex – NuRD – to regulate Nanog expression (Fidalgo *et al.,* 2012*)*. However, no NuRD-complex member was identified in the primary siRNA screen.

### *3.4.1 Reproducing Zfp281 Knockdown Phenotype in ESC with Possible Interaction Partners*

The first experiment carried out to identify genes, which might have a similar phenotype as Zfp281, is the differentiation assay using *O4GIP* ESC described above (see 3.1.1, page 29), using negative, GFP, Tcf3 and no siRNA as controls. As expected knockdown of Tcf3 and Zfp281 leads to a massive increase, ten times for Tcf3 and eight times for Zfp281, in surviving cells. This result is also visible in the performed AP stain (Figure 14B). Negative controls do not show a mentionable number in uncommitted cells, apparent in both assays (Figure 14A-B). The plausible interaction partners belonging to the SWI/SNF complex do not show a convincing phenotype in these assays even if they are slightly above the negative controls. Ews and Fus single knockdown are not able to mimic the strong Zfp281 phenotype either. Only a double knockdown of Ews and Fus leads to a phenotype similar to Zfp281 knockdown. The difference between single and double knockdown may be based on redundancy of these two genes.

As for the initial experiments identifying the Zfp281 phenotype, the effect of siRNA-based knockdown of the possible interaction partners is also tested in a second ESC line, namely *Rex1-d2EGFP*. Cells are transfected with siRNAs, differentiated for 24 hours and analysed by flow cytometry (Figure 14C). Cells transfected with negative siRNA show a heterogeneous GFP expression similar to non-manipulated ESC, equivalent to shut down of Rex1 expression, which is indicative for beginning differentiation of the cell population. All cell lines each transfected with one of the target genes show decreased GFP heterogeneity to the point of undifferentiated cells. Double knockdown of Ews+Fus leads to the strongest phenotype observed and very similar to the Zfp281 knockdown phenotype. Transfected *Rex1-d2EGFP* cells are also differentiated for 72 hours and 20% of them are replated on a Laminin coated plate in 2i conditions for 48 hours followed by two days of Blasticidin (1ng/ml) selection. Analysis of undifferentiated cells is performed with an AP stain (Figure 14D). Negative controls show very few stained, uncommitted, colonies, compared to the cells transfected with Tcf3 and Zfp281 siRNAs where most of the cells are undifferentiated. Cells with Arid1a, Smarca4 or Smarcb1 knockdown show a similar result as the negative controls. Nearly no cells are undifferentiated. The same observation is made for Fus siRNA knockdown cells. Only cells with Ews or Ews+Fus knockdown show stained colonies to an amount clearly over negative controls but not as strong as the Zfp281 knockdown.

An observation, which has been made during the time course of differentiation, is the morphology of cells transfected with siRNAs targeting SW/SNF family members (Figure 14E). Cells transfected with negative siRNA show the morphology of differentiating cells, whereas cells with Tcf3 knockdown look similar to cells cultured in 2i. Zfp281 knockdown leads to a cell morphology, which is not as clear as Tcf3, but there is a definite difference to differentiating cells. Arid1a, Smarca4 or Smarcb1 knockdown cells form abnormal clusters of cells with visible attachment problems and unusual high cell death is observable as well. This might be the reason for the result from the replating assay, as during differentiation many cells died and the other cells lose their ability to attach already in the plate where the differentiation step is carried out, so there are hardly any cells left for replating.

The results from the differentiation assays using two different ES cell lines suggest that Ews knockdown might have a similar phenotype to the knockdown of Zfp281, which can be increased by additional knockdown of Fus. This phenotype can be observed in *O4GIP* ESC commitment assays as well as in the Rex1 flow cytometry experiment and the replating assay performed with *Rex1-d2EGFP*. The SWI/SNF complex members do not seem to have a Zfp281 knockdown like phenotype due to the unusual morphology during *Rex1-d2EGFP* differentiation, the resulting replating experiment outcome and the weak results from differentiation assay performed in *O4GIP* ESC, even if the Rex1 profile established by flow cytometry shows delayed differentiation. For these reasons Arid1a, Smarca4 and Smarcb1 are discarded as possible interaction partners of Zfp281.

### *3.4.2 Influence of Interaction Partners on EpiSC Reprogramming*

As Ews seems to have a similar phenotype to Zfp281 in ESC commitment assays, especially in combination with its family member Fus, it has to be determined, if a knockdown of these genes also influences EpiSC reprogramming. For this reason reprogramming assays, using negative siRNA as negative control, Zfp281 siRNA as reference and pooled Ews and Fus siRNAs are carried out as described in Results page 49 *et seq*.

For the initial reprogramming assay the chimeric LIF receptor EpiSC are used. Through the hyperactive JAK/STAT signalling, reprogramming is facilitated which allows easier observations of even small effects of gene knockdown on

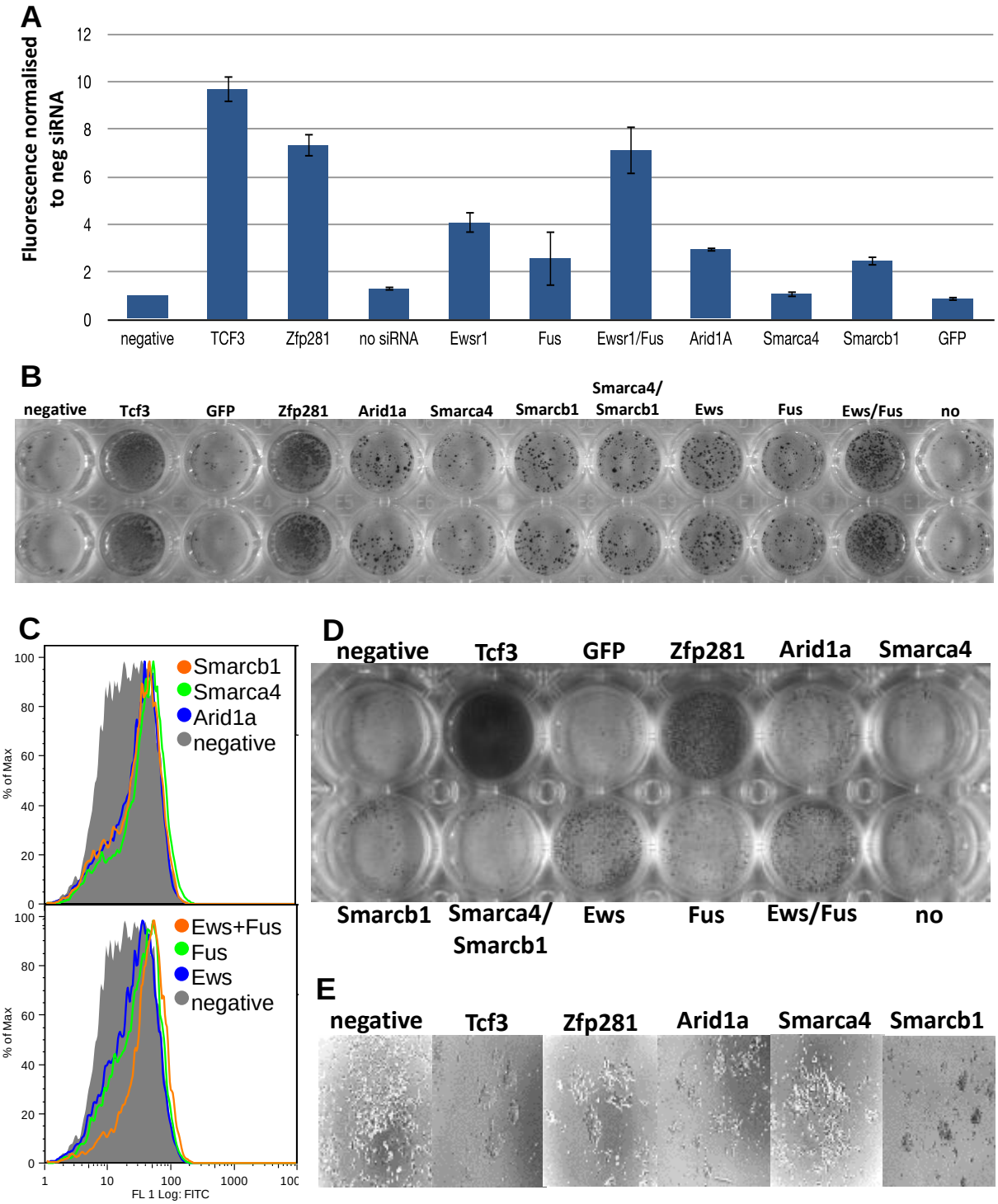


**Figure 14. Phenotype of possible Zfp281 interaction partners in ESC commitment assays**
**(A) Alamar Blue statistics from *O4GIP* ESC commitment assay investigating the influence of gene knockdown in commitment by quantification of living and therefore uncommitted cells. Size of bars indicates amount of living cells relative to living cells transfected with negative siRNA. This correlates with the intensity of commitment delay caused by gene knockdown. Experiment is done in duplicates. Results are the mean of three replicates, expressed as mean ± S.E. (B) Photograph of AP stained plate from *O4GIP* commitment assay. Intensity of dark spots indicates amount of uncommitted cells; experiment done in two replicates. Three individual experiments were carried out with similar results. (C) Flow cytometry histograms showing GFP levels of siRNA transfected *Rex1-d2EGFP* ESC; grey, negative siRNA; upper panel: blue, Arid1a siRNA; green, Smarca4 siRNA; orange, Smarcb1 siRNA; lower panel: blue, Ews siRNA; green, Fus siRNA; orange, Ews+Fus siRNA (D) Photograph of AP stained plate from replated cells after 72 hours of differentiation. Black dots represent stained, therefore undifferentiated, colonies; (E) Photograph of *transfected Rex1-d2EGFP* ESC fifty hours after transfection with indicated siRNAs.**

reprogramming (Figure 15A). Only cells expressing the chimeric LIF receptor stimulated with G-CSF show reprogrammed cells, indicating the dependence on this additional signalling. Compared to negative siRNA the Zfp281 knockdown leads to a 14-fold increase of AP positive colonies. Knockdown of Ews, Fus or Ews+Fus does not lead to an increase of reprogrammed cells compared to negative control. The observed AP positive colonies for Zfp281 and Ews in the cells transfected with empty vector might be false positive stained colonies, as no colonies appear in the chimeric LIF receptor cells without G-CSF, and the empty control cells cannot respond to G-CSF. Ews and Fus knockdown do not seem to enhance reprogramming in the hyperactive JAK/STAT signalling EpiSC. Therefore, a possible influence of the two genes on reprogramming is investigated in the two EpiSC lines (*OEC-2* and *O4G-7*), which express transgenic pluripotency factors to facilitate reprogramming. For this assay, the same siRNAs as for the chimeric LIF receptor EpiSC assay are used (Figure 15B-C). As shown before, knockdown of Zfp281 massively enhances reprogramming efficiency in both cell lines with all transgenic pluripotency factors. Reprogramming efficiency of *OEC-2* EpiSC (Figure 15B) transgenic expressing Klf4 is enhanced with knockdown of Ews (seven times), Fus (six times) and double knockdown (11 times). The double knockdown of Ews+Fus leads to an increase nearly as strong as for Zfp281. Nanog expression in *OEC*-2 EpiSC leads to reprogrammed cells with Ews and Ews+Fus knockdown, whereas the double knockdown leads to the same result as the Zfp281 knockdown, but not with knockdown of Fus alone. Reprogramming of transgenic *O4G-7* EpiSC does not show such a clear result (Figure 15C). Cells expressing Esrrb show a similar amount of AP positive colonies with knockdown of Zfp281, Ews and Ews+Fus double knockdown. However, the absolute amount of AP positive colonies is much lower compared to *OEC-2* EpiSC, which has been shown before. Transgenic expression of Klf4 only increases reprogramming efficiency in combination with Zfp281 knockdown. Knockdown of Ews and/or Fus do not give conclusive results as the amount of reprogrammed colonies is only a little bit higher compared to the negative control. This result suggests that Ews, especially in combination with Fus, knockdown is able to mimic the Zfp281 knockdown phenotype, at least in transgenic *OEC-2* EpiSC, which have shown to be more susceptible for reprogramming. To complete this investigation the influence on reprogramming of non manipulated EpiSC lines, the same as used before, is studied (Figure 15D). Zfp281 knockdown has already been

discussed on page 53 *et seq*. Compared to Zfp281 none of the other knockdowns is able to initiate reprogramming in *OEC-2*, despite the assumption, based on Zfp281 knockdown results, that this cell line is more susceptible for reprogramming. Ews and Ews+Fus knockdown are only able to enhance reprogramming in the *OEC-4* EpiSC line with four respectivly three reprogrammed colonies. In all other cell lines only one reprogrammed colony appears, which is no conclusive result.

These results suggest that Ews may have a similar function in reprogramming to Zfp281. The outcome is less conclusive and real enhancement in reprogramming has only been shown in combination with transgenic expression of pluriptotency factors and only in the *OEC-2* EpiSC line which is easier to reprogram compared to others.

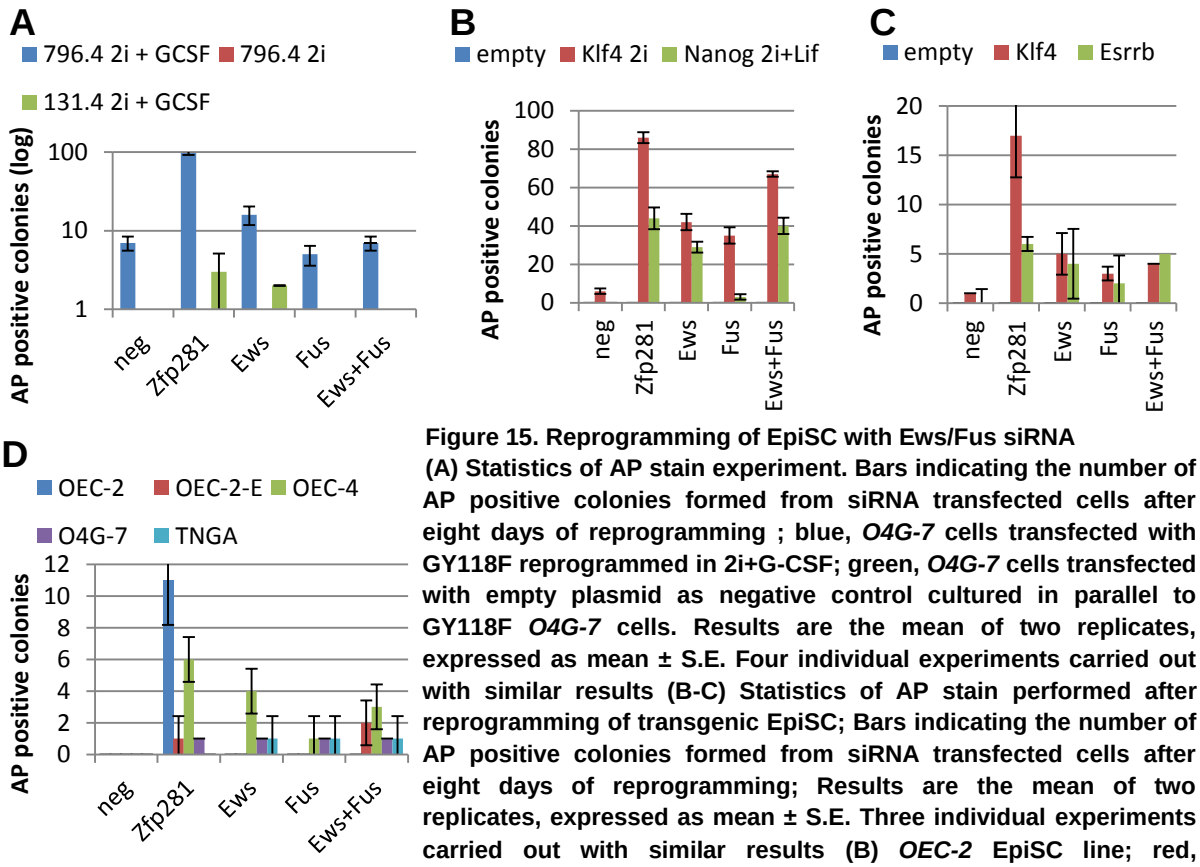


**Figure 15. Reprogramming of EpiSC with Ews/Fus siRNA**
**(A) Statistics of AP stain experiment. Bars indicating the number of AP positive colonies formed from siRNA transfected cells after eight days of reprogramming ; blue, *O4G-7* cells transfected with GY118F reprogrammed in 2i+G-CSF; green, *O4G-7* cells transfected with empty plasmid as negative control cultured in parallel to GY118F *O4G-7* cells. Results are the mean of two replicates, expressed as mean ± S.E. Four individual experiments carried out with similar results (B-C) Statistics of AP stain performed after reprogramming of transgenic EpiSC; Bars indicating the number of AP positive colonies formed from siRNA transfected cells after eight days of reprogramming; Results are the mean of two replicates, expressed as mean ± S.E. Three individual experiments carried out with similar results (B) *OEC-2* EpiSC line; red, transgenic expression of Klf4, reprogramming performed in 2i; green, transgenic expression of Nanog, reprogramming performed in 2i+LIF (10ng/ml); (C) *O4G-7* EpiSC line; reprogramming performed in 2i conditions; red, transgenic expression of Klf4; green, transgenic Esrrb expression. (D) Statistics of AP stain performed after reprogramming of different EpiSC lines; Bars indicating the number of AP positive colonies formed from siRNA transfected cells after ten days of reprogramming; Results are the mean of two replicates, expressed as mean ± S.E. Experiment carried out once; blue, *OEC-2* EpiSC; red, *OEC-2-E* EpiSC; green, *OEC-4* EpiSC; violet, *O4G-7* EpiSC; light blue, *TNGA* EpiSC**

### *3.4.3 Identification of direct Zfp281 Interaction Partners*

Previous experiments have shown that Ews and Zfp281 phenotypes overlap. A Co-Immunoprecipitation is performed, as described in Material and Methods page 22 *et seq.,* to prove if these similarities are based on a direct interaction between those two proteins. For these experiments, the same cell lines used in ChIP experiments (see 3.1.8, page 46 *et seq.*) are used. *Rex1-d2EGFP* ESC expressing 3xFlag-Zfp281 and the Z2.31 3xFlag-Zfp281 rescue cell line, but not an empty vector, show expression of the 3xFlag Zfp281 protein by western blot analysis (protocol in Material and Methods page 24 *et seq.*) using the ANTI-FLAG M2 antibody (Sigma Aldrich, F1804) (Figure 16A). Immunoprecipitation using the anti-Flag antibodies followed by elution with Flag peptide (0.2mg/ml) shows the specificity of the eluation process as only in the cell lines with the 3xFlag construct a band is visible compared to the empty control (Figure 16A). It has been published that Ews is a direct interaction partner of Zfp281 based on mass spectroscopy data (Wang *et al.*, 2008). Furthermore this study and another (Fidalgo *et al.*, 2011) assume that Zfp281 directly interacts with Nanog. Based on this study Nanog is used as a positive control, as for this protein a specific working antibody is available (Anti mouse Nanog; eBioscience 14-5761), which can be used for western blot analysis. For Ews detection a polyclonal antibody is used (C19 Anti-EWS, Santa Cruz, Sc-6532).

Despite repeated experiments, no co-precipitation of 3xFlag Zfp281 with Ews or Nanog can be achieved (Figure 16B). Nanog can be detected in the whole cell lysate of *Rex1-d2EGFP* ESC, which is used as input but not in the eluated fractions. The same result can be obtained with the Z2.31 knockdown rescue cells (data not shown). Ews cannot be detected as none of the bands detected by the antibodies migrates at the predicted molecular weight. To identify the Ews band a western blot is performed with cells after siRNA mediated knockdown (data not shown). This assay does not give conclusive data either, suggesting that the antibodies do not recognise Ews. Because of the inconclusive results from the western blot analysis a silver stain, according to Material and Methods page 25 *et seq*., is performed with Co-IPs from the Z2.31 rescue cell line. This detection method allows for unbiased identification of protein complexes. No specific reproducible bands at the molecular weights of Nanog or Ews are detectable, although 3xFlag-Zfp281 is precipitated (arrow, Figure 16C).

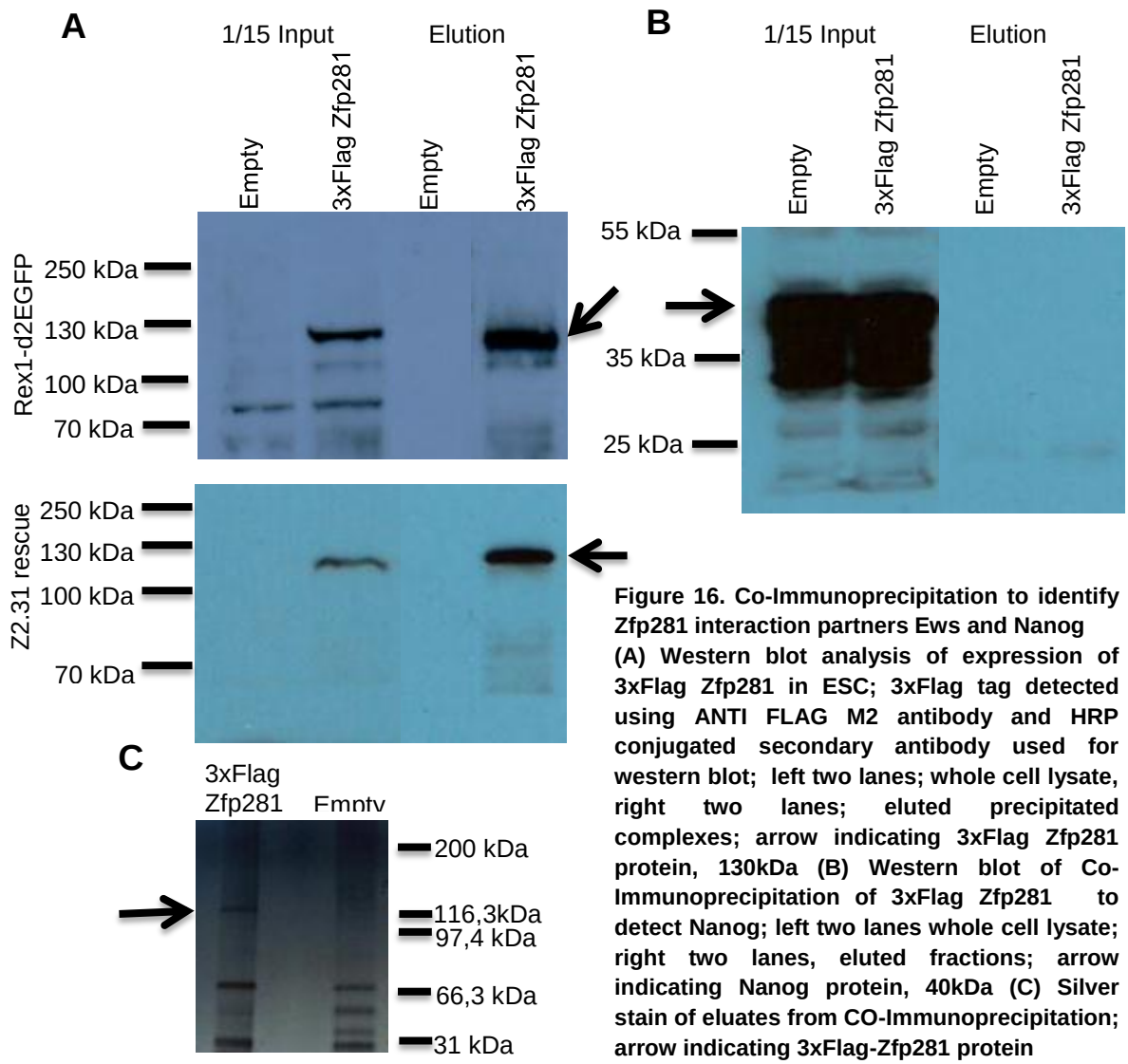


**Figure 16. Co-Immunoprecipitation to identify Zfp281 interaction partners Ews and Nanog**
**(A) Western blot analysis of expression of 3xFlag Zfp281 in ESC; 3xFlag tag detected using ANTI FLAG M2 antibody and HRP conjugated secondary antibody used for western blot; left two lanes; whole cell lysate, right two lanes; eluted precipitated complexes; arrow indicating 3xFlag Zfp281 protein, 130kDa (B) Western blot of Co-Immunoprecipitation of 3xFlag Zfp281 to detect Nanog; left two lanes whole cell lysate; right two lanes, eluted fractions; arrow indicating Nanog protein, 40kDa (C) Silver stain of eluates from CO-Immunoprecipitation; arrow indicating 3xFlag-Zfp281 protein**

# 4 DISCUSSION

## 4.1 Exit from Pluripotency

Transient knockdown of Zfp281 is shown to impair commitment of differentiating ESC. This phenotype is observed with two different ESC lines in different assays. In *O4GiP* cells using a live/dead assay and AP staining and in *Rex1-d2EGFP* cells using quantitation of GFP down regulation during differentiation and replating assays coupled to AP staining. As individual siRNAs also lead to a similar phenotype an off-target effect can be ruled out. While control cells exit naïve pluripotency after 72 hours of differentiation, upon Zfp281 knockdown about 10% of the cells are still uncommitted, as determined by clonogenicity experiments. Impaired differentiation is also observed with stable Zfp281 knockdown in *Rex1-d2EGFP* cells. Flow cytometry and replating assays of differentiating knockdown cells shows a dose dependent effect of Zfp281 knockdown on exit from the ESC state and reveal similar phenotypes compared to transient Zfp281 knockdown. The phenotype observed in the stable knockdown cells is specific for Zfp281, as ectopic expression of a shRNA “immune” Zfp281 transgene partially rescues the phenotypes. This is demonstrated by heterogeneous Rex1 expression and more efficient exit from pluripotency during differentiation. A reason why no complete rescue is observed may be the moderate expression level of ectopic Zfp281. This may be due to the use of cell populations with differences in Zfp281 expression levels in individual cells that may not be sufficient to rescue the phenotypes or cause overexpression-artefacts. Taken together these results suggest that Zfp281 is required for exit from naïve pluripotency.

To place Zfp281 functionally into pluripotency pathways, the impact of Zfp281 knockdown in sensitised ESC culture conditions was determined.

The majority of ESC cultured in Serum+LIF stably expressing Zfp281 shRNA express Rex1 similar to 2i-cultured ESC. This is contradictory to a previous study (Wang *et al.*, 2008) which proposes that Zfp281 is required to maintain pluripotency in cells cultured in Serum+LIF. Control ESC exhibit heterogeneity in Rex1 expression (Wray *et al.*, 2010). Similarly, compared to controls, Zfp281 knockdown cells have a ten times higher propensity to form undifferentiated, homogeneously AP positive, colonies in clonogenicity assays. Expression of the Zfp281 rescue construct reintroduces heterogeneity to control levels. Overexpression of Zfp281 caused by

ectopic expression of Zfp281 in control clones leads to an enrichment of fully differentiated colonies. To determine if Zfp281 genetically interacts with the LIF-signalling pathway, ESC self-renewal is quantified upon reduction of LIF concentrations. Control clones show an increased formation of fully differentiated colonies as a function of reduced LIF concentrations. By comparison, Zfp281 knockdown cells show a higher frequency to form partially differentiated, AP mixed, colonies under limiting LIF concentrations. Even in the absence of LIF, Zfp281 depleted cells mostly form partially differentiated colonies, whereas control cells are not even able to survive in these culture conditions. These findings are consistent with a study using Zfp281 knockout cells (Fidalgo *et al.*, 2011). STAT3 is a potential direct Zfp281 target since Zfp281 has been reported to bind to the STAT3 promoter and STAT3 is derepressed after Zfp281 knockdown (Wang *et al.*, 2008). Furthermore, another study revealed the human ortholog of Zfp281, Znf281, as an interaction partner of the oncogene c-Myc (Koch *et al.*, 2007), which is known to be an important factor in stem cell maintenance (Cartwright *et al.*, 2005) and was identified as one of the original factors mediating induced pluripotency (Takahashi and Yamanaka, 2006). Similar to Oct4, Sox2 and Nanog, c-Myc is a downstream target of STAT3 (Kiuchi *et al.*, 1999; Cartwright *et al.*, 2005; Niwa *et al.*, 2009), suggesting that Zfp281 may coordinate transcription of the pluripotency network through repression of STAT3, and explain reduced LIF dependence on self-renewal observed here. To determine the main signals responsible for the observed heterogeneity and a possible shift of those after Zfp281 knockdown, gene expression profiling of c-Myc, STAT3 and the core pluripotency factors, as well as for lineage markers, which could be repressed after Zfp281 knockdown and therefore lead to reduced heterogeneity, will have to be performed. Additionally, determination of the STAT3/ phospho-STAT3 ratio using western blotting in Zfp281 knockdown cells cultured in Serum+LIF, will possibly reveal the involvement of Zfp281 in activation of STAT3 and explain how Zfp281 interacts with LIF concentration dependence.

A Zfp281 knockdown phenotype is also observed upon withdrawal of either of the two 2i inhibitors. Knockdown cells cultured in the presence of the GSK3 inhibitor only, self-renew as efficiently as ESC in complete 2i medium. This is quantified by flow cytometry and clonogenicity assays where the majority of Zfp281 depleted cells express Rex1-d2GFP and form undifferentiated, homogeneously AP positive, colonies. In contrast, control cells show two distinct Rex1-d2GFP cell populations and

form mostly partially differentiated colonies. These phenotypes can be rescued by expression of a shRNA-resistant version of the Zfp281 transgene. As mentioned for Serum+LIF culture conditions, rescue of the control favours heterogeneity compared to non-manipulated ESC and promotes differentiation. The independence of Zfp281 knockdown cell self-renewal for Erk inhibition may be explained by the already mentioned overexpression of c-Myc, which is able to block Erk signal transduction (Hishida *et al.*, 2011). However, this may also suggest that Zfp281 acts in the MAPK/Erk signalling pathway and it will be interesting to test activation and phosphorylation of Erk as well as transcriptional regulation of MAPK targets.

Knockdown of Zfp281 also promotes self-renewal after withdrawal of the GSK3 inhibitor Chiron, even though cells are not able to survive for more than three passages in the absence of GSK3 inhibition. This suggests that Zfp281 knockdown cannot fully substitute for the GSK inhibition, which would be necessary for cells to survive in these ESC culture conditions. Different from the flow profiles observed in cells cultured with Chiron alone, cells cultured in PD03 do not give conclusive results, indicating that in these conditions cells are not able to maintain their ground state pluripotency. This observation is validated by a clonogenicity assay in which neither cell line is able to form undifferentiated colonies. However, the level of partially differentiated colonies is higher in knockdown cell lines compared to control cells and can be rescued by expression of shRNA resistant Zfp281. The observed differentiation-favouring role of slight Zfp281 overexpression in the controls across the different experiments mentioned above, based on rescue construct expression, have to be confirmed by gain of function experiments.

Taken together, these results show that Zfp281 genetically interacts with LIF and Erk signalling. This suggests that Zfp281 does not act downstream of known pluripotency promoting pathways but may rather act in parallel, thus defining a new module that drives exit from pluripotency and differentiation.

It has been shown that Zfp281 binds the promoter regions of *Nanog* and *Oct4* (Wang *et al.*, 2008; Fidalgo *et al.*, 2011). Therefore, the influence of Zfp281 on different pluripotency transcription factors in 2i conditions and after 24 hours of differentiation was investigated. Unlike a previous study, which proposed upregulation of Nanog and Oct4 after Zfp281 knockout in ESC (Fidalgo *et al.*, 2011) the experiments in this study, similar to Wang *et al.*, 2008, show a decrease in the

expression of all tested transcription factors (Nanog, Oct4, Klf2, Klf4) after Zfp281 knockdown in naïve pluripotent ESC. The differences may be due to different culture conditions. Compared to the Serum+LIF conditions used by Fidalgo and colleagues, experiments in this study were performed in 2i ground state conditions. In Serum+LIF ESC cultures are heterogeneous, reflected in varying expression levels of pluripotency marker genes such as Nanog. In 2i conditions this heterogeneity is reduced significantly (Wray *et al.*, 2011) and therefore the increase in Nanog expression in Serum+LIF cultured Zfp281 knockout ESC (Fidalgo *et al.*, 2011) may be due to decreased differentiation, see Figure 6A, rather than derepression of the Nanog promoter. The reduction in pluripotency factor transcripts after knockdown of Zfp281 in ground state conditions, suggests that Zfp281 rather acts as an activator of the pluripotency network. To test this further it will be necessary to compare gene expression profiling in Serum+LIF and 2i culture. Surprisingly, the same pluripotency factor transcripts, with the exception of Klf4, are increased 24 hours after inhibitor withdrawal compared to controls. It remains to be determined if this is an indirect consequence of blocking exit from pluripotency in the absence of Zfp281, or due to a direct repressive function of Zfp281 on those genes. Consistent with the first hypothesis it is noteworthy that mRNA levels decrease during differentiation compared to 2i conditions even upon knockdown of Zfp281. However, expression of the Zfp281 shRNA immune transgene rescues mRNA expression defects, but in the case of Klf2 and Oct4 mRNA levels are even lower than observed in control cells. This may be consistent with Zfp281 repressing transcription directly.

Taken together it might be possible that Zfp281 fulfils a dual function depending on the cellular context. During self-renewal, Zfp281 may activate and during differentiation repress target genes. Indeed, ChIP-qPCR shows binding of Zfp281 to enhancer regions in selected pluripotency transcription factors, but these studies have to be repeated on a genome-wide level using ChIP-seq in ground state and differentiating cells. The molecular mechanism how Zfp281 may switch from an activator to a repressor will be encoded in cell state specific interaction partners. These will have to be identified in future studies for both ground state and differentiating cells after Zfp281 knockdown. For this reason, CoIPs followed by mass spectrometry will have to be performed to identify even weak interaction partner. The reason for this technique is that western blot detection failed to identify already published interaction partner like Nanog (Wang *et al.*, 2006). It is unlikely that the

used 3xFlag tag interferes with the Nanog – Zfp281 interaction, as it is, similar to the biotinylated tag used in the former study, located at the N-terminus. Furthermore, 3xFlag Zfp281 rescues defects in exit from the ESC state of Zfp281 knockdown cells similar to the wildtype protein. A reason for the discrepancies may be different culture conditions since Wang and colleagues used Serum+LIF compared to the 2i conditions in this study. To exclude this possibility mass spectrometry should also be performed in Serum+LIF conditions.

Above all other experiments, which have to be carried out in the future to determine the exact function of Zfp281 in exit from pluripotency and differentiation, the establishment of knockout ESC and re-performance of the above mentioned commitment experiments using these knockout cells is most important, as these cells do not have any functional Zfp281 left, which could interfere with the results.

## 4.2 Reprogramming

Additionally to impaired differentiation caused by Zfp281 knockdown in ESC, Zfp281 also inhibits reprogramming of EpiSCs. EpiSC, ectopically expressing a chimeric LIF receptor (GY118F) leading to G-CSF dependent activation of STAT3, reprogram to naïve pluripotency in the presence of G-CSF at low frequency, which is dramatically increased after transient Zfp281 knockdown. Thus, Zfp281 coordinates differentiation with exit from pluripotency and similarly stabilises EpiSC against conversion into naïve pluripotency.

Apart from hyperactivated STAT3, ectopic expression of Nanog, Klf4 or Esrrb in combination with Zfp281 knockdown also leads to an enhanced reprogramming efficiency. The additional reprogramming input contributed by Zfp281 knockdown might be based on the already mentioned deregulation of STAT3 and/or c-Myc. It has to be investigated, by gene expression profiling and western blotting, if knockdown of Zfp281 has an enhancing effect on STAT3 activation. Furthermore, since over activation of STAT3-regulation would require an increase in its phosphorylation, Zfp281 may also play a repressive role in the JAK/STAT pathway. Since ectopic expression of c-Myc is not essential for reprogramming from somatic cells, but greatly increases reprogramming efficiency (Nakagawa *et al.*, 2008), it may be conceivable that Zfp281 reprogramming phenotypes are due to transcriptional regulation of c-

Myc. This could be assessed by determining the mRNA levels of c-Myc in control and knockdown EpiSC.

Reprogramming of EpiSC with Zfp281 knockdown alone does not give conclusive results. A great variation in reprogramming efficiencies from different tested EpiSC lines can be observed. None of them shows a reprogramming phenotype nearly as strong as compared with sensitised conditions in the presence of STAT3 signalling or pluripotency factor overexpression. This variation may be due to differences between diverse EpiSC lines. Although EpiSC are classified as a specific cell type derived from the post implantation epiblast (Brons *et al.*, 2007; Tesar *et al.*, 2007), their derivation requires extended culture periods suggestive of culture adaption, which may lead to distinct cell states. This variation is apparent in reprogramming assays using two distinct EpiSC lines derived from the same mouse strain. Ectopic expression of the same pluripotency factors lead to significantly different reprogramming efficiencies. It has also been shown that different batches of culture medium have an influence on EpiSC behaviour, which could be another reason for the varying results in the reprogramming assays. Disregarding the differences observed, it seems likely that Zfp281 blocks reprogramming of EpiSC into naïve pluripotency but that knockdown on its own is not sufficient. It seems likely that Zfp281 acts as a trigger that however needs additional signals for efficient reprograming. To test this further, gain of function experiments have to be carried out to test if Zfp281 overexpression blocks reprogramming in STAT3 activated or ectopic pluripotency factor expressing EpiSC, especially ectopic expression of Nanog as it has been proposed to be repressed by Zfp281 (Fidalgo *et al.*, 2011; Fidalgo *et al.*, 2012). Furthermore, ChIP assays, ideally ChIP-seq, should be performed to see if Zfp281 binds to pluripotency genes, as it has been shown for ESC, and therefore directly acts as a transcriptional repressor, which subsequently stabilises the EpiSC state.

While EpiSC are a convenient cell type for reprogramming to the ESC state, the inhibitory function of Zfp281 needs to be extended to somatic cells. Therefore, the influence of Zfp281 knockdown on reprogramming of NSC was analysed. Due to the duration of somatic cell reprogramming, retroviral transfection with Yamanaka's reprogramming factors (Oct4, Sox2, Klf4 and c-Myc) in stable Zfp281 knockdown NSC had to be performed. Surprisingly a small percentage of knockdown NSC, even in the absence of reprogramming factors, show expression of Oct4, observable with a

transgenic GFP reporter gene under the control of the *Oct4* promoter, Oct4 immunostaining and Oct4 mRNA levels. These observations would indicate that Zfp281 represses Oct4 transcription, consistent with results from ESC commitment and EpiSC reprogramming experiments. However, after sorting Oct4-GFP positive cells, gene expression profiling revealed an increase in Zfp281 mRNA levels, which should not have happened after a gene knockdown. These findings will have to be reproduced and if similar results will be obtained further investigation will be needed. Moreover, ChIP experiments, as contemplated for ESC and EpiSC, would show if Zfp281 could act as a transcriptional Oct4 regulator in NSC. In this study, these sorted cells were discarded, as the mentioned results seem to be debatable and not validated. The observed induction of Oct4 expression is expected to cause higher reprogramming efficiency, as Oct4 overexpression is sufficient to induce reprogramming of NSC (Schoeler *et al.*, 2009). In the first days after transduction with reprogramming factors an increase in GFP positive, therefore Oct4 expressing, cells was observe, suggestive of a reprogramming increase upon reduction of Zfp281. However, while control NSC eventually gave rise to many Oct4-GFP positive iPS cells, Zfp281 knockdown NSC failed to do so. Zfp281 knockdown NSC are only able to reprogram into pre-IPS cells, which are characterised by alkaline phosphatase activity, but negative for reactivation of Oct4. This result suggests that Zfp281 may be necessary for complete reprogramming of NSC, which is contradictory to a recent study, which shows the influence of Zfp281 knockout for final dedifferentiation from pre-iPS to iPS cells (Fidalgo *et al.*, 2012). However, the initial increase in Oct4-GFP expression indicates that absence of Zfp281 facilitates initial steps of NSC reprogramming. To test this hypothesis, an inducible dominant negative form of Zfp281 will be required to delineate the time window Zfp281 is acting. With this knowledge, further reprogramming experiments with NSC and other cell types will be possible and subsequently will help to reveal the cellular function of Zfp281. Furthermore, it has to be validated if the activation of the Oct4 reporter in Zfp281 shRNA NSC is due to knockdown of Zfp281.

## 4.3 Interaction Partners

As the molecular function of Zfp281 is ill defined, interaction partners or genes displaying a similar phenotype would be informative to reveal how, on a molecular level, Zfp281 is involved in the exit of ground state pluripotency. Putative interaction

partners, which have been tested are Ews as it has been previously shown by mass spectrometry to directly interact with Zfp281 and Oct4 (Wang *et al.*, 2008) and its family member Fus, which has been additionally tested to exclude redundancy. Members of this family are multifunctional proteins, with the capability to bind RNA and regulate initiation of transcription via interaction with members of the transcriptional pre-initiation complex as well as with transcription factors. These genes might be able to modulate gene expression from transcription via RNA processing up to transport of mature RNA to the cytoplasm. Furthermore, these genes are frequently found in chromosomal translocations contributing their transcriptional activation domain to the respective oncogenes (Law *et al.*, 2006). The other genes, possibly interacting with Zfp281, belong to the SWI/SNF chromatin-remodelling complex. Their possible interaction seems very similar as Zfp281 interacts with another chromatin-remodelling complex – NuRD – to ensure its physical integrity and to recruit it to the *Nanog* promoter (Fidalgo *et al*., 2012). The tested SWI/SNF complex members are Arid1a, Smarca4 and Smarcb1. All three genes have been hits in the large-scale screen, even if only Arid1a was above the threshold defined for selection of high confidence hits (Betschinger J., unpublished data). Furthermore, acute knockout of Arid1a in ESC leads to down regulation of predicted Zfp281 target genes (Peri Tate, unpublished observation).

Exit from pluripotency assays have shown that a double knockdown of Ews and Fus causes impaired differentiation of ESC similar to knockdown of Zfp281. Yet this result needs to be investigated further as this phenotype has only be achieved using pooled siRNAs targeting both genes, so an off target effect cannot be ruled out at the moment. The double knockdown also enhances reprogramming in an EpiSC line expressing pluripotency factors. However, the phenotype cannot be observed in EpiSC with hyperactivated JAK/STAT signalling. The reprogramming effect has only been validated in one EpiSC line, which has been shown to be more susceptible of reprogramming. The second EpiSC does not show any change in reprogramming efficiency after Ews/Fus knockdown. These results suggests that Ews and/or its family member Fus might be involved in exit from pluripotency, but it is unlikely that it is a direct interaction partner of Zfp281 as it does not recapitulate its full reprogramming phenotype. Additionally the proposed physical interaction between Zfp281 and Ews could not be verified despite repeated experiments, which is most likely because of a non-functional anti-EWS antibody or because of different culture

conditions compared to Wang and colleagues (Wang *et al.*, 2008). Knockdown of the SWI/SNF complex genes during ESC differentiation causes cell death and abnormal cell morphology, which is different to knockdown of Zfp281. Furthermore, another study proposed that overexpression, rather than knockdown of these genes, facilitates reprogramming (Singhal *et al.*, 2010). This suggests that SWI/SNF is unlikely to interact functionally with Zfp281.

To identify functional interaction partners of Zfp281 CoIP experiments followed by mass spectrometry will have to be performed. Potential interaction partner will then have to be tested for their role in commitment and reprogramming in order to delineate the molecular pathways Zfp281 acts in.

## 4.4 Conclusion

In conclusion, this study shows that Zfp281 is required for exit from pluripotency during differentiation of ESC and stabilises the EpiSC state against conversion into naive pluripotency. Due to this dual function, Zfp281 may act as a central checkpoint regulating the interconversion of ESC and EpiSC states. While the molecular function in differentiation and dedifferentiation remains to be determined, it is conceivable that Zfp281 acts as a transcriptional repressor during differentiation to drive down-regulation of pluripotency factors and, consequently, shutdown of the pluripotency network. Similarly, Zfp281 may restrict expression of naïve pluripotent network members in EpiSC to prevent acquisition of ground state ESC pluripotency. Furthermore, this work provides evidence that Zfp281, in ESC, acts as a transcriptional activator, but switches its function to a repressor upon differentiation.

Future studies will have to investigate how Zfp281 acts, for example, with JAK/STAT signalling or c-Myc and how these interactions regulate the pluripotency network. Identification of Zfp281 chromatin-targets, regulation of the ESC/EpiSC transcriptome and determination of interaction partners will be necessary to reveal the cellular function of Zfp281 during differentiation and reprogramming. By extension, deciphering the role of Zfp281 during reprogramming of somatic cell types may contribute to exploit their therapeutic potential and to understand the complex cell fate transition underlying induced pluripotency.

# APPENDIX

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## List of Figures

## Abbreviations

| | |
|---|---|
| 2i | combination of the two inhibitors PD0325901 and CHIR99021 |
| AP | alkaline phosphatase |
| BMP | bone morphogenic protein |
| CMV | *cytomegalic virus* |
| d2EGFP | destabilised enhanced green fluorescent protein |
| DAPI | 4,6-diamidino-2-phenylindole |
| DMEM | Dulbecco's modified Eagle's medium |
| DTT | Dithiothreitol |
| EGF | epidermal growth factor |
| EpiSC | epiblast stem cell |
| Erk | extracellular signal-regulated kinase |
| ESC | embryonic stem cell |
| FGF2/4 | fibroblast growth factor 2/4 |
| G-CSF | granulocyte colony-stimulating factor |
| GFP | green fluorescent protein |
| GMEM | Glasgow minimum essential medium |
| GP130 | glycoprotein 130 |
| GSK3 | glycogen synthase kinase 3 |
| iPS | induced pluripotent stem cell |
| IRES | internal ribosome entry site |
| JAK | Janus kinase |
| LIF | leukaemia inhibitory factor |
| MAPK | mitogen activated protein kinase |
| MEK | mitogen activated protein kinase kinase |
| NSC | neuronal stem cell |
| PBS | phosphate buffered saline |
| PI3K | phosphatidylinositol 3-kinase |
| qRT-PCR | quantitative reverse-transcriptase polymerase chain reaction |
| RISC | RNA-induced silencing complex |
| RNAi | RNA interference |
| SDS | Sodium dodecyl sulphate |
| shRNA | small hairpin RNA |
| siRNA | small interfering RNA |
| SMAD2/3 | mothers against decapentaplegic homolog 2/3 |
| SOCS3 | suppressor of cytokine signalling 3 |
| STAT3 | signal transducer and activator of transcription 3 |
| SWI/SNF | SWItch/Sucrose Non Fermenting |
| TBST | Tris-buffered saline Tween 20 |

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|---|---|
| **May 2011 - Feb 2012** | Diploma Thesis at the WT-CSCR with Professor Austin G. Smith, Title: “Zfp281: A Key Player in Embryonic Stem Cell Commitment?” |
| **Oct 2007 - May 2011** | Studies of Genetics and Molecular Pathology Additional specialisation in Immunology, Pathological Cell behaviour from a Medical Point of View and Hereditary Diseases |
| **Oct 2005 – Sept 2007** | Basic studies in Biology |

## School Education

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| **Sept 1996 – June 2004** | Grammar School, BRG Michaelerplatz 4400 Steyr |
| **Sept 1992 – June 1996** | Primary School, VS Christkindl/Steyr |

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