Identification of stem cell factors

Novel protein-protein interactions and their functions

Helena Johansson
Cover picture: Illustration of protein-protein interactions between Tpt1 and Npm1 in embryonic stem cells using *in situ* proximity ligation assay. Each Tpt1/Npm1 complex detected is visualized by a red dot and DNA is counterstained with Hoescht 33342 (blue). The Tpt1/Npm1 interaction shows a peak during mitosis, as seen in the mitotic cell at the top.

**ISBN**: 978-91-628-8135-1  
**URL**: http://hdl.handle.net/2077/23135

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Printed by Intellecta Infolog AB  
Göteborg, Sweden, 2010
Till alla de som betyder mest för mig

Palmam qui mervit ferat
Abstract

Embryonic stem (ES) cells provide an unlimited source of cells potentially useful for regenerative medicine, however, prior to clinical implementation, additional basic research is needed. This thesis is focused upon different molecular aspects regarding ES cells, primarily by finding novel stem cell protein-protein interactions and their functions.

As functions of a specific protein may be dependent on its interacting partner, identification of such protein-protein interactions is important. Using several different methods, for example in situ proximity ligation assay and co-immunoprecipitation, numerous novel protein-protein interactions occurring in ES cells were found. The same proteins were shown to be involved in several different protein complexes, some of them likely to be part of bigger complexes. Tpt1 and Npm1 were two such proteins found in several different interactions. Tpt1/Npm1 interacted with a prominent peak during mitosis and were proven to be involved in cell proliferation. Individual depletion of Tpt1 and Npm1 resulted in increased levels of markers of the neural and mesodermal lineages, respectively. Further, Npm1 also associated with all three core transcription factors, namely Oct4, Sox2 and Nanog, signifying the importance of Npm1 in ES cells. The Npm1/Sox2 interaction was shown to remain while cells were induced to differentiate into neural lineage, while decreasing in the other differentiation pathways, indicating of an additional role of this protein complex during differentiation to ectoderm. Phosphorylated Ncl was found to interact individually with Tpt1 and Oct4 in a cell cycle dependent manner, speculatively involved in cell proliferation and transcription.

In screening for factors binding to Oct4 proximal promoter, SAF-A was found and subsequently shown to be involved in the transcriptional regulation of Oct4. The binding occurred preferentially to unmethylated Oct4 promoter and was reduced when ES cells were induced to differentiate. SAF-A was also found to interact with RNA pol II as well as STAT3, Oct4 and Sox2.

In conclusion: twelve novel protein-protein interactions, involved in cell proliferation, differentiation and transcriptional regulation, are presented in this thesis.

Key words: embryonic stem cells, Tpt1, Npm1, Ncl, Oct4, Sox2, Nanog, SAF-A, cell proliferation, transcriptional regulation
List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Johansson, H.,** Vizlin-Hodzic, D., Simonsson, T., Simonsson, S.
   Translationally controlled tumor protein interacts with nucleophosmin during mitosis in ES cells.
   *Cell Cycle, 2010:* 9, 2160-2169

II. **Johansson, H.,** Svensson, F., Simonsson, T., Simonsson, S.
    Phosphorylated nucleolin interacts with translationally controlled tumor protein during mitosis and with Oct4 during interphase in ES cells.
    *Submitted manuscript, under revision*

III. Vizlin-Hodzic, D., **Johansson, H.,** Simonsson, T., Simonsson, S.
    SAF-A has a role in transcriptional regulation of *Oct4* in ES cells through promoter binding.
    *Submitted manuscript*

IV. **Johansson, H.,** and Simonsson, S.
    Nucleophosmin is in complex with Oct4, Sox2 and Nanog in ES cells.
    *Manuscript*

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# ACKNOWLEDGEMENTS

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Abbreviations

BrdU 5-bromo-2-deoxyuridine
Cdk cyclin dependent kinases
ChIP chromatin immunoprecipitation
DAPI 4,6-diamidino-2-phenylidole
DMSO dimethyl sulfoxide
EdU 5-ethynyl-2’-deoxyuridine
ES embryonic stem
GFP green fluorescent protein
HMG high mobility group
hnRNP heterogeneous nuclear ribonucleoprotein
ICM inner cell mass
IP immunoprecipitation
iPS induced pluripotent stem
JAK/STAT Janus kinase-signal transducer and activator
LIF leukemia inhibitory factor
Ncl nucleolin
Ncl-P phosphorylated nucleolin
Npm1 nucleophosmin
Npm1-P phosphorylated nucleophosmin
PLA proximity ligation assay
Plk1 polo-like kinase 1
POU pit-oct-unc
qPCR quantitative real-time polymerase chain reaction
RA retinoic acid
Rb retinoblastoma
SAF-A scaffold attachment factor A
SCNT somatic cell nuclear transfer
shRNA short hairpin ribonucleic acid
SRR sox regulatory region
SRY sex determining region Y
SSEA stage-specific embryonic antigen
Tpt1 translationally controlled tumor protein
Introduction

A typical specialized somatic cell has its destiny set to a specific and generally irreversible cell fate. An important question in medicine and cancer biology is whether and how these cells could be programmed to adopt a different cellular fate. The answer would provide knowledge about how cell specialization is reset or maintained, which is of great interest in understanding cancer, and might open the way to personalized cell- and tissue-based treatments for Diabetes Mellitus and degenerative diseases like Alzheimer’s and Parkinson’s. The first step in this process is to reprogram cells toward a stem cell like state. The first experiment demonstrating that a vertebrate somatic cell nucleus could be reprogrammed back into an embryonic state was performed in 1962, when a nucleus was transferred from a differentiated intestinal epithelial cell into a *Xenopus laevis* egg, and generated a cloned embryo that developed into an adult frog\(^1\). This experimental approach was successfully repeated in mammals with Dolly the sheep in 1997\(^2\).

It is equally important to understand how pluripotent stem cells can be developed into desired cell types. Embryonic stem (ES) cells are the perfect model for such experiments due to their capacity of unlimited self-renewal and pluripotency, i.e. having the possibility to give rise to all three embryonic germ layers: ectoderm, mesoderm and endoderm, as well as primordial germ cells. Identification of novel signaling pathways and transcriptional regulatory circuitry, and their role in self-renewal capacity and pluripotency are essential for understanding early development as well as discovering the therapeutic potential of ES cells. In this thesis, we have identified important stem cell factors by finding novel protein-protein interactions and their functions. Depending on interaction partners, most proteins can be involved in several different cellular mechanisms, and it is therefore important to find and characterize these interactions to explore more aspects regarding pluripotency.

Embryonic stem cells

ES cells offer a unique opportunity to study early development and hold great promise for regenerative medicine, drug development and delivering gene therapies. ES cells are derived from the inner cell mass (ICM) of developing blastocysts of pre-implantation embryos, and can be propagated indefinitely in culture in an undifferentiated state while maintaining the capacity to produce any cell type in the body, i.e. they are pluripotent (Figure 1). The first generated ES cell line originated from a mouse\(^3\)\(^-\)\(^4\) and it took almost 20 years before human ES cell lines were derived from donated *in vitro* fertilization leftover embryos\(^5\). Ethical issues about terminating embryos for creating ES cells lines is a major concern, which led to the development of a technique to create murine ES cell lines from single-cell embryo biopsy\(^6\), similar
to the method used for genetic defects screening. The same technique has also been proven successful to create human ES cells without embryo destruction. Furthermore, single-cell embryo biopsy provides an important route to obtain ES cell lines with specific genetic defects, which can be used for drug screening and potentially in the future also for genetic therapies.

The generation of murine ES cells led to an enormous breakthrough in determining gene functions, with site-directed mutagenesis by gene targeting in ES cells, that could be reintroduced back into an embryo, giving the opportunity to create null mutants, hypomorphic mutants, introduce reporter genes to follow gene expression, as well as the possibility to study deletion of a gene in a specific organ or at a specific period during development. Equally important was the fact that one could also do site-directed corrections in ES cells with specific mutations. Some indications of what ES cells might be able to do for us in the future arise from different mouse disease models. For example, by transplanting ES cell derived dopaminergic neurons into a Parkinson’s disease mouse model, the disease phenotype was corrected. One must bear in mind that ES cells need to be efficiently differentiated into the desired cell type and undifferentiated ES cells need to be eliminated from the differentiated cells, to reduce the risk of tumor development. It is therefore important to find distinctions between pluripotent and differentiated cell states.

![Figure 1. Derivation of ES cells from blastocysts and their pluripotent capacity. After fertilization the egg cell begins to divide, first synchronized i.e. all cells are similar, creating a morula. Then two different cell types emerge giving rise to the blastocyst, namely trophoderm and ICM. In vitro culture of ICM results in undifferentiated ES cells. Such cells have unlimited self-renewal capacity and ability to differentiate toward all three embryonic germ layers.](image)
ES cell characteristics

ES cells have three distinct hallmarks, 1: unlimited self-renewal by symmetrical cell-division, 2: capacity to differentiate into all cell types in the body, and 3: ability to contribute to functional tissue generation when incorporated back into embryos. Beside the above, ES cells have several additional characteristics: high nucleo-cytoplasmic ratios, large nucleoli, rapid cell proliferation, positive staining for alkaline phosphatase, high telomerase activity, expression of specific pluripotency markers such as Oct4, Nanog, Sox2, Rex1, SSEA1 (murine), SSEA3 (human), SSEA4 (human), TRA-1-60 (human), TRA-1-81 (human)\textsuperscript{12}.

ES cells are tumorigenic when injected into severe combined immunodeficient mice, a property that is lost when ES cells have started to differentiate. In culture, murine ES cells form tight, rounded and multi-layer colonies, while human ES cells form flat and loose colonies\textsuperscript{12}. As mentioned previously, the unlimited self-renewal occurs via symmetrical cell-division (Figure 2). That is a cell-division producing two new identical pluripotent ES cells without any differentiation. Adult stem cells often use asymmetrical cell-division to give rise to both a new stem cell as well as a differentiated cell. It is not known if ES cells ever divide asymmetrically or not.

Signal transduction pathways

Signal transduction pathways, sustained through addition of external factors, are required to keep ES cells in an undifferentiated state. First successful generation of murine ES cell lines required a feeder layer of mitotically inactivated fibroblast in combination with medium supplemented with fetal calf serum\textsuperscript{3-4}. The feeder cells were later shown to be substitutable with the cytokine LIF\textsuperscript{13-14}, while serum could be replaced with bone morphogenetic protein 4\textsuperscript{15}. LIF has been shown to work through the JAK/STAT pathway, where activation of STAT3 plays a central role in the maintenance of pluripotency\textsuperscript{16}. Wnt signaling, through the canonical pathway, has

Figure 2. To maintain an undifferentiated state, ES cells divide symmetrically, giving rise to two new identical ES cells. Asymmetrical cell-division, which is common with adult stem cells, might also occur in ES cells, which would result in one ES cell and one desired differentiated cell.
been shown to enhance the effect of LIF to maintain pluripotent ES cells. Additionally, the transcription factors Klf4 and Tbx3 were recently shown to be involved in connecting a parallel circuit of LIF signaling to the core transcription factors. Murine and human ES cells are shown to respond to different signal transduction pathways. Unlike murine ES cells, human ES cells are maintaining pluripotency without involvement of LIF and instead need supplements of basic fibroblast growth factor 4 and activin as well as suppression of bone morphogenetic protein signaling, to stay in an undifferentiated state. Recently it was reported that the external factors required for ES cell maintenance are used in signal transduction pathways to inhibit differentiation routes rather than for keeping the pluripotent state.

**Human versus murine ES cells**

Differences between murine and human ES cells, like specific markers and signal transduction pathways, might be attributed to cell origins from different developmental stages. Murine epiblast stem cells derived from late epiblast layers of pre-gastrulation stage embryos share many characteristics with human ES cells, like growth requirements of added fibroblast growth factor and activin instead of LIF, and the ability to express trophoblast markers *in vitro*. These similarities could be due to the fact that human ES cell isolation protocols use fibroblast growth factor and activin, which might select against “true” ES cells and instead result in epiblast stem cells. Murine ES cells may therefore originate from an earlier embryonic stage (ICM) compared to human ES cells (late epiblast), even though both types of ES cells are obtained from the blastocyst stage.

**Similarities between ES and cancer cells**

Cancer cells share many properties with ES cells including the phenotype with large nucleoli and self-renewal in an immortalized fashion. Signal transduction pathways that promote ES cell self-renewal might very well exist also in cancer cells. All the similarities have led to the definition of the term cancer stem cells, with the hypothesis that cancer cells emerge from adult stem cells that become immortalized and taken back towards an ES cell like state. ES cell specific proteins, like Oct4, have been reported to be expressed in certain human tumors, and embryonic gene expression patterns were shown to become reactivated in malignant tumors, additionally strengthening the theory that cancer cells have a stem cell origin. Further, Oct4 is often found in testicular germ cell tumors and was proposed to be a dose-dependent oncogene, since high Oct4 levels were coupled with increased malignancy.
Core transcription factors

Three different core transcription factors have been proven essential for ES cell maintenance, namely Oct4, Sox2 and Nanog. They co-occupy a substantial portion of their target genes and form together a gene expression program consisting of both induction as well as repression of genes necessary for self-renewal, pluripotency or differentiation\(^{28-29}\). Besides their strict regulation pattern with feedforward loops they also perform autoregulatory loops on their own expression (Figure 3). In the subsequent sections, these three core transcription factors will be presented one by one.

![Figure 3. Autoregulation of the three core transcription factors. All three core transcription factors are involved both in their own as well as the others regulation of expression. Oct4/Sox2 bind and regulate all three proteins as a complex, while Nanog binds and regulates all three proteins by itself.]

Oct4

Oct4 (also known as Pou5f1, Oct-3, Oct3/4, Otf3 or NF-A3) belongs to the pit-oct-unc (POU) transcription factor family. POU transcription factors were originally identified as DNA-binding proteins that are able to activate the transcription of genes containing an octameric sequence, called the octameric motif 5’-AGTCAAAT-3’, within the regulatory regions\(^{30}\). Oct4 is the first transcription factor to be expressed during mammalian embryonic development. Oct4 is detected already in oocytes, stays activated during morula stage, gets restricted and peaks in ICM at blastocyst stage, and from E5.5 only being present at low levels in primitive ectoderm, to totally disappear from E9, with the exception of primordial germ cells\(^{31}\). These observations were confirmed at the protein level\(^{32}\). However, instead of decreased Oct4 levels at E5.5, higher Oct4 levels were observed compared to ICM, as well as that maternally expressed Oct4 was degraded before the end of the two-cell stage\(^{32}\).

Knockout mice deficient in both Oct4 alleles result in peri-implantation embryonic lethality and even though blastocyst embryos appear to be normal, they lack the pluripotent ICM and instead only develop into trophoblast cells when outgrown\(^{33}\). Oct4 is therefore considered to be essential for generating the pluripotent cell population in mammalian embryos, where it is involved in the maintenance of an undifferentiated, pluripotent ES cell state. Surprisingly, Oct4 expression is not
sufficient to maintain undifferentiated ES cells in the absence of LIF\textsuperscript{34}. On the other hand, a precise level of Oct4 is essential to maintain ES cell pluripotency, given that a twofold increase or decrease in Oct4 triggers differentiation towards endoderm/mesoderm or trophectoderm, respectively\textsuperscript{34}.

Although Oct4’s endogenous expression normally is thought to be tightly restricted to ES cells, germ cells and embryonic carcinoma cells\textsuperscript{31,33}, Oct4 expression has also been reported in various types of cancer cells\textsuperscript{25}. When ectopically expressing Oct4 in adult mice somatic tissues it results in dysplastic growths, which are dependent on continuous Oct4 expression\textsuperscript{35}. Otherwise the tumor growths are fully reversible. Immature cells were required for Oct4 to be able to function similarly as it does in ES cells, that is maintaining a stem cell state and preventing differentiation\textsuperscript{35}. Oct4 has also been reported to be important for successful nuclear reprogramming, where distribution and level of Oct4 showed consequences for pluripotency and development of somatic cell clones\textsuperscript{36}. A few years ago, a new method to reprogram somatic cells using retroviral transduction of specific transcription factors further proved that Oct4 is one of the necessary components to succeed with reprogramming cells to a pluripotent state\textsuperscript{37-40}.

**Sox2**

Sox2 belongs to the SRY-related HMG-box family of genes, which are expressed during different embryonic developmental phases, with the HMG domain binding to a specific DNA motif 5′-(A/T)(A/T)CAA(A/T)G-3′\textsuperscript{41-42}. The Sox family of proteins are divided into several groups and subgroups, where Sox2 belongs to B1, together with Sox1 and Sox3\textsuperscript{43}. HMG proteins bind DNA in a unique way at the minor groove of the DNA-helix, resulting in an induction of a strong DNA bend\textsuperscript{42}. This property has led to the hypothesis that Sox proteins function partly by organizing local chromatin structures, enabling other transcription factors bound at adjacent DNA sites into biologically active complexes, or facilitating the interaction between distant enhancer nucleoprotein complexes with the basal transcription sites\textsuperscript{41-42}. To perform this function, the Sox proteins seem to require interactions with different partners and only then can they form a stable complex with the target DNA\textsuperscript{43}. The different Sox-interaction partner complexes are likely to be involved in the regulated fashion of Sox proteins being expressed and active during most of the embryonic developmental phases\textsuperscript{41-43}.

During embryonic development Sox2 is first detected at morula stage, followed by specific expression in ICM during blastocyst stage, and from E7.5 the expression is further restricted to neuroectodermal progenitor cells and primordial germ cells\textsuperscript{44}. Knockout mice depleted of both Sox2 alleles are embryonic lethal and die shortly after implantation, although appearing normal at blastocyst stage\textsuperscript{44}. However, without
Sox2 the epiblast does not form and only trophoblast giant cells survive from what normally would be ICM\textsuperscript{44}. In accordance with this, it has been shown that Sox2 depleted ES cells differentiate to mature trophoblast cells, but this could be inhibited by overexpressing Oct4\textsuperscript{45}.

Somewhat contradictory reports about Sox2 overexpression exist, one claiming that overexpression of Sox2 does not affect self-renewal or the undifferentiated state of ES cells, nor affect their differentiation capacity when induced to differentiate by LIF deprivation\textsuperscript{46}. However, withdrawal of both serum and LIF suppressed mesodermal and endodermal lineage formation, and only neural phenotypes were developed\textsuperscript{46}. Contradictory, it has been shown that just a small increase in Sox2 levels induced ES cells to differentiate toward several different cell types even in presence of LIF\textsuperscript{47}. This is supported by that elevation of Sox2 levels inhibited endogenous expression of several Sox2:Oct4 target genes, including all three core transcription factors\textsuperscript{48}.

**Nanog**

Nanog is the latest addition of the three core transcription factors, found by two independent groups in 2003\textsuperscript{49,50}. It is named after “Tir nan Og”, which comes from the Celtic mythology, with the meaning: land of the ever young\textsuperscript{49}. Nanog is a homeobox domain containing protein with a serine rich motif in the N-terminal and a tryptophan at every fifth position in the C-terminal\textsuperscript{49}. These pentapeptide repeats constitute a potent transactivation subdomain, that together with another subdomain in the C-terminal are essential for Nanog to act as a transcription activator\textsuperscript{51}. Nanog shows low homology with other homeobox domain protein families, with the NK2 family of homeoproteins as its closest relative\textsuperscript{49}.

During embryonic development, Nanog expression is first detected in the interior cells of the morula, get maximal levels of expression between late morula and mid blastocyst stage, where Nanog first is located to ICM and absent from trophectoderm, to get further restricted into epiblast cells, and disappears at implantation\textsuperscript{49}. Knockout mice deficient in both Nanog alleles are embryonic lethal and fail to develop beyond blastocyst stage since they cannot develop the epiblast\textsuperscript{50}. At E3.5 they are indistinguishable from normal embryos but ES cells derived from Nanog null blastocysts do not stay as undifferentiated cells, instead completely differentiate into endoderm-like cells\textsuperscript{50}. In accordance with this, it has been shown that RNAi depletion of Nanog in human\textsuperscript{52} and murine\textsuperscript{53} ES cells result in induction of extraembryonic endodermal lineages. However, another study found that depletion of Nanog in ES cells did not affect the undifferentiated cell state upon continous passaging, although slower proliferation and flatter morphology were observed\textsuperscript{54}. The reduced proliferation could be explained with that Nanog has been implicated in G1 to S transition, where Nanog overexpression results in quicker cell cycle
progression through accelerated S-phase entry by direct binding and regulation of two proteins important for this process\textsuperscript{55}.

Nanog is to some extent very different from the other two core transcription factors. Heterogeneous expression of Nanog is observed in ES cells, where Nanog-high populations only express pluripotency markers, whereas Nanog-low populations also express markers for primitive endoderm\textsuperscript{56}. It has also been shown that overexpression of Nanog is enough to maintain murine and human ES cells undifferentiated in absence of LIF\textsuperscript{49,50} or feeder layer\textsuperscript{57}, respectively. The central role for Nanog in the transcription factor hierarchy for maintaining ES cell identity is further confirmed with its involvement in different nuclear reprogramming aspects\textsuperscript{38,58}, discussed later.

**Transcriptional regulation**

Most cells in the human body have identical genomes, and the thing making a heart cell differ from a skin cell mostly depends on which genes they express. Different genes are active at different stages during development and an adult specialized somatic cell only expresses a minority of genes from the genome. The reason for this divergent expression in different cell types depend on for example: DNA methylations\textsuperscript{59}, chromatin modifications\textsuperscript{60}, and transcription factors either activating or repressing transcription. Gene transcription is regulated at so called \textit{cis}-regulatory regions situated upstream of the transcription start site. These regions contain different binding sites where transcription factors can bind if they are accessible and unmethylated. Aspects about the transcriptional regulation of the three core transcription factors, with focus on \textit{Oct4}, are summarized below.

**\textit{Oct4}**

Regulation of \textit{Oct4} expression has been proven dependent on three upstream \textit{cis}-regulatory regions: the TATA-less proximal promoter, the proximal enhancer and the distal enhancer\textsuperscript{61-62}. The two enhancer regions have been shown to control \textit{Oct4} expression in different types of murine cells, with the distal enhancer active in ICM, ES cells and primordial germ cells, while the proximal enhancer functions in epiblast and embryonic carcinoma cells\textsuperscript{62}. Four conserved regions of homology (termed CR1-CR4) can be found within these three regulatory regions\textsuperscript{61}. CR1 is positioned in the proximal enhancer and consists of a putative Sp1/Sp3 site, a retinoic acid-responsive element (RARE), a steroidogenic factor-1 (SF-1) binding site and a 1A-like site\textsuperscript{61,63-65}. CR4 is located in the distal enhancer and contains an Octamer/Sox \textit{cis}-regulatory element, proven to be required for \textit{Oct4} expression, where Sox2 and Oct4 together regulate the transcription of \textit{Oct4}\textsuperscript{66}. 
Epigenetic control of *Oct4* has also been proven important for its transcriptional regulation. By comparing the DNA methylation status of the upstream region of *Oct4* between ES cells and trophoblast stem cells it was revealed that this region was hypomethylated in ES cells, while hypermethylated in trophoblast stem cells\(^67\). This together with that ES cell regulatory regions were hyperacetylated, and that hyperacetylation and demethylation of NIH 3T3 cells were enough to activate *Oct4*, demonstrate the power of the epigenetic control on *Oct4* expression\(^67\).

**Sox2 and Nanog**

*Sox2* contains at least two regulatory regions, named SRR1 and SRR2\(^68\). The latter has been shown to contain a somewhat unique Octamer/Sox regulatory element, with both of the sequences being different in some aspects to their corresponding consensus sequences\(^68\). Both *Sox2*:Oct4 and *Sox2*:Oct6 can bind to the SRR2 and activate the transcription of *Sox2*\(^68\). Oct6 has increased expression in embryonic ectoderm compared to ES cells, consequently, the *Sox2*:Oct6 complex is probably involved in the regulation of *Sox2* in embryonic ectoderm\(^68\).

*Nanog* also contains an Octamer/Sox *cis*-regulatory element, proven to be essential for its transcription, and *Oct4* together in a complex with either *Sox2*\(^69-70\) or a yet unidentified factor\(^70\) were shown to regulate this transcription in ES cells. Other still unknown regulatory factors are also important for *Nanog* transcription given that *Oct4* knockout embryos still express *Nanog*\(^49\).

**Cell cycle**

ES cells have a very unique cell cycle in comparison with differentiated somatic cells, with their unusual cell cycle structure and rapid cell proliferation as characteristics. The cell cycle consists of four distinct phases: G1, S (DNA replication), G2 and M (chromosome segregation). Generation time for murine ES cells are extremely short and the cells go through the cell cycle in approximately 8-12 hours, primarily owing to a truncated G1 phase giving an ES cell cycle mostly consisting of S phase cells\(^71-72\). Murine ES cells also share many features with transformed cells: they do not undergo senescence or quiescence, are not subject to contact inhibition or anchorage dependence and can multiply in the absence of serum. Human ES cells share most of these properties, but they are less studied than their murine counterpart. Human ES cells also have a truncated G1 phase, but a somewhat longer cell cycle in comparison with murine ES cells, of approximately 15-16 hours\(^73\).
Cdk-cyclin complexes

Somatic cells show a strict activation pattern of Cdk-cyclin complexes to obtain proper transitions from one cell cycle phase to the next in the correct order\(^74\). Each of the gap phases (G1 and G2, respectively) also contain checkpoints to enable detection of DNA damage or nucleotide depletion (Figure 4A). These properties are not shared with murine ES cells. The latter show constitutively high Cdk2-cyclin E and Cdk2-cyclin A levels, undetectable levels of Cdk4-cyclin D, modest levels of cyclin D1 and D3 in complex with Cdk6 and only Cdk1-cyclin B has an activity that is changing during the cell cycle phases\(^71,75-76\) (Figure 4B). Inhibition of Cdk2 activity in ES cells is rate-limiting for cell cycle progression, but surprisingly not affecting the cell cycle structure\(^71\).

Human ES cells differ from murine ES cells in level and distribution of different Cdk-cyclin complexes during the cell cycle with most Cdk-cyclin complexes showing a cell cycle dependent expression\(^77\). Cyclin D2 levels, with a peak during G1/S transition, are rate-limiting for their cell cycle progression\(^78\). Cdk2 has important roles both in cell cycle regulation as well as maintaining the pluripotent phenotype, given that Cdk2 depletion results in cells arrested in G1 as well as decreased levels of several pluripotency markers\(^77\).

Retinoblastoma (Rb) is often considered as a negative regulator of the cell cycle progression and a positive regulator of cellular differentiation. In somatic cells, Rb functions as an obstacle in G1, and Rb needs to be phosphorylated by Cdk2-cyclin E before the cells are allowed to enter S phase\(^79\). In contrast, most Rb in ES cells are hyperphosphorylated during the whole cell cycle and total Rb content is also decreasing from exit of mitosis to entry of S phase\(^72\). This effectively omits early G1 phase by bypassing the restriction point that separates early G1 from late G1 and partly explains the truncated G1 phase of ES cells.

**Figure 4.** Comparison of Cdk-cyclin patterns between somatic cells and murine ES cells. (A) Somatic cells use Cdk-cyclin complexes to get through the cell cycle by a strict activation pattern. (B) Murine ES cells on the other hand have continuously high levels of certain Cdk-cyclin complexes and only Cdk1-cyclin B has a cell cycle changing pattern.
Nuclear reprogramming

There are several possible procedures to achieve reprogramming of an adult somatic nucleus to either an embryonic like state or directly towards a different cell type. The most studied procedures are: somatic cell nuclear transfer, fusion of somatic cells with ES cells and induction of pluripotent stem cells with viral-mediated transduction of different sets of transcription factors. Below, each of these methods will be discussed in detail.

Somatic cell nuclear transfer

The most efficient nuclear reprogramming method is the somatic cell nuclear transfer (SCNT). The first successful cloning experiments in *Xenopus laevis* as well as the creation of Dolly the sheep were obtained using SCNT. This method also offers a potential route to produce patient specific ES cells. This would be accomplished by inserting the nucleus of an already differentiated adult cell, like a patient’s skin cell, into a donated enucleated egg. The egg cytoplasm contains the necessary components that are needed to reprogram the genetic material of the skin cell back towards a pluripotent state and can then be stimulated to divide to blastocyst stage. This is followed by isolation and culturing of ICM resulting in ES cells that are genetically identical with the original skin cell (Figure 5). If the blastocyst instead is implanted into a uterus, known as reproductive cloning, it can result in a cloned embryo as in the case of Dolly the sheep.

*Figure 5.* Illustration of SCNT procedure to create personalized ES cells. A nucleus from a somatic adult cell is placed into an enucleated egg, where it becomes reprogrammed back towards a pluripotent state and then can start dividing, forming a blastocyst, where ICM can be removed for ES cell culture.
Even though this seems really promising and quite easily done, SCNT has certain limitations that require improvement before this method can be used in clinical applications. Depending on how efficiency of SCNT is calculated, it differs from less than 1% up to 80%\(^\text{80}\). A large part of this difference was proven to be the differentiation and epigenetic state of the donor nuclei\(^\text{83}\). A major drawback is also that the SCNT method has not yet been successful with human donor cells to produce ES cell lines. However, a Korean group reported that they had succeeded in generating cloned human ES cells\(^{82-83}\), but it was later shown that these reports were fraudulent with fabricated data. Human cloned blastocyst stage embryos produced via SCNT experiments have been obtained\(^\text{84}\), but non-human primates are still the highest mammal where SCNT has been successful in generating ES cells\(^\text{85}\).

Besides the lack of success in human SCNT, the largest drawback of this method is the need of donated oocytes and the ethical aspects that arise from this. Is it ethically correct to do therapeutic cloning, when the terminated blastocyst could develop into a cloned embryo if implanted into a uterus? Reproductive cloning of humans is banned in most countries and how can anyone be certain that SCNT will not be used to create human clones? Truly difficult and important questions to consider. Hopefully will the benefits, if successful with human cells, overcome the drawbacks. Reports showing the therapeutic potentials of SCNT in mouse models, for example that genetic immune defects could be corrected\(^\text{86}\), imply what may be done in the future of regenerative medicine using SCNT.

**Fusion of somatic cells with ES cells**

Cell-cell fusion occurs spontaneously in both developmental and pathological processes *in vivo* and can either be homo- or heterotypic. This method can also be used *in vitro* to study reprogramming mechanisms, when cell fusion is either chemically induced with polyethylene glycol or occurring spontaneously. Both human and murine ES cells have been shown to possess the capacity of reprogramming somatic cells after cell fusion\(^\text{87-88}\). By fusing an adult somatic cell with an ES cell, one can get hybrid cells that share many characteristics with ES cells. This route could be a good way of creating genetically tailored patient specific ES cell lines to use for biochemical or genetic studies as well as the study and treatment of human diseases. Before it can be used in regenerative medicine one major obstacle needs to be solved, which is that the hybrid cells get a tetraploid DNA content. A pathway of removing single whole chromosomes has recently been developed\(^\text{89}\), but not yet shown to eliminate the obstacle of tetraploidy with hybrid cells. Controversial reports argue about whether the ES cell factors needed for reprogramming of somatic cells exist in the nucleus\(^\text{90}\) or in the cytoplasm\(^\text{91}\), if the latter is true it would remove the problem with tetraploidy. Poor efficiency is, as with all reprogramming methods, another drawback that needs improvement. The number
of formed hybrid colonies were shown to increase by fusing neural stem cells with Nanog overexpressing ES cells, but overexpression of Nanog alone in neural stem cells was not enough to promote reprogramming\(^5\).

**Induced pluripotent stem cells**

The latest breakthrough in reprogramming somatic cells came a few years ago when Takahashi and Yamanaka did the first successful experiments with reprogramming mouse embryonic and adult fibroblasts using viral-mediated transduction of only four transcription factors: Oct4, Sox2, c-Myc and Klf4\(^9\). Soon after, successful experiments were performed with human fibroblasts and other more terminated somatic cells, as well as different sets of transcription factors were shown to work\(^{37-40}\) (Figure 6). These induced pluripotent stem (iPS) cells are similar to ES cells in epigenetic states, morphology, expression of pluripotency markers, cell proliferation and ability to differentiate to all three embryonic germ layers. Pluripotency markers become reactivated at different time-points during iPS cell creation with alkaline phosphatase occurring first, followed by SSEA1, and fully reprogrammed cells occurring with Oct4 or Nanog expression\(^9\).

The major advantages with this method are that no oocytes are needed and that the procedure works with human cells. Even though a major breakthrough and step toward personalized regenerative medicine, some major issues require to be solved before it can be used in clinics. Low efficiency (average 0.001-0.2\%) is one of these problems. A recent report showed that the differentiation stage influenced the reprogramming potential of immature and mature hematopoietic cells\(^9\). The poor efficiency was also shown to partly depend on that reprogramming triggers a stress response with characteristics of senescence\(^9\). Epigenetic silencing of In\(k4a/Arf\) locus\(^9\), or usage of p53 siRNA\(^9\), both strategies leading to immortalization, were also shown to improve the potential to generate iPS cells. The use of oncogenes and retroviral transduction to induce reprogramming factors represent additional serious barriers that need to be solved before it potentially can be used in regenerative medicine. The oncogene c-Myc has been shown to be dispensable for iPS cell creation, although resulting in even lower efficiency\(^9\). New approaches to avoid retroviral transductions are reported continuously: replacing factors with small molecules\(^1\), using adenoviral vectors\(^1\), plasmid transfection\(^1\), recombinant proteins\(^1\) or starting with cells endogenously expressing three of the factors, like neural stem cells\(^1\), are just some examples.

Not long after the initial successful iPS cell creation, the first report on the potential of such cells in personalized treatment of degenerative diseases was published\(^1\). A sickle-cell anemia mouse model was rescued after transplantation with its own corrected hematopoietic progenitors obtained from iPS cell creation\(^1\).
Another report showed that iPS cell derived neurons injected into the fetal brain of rats with Parkinson’s disease improved their symptoms. One must bear in mind that a lot of safety issues, besides the ones already mentioned above, need to be solved before this method can be used in clinics, like elimination of ES cells from the differentiated cells to minimize the risk of tumor formation.

**Specific proteins of interest**

In this thesis we have come to focus upon four different proteins beside the core transcription factors, i.e. translationally controlled tumor protein, nucleophosmin, nucleolin and scaffold attachment factor A, and these following sections will highlight some parts of what previously are known about these proteins, both generally and specifically in ES cells.

**Translationally controlled tumor protein**

Translationally controlled tumor protein (Tpt1, also denoted TCTP, Histamine-releasing factor HRF, Fortilin or P23) shows very low sequence homology with other known proteins. However, a relationship to the guanine nucleotide-free chaperone Mss4 has been observed. Tpt1 is highly conserved and expressed in all eukaryotes and encodes for a hydrophilic protein of 21-23 kDa. It was initially identified due to an abundance in ribonucleoprotein particles in mouse tumor cell lines. Tpt1 contains a highly mobile polypeptide sequence (aa. 39-65), which contains two serine residues that are subject to phosphorylations by polo-like kinase 1 (Plk1).
Tpt1 has been shown to be important for embryonic development. Knockout mice deficient in both Tpt1 alleles are embryonic lethal and dependent on deletion of the entire gene or part of the gene, death occurs around E3.5 or E6.5-E9.5, respectively. The homozygous mutant embryos showed a general growth deficiency in combination with a severely disorganized structure, due both to an increase in apoptosis and a decrease in cell proliferation. Further, proteome analysis of murine ES cell lines has revealed that the presence of Tpt1 is a characteristic of undifferentiated ES cells, with high Tpt1 expression levels being reported in both murine and human ES cells, while neural differentiation is accompanied with a decrease of Tpt1. Tpt1 has also been found to influence Oct4 expression in transplanted somatic cell nuclei. In addition, pretreatment of bovine somatic donor cells with phosphorylated Tpt1 gave a beneficial effect in SCNT experiments, resulting in an increased efficiency to develop normal cloned calves.

No primary function of Tpt1 is known, but it has been shown to be calcium and microtubule binding, important for cell growth, cell cycle progression, malignant transformation, inflammatory processes and anti-apoptotic. Accumulating evidence suggests that Tpt1 has an essential role in cell cycle progression. Overexpression of Tpt1 results in decreased growth rates together with a delayed cell cycle progression, whereas overexpression of a Tpt1 double mutant, in which the two Plk1 phosphorylation sites have been substituted for alanines, induced an increase in multinucleated cells, rounded cells with condensed ball-like nuclei and apoptotic cells. Lower cell proliferation was also observed in prostate and colon cancer cell lines after knockdown of Tpt1 using RNA-interference. High levels of Tpt1 are often found in several cancer tissues or cell lines and human Tpt1 has been demonstrated to make cancer cells adopt more malignant phenotypes. It is the one gene that exhibits the strongest differential expression between tumor and tumor-reversed states in human leukemia and breast cancer cells and inhibition of Tpt1 expression results in suppression of the malignant phenotypes. Further analysis strengthened the idea to use Tpt1 as a target for tumor reversion and is now considered a good candidate to use in cancer treatments.

A possible reason for the tumorigenic properties, besides its role in cell proliferation, is that Tpt1 has been proven to be anti-apoptotic. Tpt1 shows a physical and functional interaction with the anti-apoptotic proteins Mcl-1 and Bcl-xL. A possible mechanism for the anti-apoptotic role of Tpt1 together with its two interaction partners have been proposed, where they function through inhibiting the apoptotic protein Bax to perform dimerization.
Nucleophosmin

Nucleophosmin (Npm1, also denoted B23, NO38 or numatrin) belongs to the nucleophosmin/nucleoplasmin family of proteins (together with Npm2 and Npm3) and is a multifunctional nucleolar phosphoprotein. Some of the functions reported for Npm1 are: ribosome biogenesis, nucleo-cytoplasmic transportation, centrosome duplication, embryonic development, cell proliferation, transcriptional regulation, and histone chaperoning. Cell cycle dependent posttranslational modifications, together with different interaction partners, are responsible for Npm1’s involvement in the different functions mentioned above. Cdk2-cyclin E phosphorylates Npm1 during G1, which is proven to be required for correct centrosome duplication. Npm1 residue S4 becomes phosphorylated by Plk1 during mitosis, and interference with this phosphorylation induced multiple mitotic defects, including fragmentation of nuclei and incomplete cytokinesis. Further, acetylation of Npm1 by p300 is involved in enhanced chromatin transcriptional activation.

Npm1 has been proven essential for embryonic development given that knockout mice deficient in both Npm1 alleles are embryonic lethal and die around E11.5-E12.5, due to severe anemia. The homozygous embryos had reduced size together with an incomplete frontal brain organogenesis with a total absence of the eye. Npm1 are expressed at high levels in both murine and human ES cells. Depletion of Npm1 in ES cells using RNA-interference, resulted in reduced cell proliferation, while Oct4 and Nanog levels were unaffected. Regarding cell proliferation, several reports in other cell systems have come to the same conclusion, namely that Npm1 levels are proportional to the cell growth rate. Npm1 is also involved in transcriptional regulation, both by activating and repressing transcription. No specific Npm1 binding site has been found, so the most likely explanation for its role in transcriptional regulation is that Npm1 functions as a histone chaperone that can remodel the local chromatin structure.

Npm1 is often found overexpressed in different types of tumors, and is therefore proposed to serve as a marker for ovarian, gastric, colon and prostatic carcinomas, at the same time as it is one of the most frequent targets for genetic alterations in hematopoietic tumors. Contradictory reports exist if Npm1 functions as a tumor suppressor or an oncogene. Overexpression of Npm1 results in malignant transformation, probably due to that Npm1 and the oncogene c-Myc directly interact, and Npm1 was proven to function as a key cofactor to c-Myc to induce hyperproliferation and transformation of normal cells. Npm1 has also been implicated in the control of chromosomal ploidy and DNA repair, as well as to function with the tumor suppressor protein p53.
Nucleolin

Nucleolin (Ncl, also denoted C23), one of the most abundant non-ribosomal proteins in the nucleoli, belongs to a large family of RNA binding proteins and is a multifunctional phosphoprotein. It has been implicated in functions like chromatin remodeling, ribosome biogenesis, cell proliferation, DNA replication, nucleogenesis, transcriptional regulation and nucleo-cytoplasmic transport. Ncl is a substrate to several kinases, which are regulating its functions and localization during the cell cycle. CK2 extensively phosphorylates Ncl serine residues during interphase, while cdc2 phosphorylates Ncl threonine residues during mitosis.

Rapidly dividing cells have functionally hyperactive Ncl compared to nondividing cells. Consequently, it is not surprising that high levels of Ncl is found in tumors and in other rapidly dividing cells, such as ES cells. A contributing explanation for this is that the stability of Ncl is increased in proliferating cells by inhibiting its self-cleaving activity. In ES cells, the nucleolar protein LYAR was shown to participate in this inhibition by interacting with Ncl. Accumulated evidence show that Ncl plays an important role in cell proliferation. Depleting Ncl using RNA-interference results in reduced cell growth rate and increased apoptosis in ES cells. In other cell systems, Ncl has been proven to be required for correct mitosis, given that the absence of Ncl shows an extended cell cycle with misaligned chromosomes and deficiency in spindle organization. Absence of Ncl has also been shown to result in growth arrest, accumulation in G2, increased apoptosis, nuclear alterations, as well as defects in centrosome duplication.

One additional function of Ncl is to participate in chromatin remodeling, where Ncl has been proposed to be involved in both induction of chromatin condensation as well as decondensation by binding to histone H1. This property might explain why Ncl has been found to interact with or be a component of several transcription factor complexes, both activating and repressing transcription. One of the transcription factor complexes is the B cell-specific transcription factor LR1, composed of Ncl and hnRNP D. LR1 has been shown to regulate c-Myc transcription in B-cell lymphomas, and Ncl has independently been found to be a c-Myc target gene, where c-Myc induces Ncl levels.

Scaffold attachment factor A

Scaffold attachment factor A (SAF-A, also known as heterogeneous nuclear ribonucleoprotein-U, hnRNP U) is a nuclear protein belonging to the hnRNP family, which constitutes of more than 20 different proteins, preferentially involved in RNA processing. Two isoforms exist of human SAF-A, yielded by alternative splicing, both binding to single- and double-stranded DNA and RNA as well as to the scaffold attached region element MII. SAF-A contains clustered repeats of Arg-Gly-Gly...
tripeptides, termed the RGG box, which were shown to be the required region for its RNA binding activity\textsuperscript{159}. During apoptosis SAF-A’s DNA binding domain becomes cleaved, resulting in loss of DNA binding activity together with detachment from nuclear substructures and possibly also contributing to nuclear breakdown\textsuperscript{160}.

No knockout mice exists with SAF-A, although a hypomorphic mutant results in embryonic lethality\textsuperscript{161}. Homozygous mutated embryos showed abnormalities at E6.5 and were resorbed by E10.5\textsuperscript{161}. Cell lines isolated from homozygous mutants showed that SAF-A levels were 2-5 times lower compared to wild-type cells, suggesting that specific amounts of SAF-A is required for normal embryonic development\textsuperscript{161}. It has been proposed that SAF-A enhances the expression of certain genes by stabilizing their mRNA\textsuperscript{162}. Other studies suggest that SAF-A is involved in transcriptional regulation, like SAF-A binding to the promoter of the ES cell important protein Klf2 together with hnRNP D, p300 and PCAF\textsuperscript{163}. SAF-A and p300 were independently found to interact, accompanied with local acetylation of nucleosomes possibly poising nontranscribed genes to get ready for transcription\textsuperscript{164}.

A functional association was reported between SAF-A and β-actin in the nuclei of HeLa cells, probably involved in the transcription of RNA pol II\textsuperscript{165}. In the same study they showed that the complex also interacts with the CTD of RNA pol II\textsuperscript{165}. PCAF was later shown to interact with SAF-A and β-actin both at the RNA pol II promoter as well as with the CTD of RNA pol II\textsuperscript{166}. The binding of SAF-A to RNA pol II have independently been shown to work as an inhibitor of CTD phosphorylation and therefore thought to function as a repressor of the elongation process\textsuperscript{167}.
Aspects of methodology

Confocal microscopy (Paper I-IV)

Confocal microscopy is an important tool for a wide range of investigations in biological and medical sciences, used for imaging thin optical sections in living and fixed samples. It offers several advantages over conventional widefield optical microscopy, including the ability to choose focal plane where the image is taken, elimination or reduction of out-of-focus background fluorescence, as well as the capability to collect serial optical sections from thick samples.

In this thesis we used an inverted Zeiss LSM 510 META confocal microscope equipped with a Zeiss image processing system to analyze all of our fluorescently stained ES cells. An 63x/1.4 NA oil objective and sequential scanning with narrow band-pass filters were used (420-480 nm for DAPI or Hoechst 33342, 505-530 nm for Alexa 488 and 560-615 nm for Alexa 555). Sequential scanning of various fluorophores reduces possible crossover and bleed-through, which can be a significant problem with simultaneous multiple-wavelength excitations.

Colocalization analysis (Paper I-II)

Colocalization is a quantifying tool used to analyze the degree of association and codistribution of stained proteins or structures in a sample, by counting their overlapping pixels.

Confocal micrographs were collected at 0.38 µm intervals to create z-series image stacks. Images rendered from the z-series were analyzed for changes/differences in colocalization with BioPix iQ 2.0 software. A minimum of five different z-series image stacks, containing at least 10 cells each, were taken for each analysis. The software calculates Pearson’s correlation coefficient, the number of overlapping pixels as well as the total number of pixels in each channel. Pearson’s correlation coefficient describes the degree of overlap between the pattern of two labeled proteins, with resulting values ranging between -1 and 1. Values between -1 and 0 indicate some form of inverse relationship between the two channels, while values between 0 and 1 indicate a proportional degree of overlap, with 1 showing a complete correlation and 0 representing no correlation. Using the obtained pixel values we calculated percentage overlap of each protein with the formula:

\[
\text{Overlapping pixels} \quad \frac{\text{Percentage overlap}}{\text{Total number of pixels}}
\]

There are a lot of different aspects that need to be taken into consideration when preparing and analyzing colocalization experiments. Antibodies need to be raised in
different species and not be cross-reactive. Fluorochromes should also have well-separated spectrums. Confocal microscope settings as well as proper control samples are needed to ensure that no bleed-through or autofluorescence will give false results.

**In situ proximity ligation assay (Paper I-IV)**

*In situ* proximity ligation assay (PLA) is a recent method to investigate protein-protein interactions and to visualize their cellular localization\(^\text{168}\). The methodology detects both heavy and light chains of IgG antibodies whereas only light chain of IgM antibodies are detected, making IgG more suitable than IgM. The major advantages with this method are the ability to visualize where the protein complexes are located within the cells, and that even low amounts of protein-protein interactions can be detected.

Prior to *in situ* PLA, cells are fixed with paraformaldehyde, permeabilized with the detergent Triton X-100 and blocked with proper serum solution. *In situ* PLA (Duolink) can be described in six different steps (Figure 7). 1, Incubation with two primary antibodies, which must have been raised in different species, specific to the proteins of interest. 2, Addition and incubation of secondary antibodies conjugated with oligonucleotides (PLA probes). 3, Hybridization step with addition of oligonucleotides that hybridize the two PLA probes together if they are in close proximity. 4, Ligation step where Ligase is added and closes the circle. 5, Amplification step where nucleotides (not shown) and Polymerase is added and starts a rolling circle amplification creating a concatameric product with one PLA probe as a template. 6, Detection step where small oligonucleotides labeled with Alexa 613 fluorescence is added and hybridize to the rolling circle amplification product, making every detected protein-protein complex appear as a red dot when visualizing with confocal microscopy. Hoechst 33342 is added in the last step to counterstain DNA.

![Figure 7. Illustration of the different steps in situ proximity ligation assay (PLA):](image)

1: Primary antibodies
2: Secondary antibodies with attached PLA probes
3: Hybridization
4: Ligation
5: Amplification
6: Detection
Inhibition assay (Paper I)

In situ PLA can be used in combination with different cellular treatments against specific posttranslational modifications to analyze if the modifications are involved in regulating the protein-protein interaction of interest.

To analyze the involvement of different kinases on newly found protein-protein interactions, ES cells were treated with two different Plk1 inhibitors (BI2536 or wortmannin) and one Cdk-cyclin inhibitor (purvalanol A) prior to in situ PLA analysis. Each experimental setup contained an internal control for individual comparison, i.e. one treated and one non-treated sample, to eliminate experimental variances from different experiments.

Cell proliferation (Paper I)

Cell proliferation assays are used to examine the number of cells still being able of synthesize new DNA after different treatments. This was examined using Click-it™ EdU Imaging Kit (Invitrogen), which is a novel alternative to BrdU for measuring DNA synthesis. BrdU requires both DNA denaturation and harsh permeabilization steps, which may affect the morphology as well as the staining with additional antibodies. EdU has the advantage of not requiring DNA denaturation since it uses small molecules for detection and consequently not affect the additional antibody staining.

EdU at final concentration of 10 µM was added and incubated in 37°C for two hours, to cells transfected with different constructs, to incorporate in newly synthesized DNA. EdU incorporation was detected according to manufacturer’s protocol with the additional antibody detection step included to visualize GFP positive cells. GFP positive cells were manually counted as either proliferating (EdU positive) or none proliferating (EdU negative) and compared to empty vector (pEPI-GFP) or GFP tagged negative shRNA control.

Transfection (Paper I and III-IV)

Transfecting cells with either vectors or shRNA is a popular tool to study the effects of overexpression or depletion of proteins.

ES cells are difficult to transfect. However, according to the manufacturer’s standard protocol, Lipofectamine LTX (Invitrogen) together with transfecting cells four hours post seeding, gave proper transfection results. Twenty four hours post transfection selection was started by adding puromycin to wells containing shRNA constructs with puromycin selection.
Aims

ES cells hold great potential in regenerative medicine, although many molecular mechanisms are still unsolved. The overall aim of this thesis has been to identify stem cell factors that are important for ES cells. Main focus has been on finding novel protein-protein interactions as well as identifying their functions.

Specific aims of the project:

- To understand more about Tpt1 regarding interaction partners as well as cellular functions (I, II, IV).
- To understand how the ES cell important protein Oct4 is regulated (III).
- To find novel ES cell specific protein-protein interactions (I-IV).
Results and discussion

Tpt1/Npm1 interaction is involved in cell proliferation of ES cells (Paper I)

Poor efficiency of reprogramming somatic cells using different methods require more basic investigations to identify additional proteins that increase the effectiveness of the reprogramming route. Tpt1 could be such a protein since it has been found to influence Oct4 expression in SCNT procedures\(^\text{117}\), as well as increasing efficiency of successful reprogramming of bovine somatic cells by pretreating donor cell nuclei with phosphorylated Tpt1\(^\text{118}\). However, not much is known about Tpt1 in ES cells and since ES cells are different compared to somatic cells in several aspects, we addressed how Tpt1 behaved in those cells.

Tpt1 subcellular localization changes during differentiation

Tpt1 cellular localization in other cell systems has shown contradictory results, with a majority reporting about cytoplasmic localization, although nuclear localization has also been observed\(^\text{125-126}\). Using western blot and immunofluorescence analysis, we found that Tpt1 is detectable in both cytoplasm and nucleus in undifferentiated ES cells, however, to some extent, slightly higher in the nucleus. When cell differentiation is induced, by either LIF withdrawal or addition of RA, both localization and amount of Tpt1 are changing. Total amount of Tpt1 gets decreased, particularly in the nucleus, giving a preferential cytoplasmic localization in differentiated cells. Embryonic carcinoma cells showed the same type of Tpt1 distribution as differentiated cells, with lower total protein levels as well as mostly cytoplasmic distribution. Our findings are in agreement with the reports that Tpt1 levels are decreasing when ES cells differentiate into neuronal lineages\(^\text{116}\), and that high Tpt1 levels are a characteristic of undifferentiated ES cells\(^\text{113}\).

Tpt1 interacts with Npm1 in a cell cycle dependent manner

To explore Tpt1’s role in ES cells, we screened for novel interaction partners using recombinant Tpt1 covalently linked to cyanogen bromide activated sepharose beads, which were incubated with ES cell extract. Potential interacting partners were eluted with increasing ionic strength, separated by SDS-PAGE and stained with coomassie brilliant blue dye. Bands of interest were excised and identified by nano-LC FT-ICR mass spectrometry. In the 0.7 M NaCl elute we identified Npm1 as a potential interaction partner.

We found that Tpt1/Npm1 colocalize in ES cells and that the colocalization is substantially higher in mitotic cells. This observation was quantified using BioPix iQ 2.0 software, which gave high values in all calculated parameters indicating good colocalization between the two proteins. However, significantly higher colocalization in mitotic cells compared to interphase cells were observed. Induced cell differentiation by treatment with RA for up to 72 hours resulted in an approximately
linear decrease in colocalization. To explore the interaction in further detail in situ PLA was used. Three different antibody combinations all gave consistent results; Tpt1/Npm1 interacts most prominently during mitosis but also exists in interphase cells. Additionally, Npm1 was co-immunoprecipitated using anti-Tpt1, further strengthening that endogenous Tpt1 physically interacts with endogenous Npm1 in ES cells.

**Tpt1/Npm1 interacts independently of Plk1 phosphorylation**

Since Plk1 previously has been shown to phosphorylate both Tpt1 and Npm1 during mitosis we wanted to investigate if the Tpt1/Npm1 interaction is regulated by Plk1 phosphorylation. To this end we treated ES cells with two different Plk1 inhibitors: wortmannin and BI2536. Wortmannin treatment, which arrest cells around metaphase, resulted in markedly weaker but also fewer PLA signals, as well as a substantial cell size increase. Contradictory, BI2536 treatment, which arrest cells in prophase, did not affect PLA signals. These contradictory results could have several possible explanations. While BI2536 is a specific Plk1 inhibitor, wortmannin also inhibits several other kinases, such as phosphoinositide-3-kinase, ATM, ATR and DNA-PKcs. The difference observed might therefore be explained by that it is one of the other kinases, inhibited by wortmannin, which is involved in regulating the Tpt1/Npm1 interaction. Another possible explanation is that Plk1 is involved in the regulation of Tpt1/Npm1 complexes, but that this regulation is occurring in later stages of mitosis, explaining the lack of effect from BI2536, since it arrest cells in the first stage of mitosis. Attempts of pre-arresting cells at metaphase, by adding demecolcine, prior to BI2536 treatment were unsuccessful since mitotic cells are attached very loosely to the chamber slides and therefore were lost when the demecolcine block was washed away. Overall, our results reveal that the Tpt1/Npm1 interaction is independent of phosphorylation by Plk1, at least during the early stages of mitosis. Given that BI2536 arrest cells early in mitosis, we cannot exclude that Plk1 has some regulatory effect in later stages of mitosis, nor that other kinases might regulate this complex although further studies are needed to evaluate this.

**Tpt1/Npm1 interaction is involved in ES cell proliferation**

Both Tpt1 and Npm1 have previously been proven to be involved in cell proliferation in different cellular systems. To investigate the involvement of Npm1 and Tpt1 alone and as a complex on cell proliferation in ES cells, we transfected cells with both pEPI-GFP-Tpt1 and pEPI-GFP-Npm1 vectors or shRNA constructs against Tpt1 and Npm1 to study effects of increased and decreased protein levels, respectively, in combination with EdU proliferation assay. Npm1 overexpression resulted in increased cell proliferation (14.7%). Tpt1 overexpression resulted in both a decreased cell proliferation (15.9%) as well as a large increase of cells with condensed ball-like nuclei (63% of all transfected cells). This is in accordance with earlier studies in other cell systems where overexpression of Tpt1 resulted in slow
-growing cells\textsuperscript{120} or increased number of cells with condensed ball-like nuclei\textsuperscript{109}.Dual overexpression of Tpt1 and Npm1 stabilized proliferation to normal ES cell rate, indicating that Tpt1/Npm1 complexes are involved in promoting cell proliferation for ES cells.

Depletion of Tpt1 or Npm1 alone resulted in significant decrease in cell proliferation compared with negative control vector at both 48 and 72 hours post transfection (Figure 8). Tpt1 depleted cells showed 9\% and 13.7\% less cell proliferation while Npm1 depleted cells resulted in 10.2\% and 18.5\% less cell proliferation compared with negative shRNA control at 48 and 72 hours post transfection, respectively. Dual Tpt1 and Npm1 depletion gave a yet larger decrease with 21.1\% and 35.8\% less cell proliferation compared with the negative shRNA control. At 72 hours post transfection, the observed reduction in proliferative cells are greater than the calculated multiplicative effect of the combination of Tpt1 and Npm1 depletion. The calculated value is 29.7\% reduction and our observed value is 35.8\%.

ES cells show many similarities with carcinogenic cells, and both Tpt1 and Npm1 are often found at elevated levels in tumors and speculatively possess carcinogenic properties\textsuperscript{119,140}. Our finding that Tpt1/Npm1 is involved in ES cell proliferation might also be true for tumor cells, where the complex very well could have the same or similar functions, making this complex a potential therapeutic target to hinder cell proliferation of carcinogenic cells. An interesting hypothesis that needs further investigations.

**Cell cycle dependent interactions involving Ncl-P, Tpt1 and Oct4 (Paper II)**

Successful reprogramming of somatic cells require proper embryonic genome activation. The nucleolus has been proposed to serve as a marker for this process since it coincides with the timing of nucleogenesis in rhesus monkey\textsuperscript{173}. Ncl, one of the most abundant non-ribosomal proteins in the nucleoli, is a multifunctional
To fill the knowledge gaps about Ncl in ES cells, we started by searching for novel Ncl interaction partners.

**Ncl interacts with Tpt1 in a cell cycle dependent manner depending on phosphorylations on Ncl**

In our previous search for Tpt1 interacting partners in ES cells (paper I), we obtained several additional proteins in the 1.0 M NaCl elute, which were identified by nano-LC FT-ICR mass spectrometry. One of the identified proteins was Ncl, and the Ncl/Tpt1 interaction was further investigated. Ncl was co-immunoprecipitated with anti-Tpt1 in nuclear, but not cytoplasmic ES cell extracts nor with IgG control. Using colocalization analysis, we found that Ncl only colocalize with Tpt1 when Ncl is phosphorylated (Ncl-P), and that the colocalization is most prominent during mitosis. This observation was quantified using BioPix iQ 2.0. To investigate if differentiation affected the Ncl-P/Tpt1 complexes, ES cells were induced to differentiate by addition of RA. Colocalization was shown to significantly decrease after 72 hours of RA induced differentiation. Also in situ PLA verified that Ncl-P and Tpt1 interact and that the interaction is most prominent during mitosis. Three different antibody combinations were tested with the PLA method, but only two antibody combinations resulted in complexes. A possible explanation of the lack of detected complexes in the third combination can be that the epitope recognized by the antibody is the part where the interaction occurs and therefore is blocked for antibody detection.

Given that we found that Ncl only interacts with Tpt1 when phosphorylated, the interaction predominantly occurs during mitosis and decreases when ES cells were induced to differentiate, all pointing toward a possible function for Ncl-P/Tpt1 in cell proliferation. Both Tpt1 and Ncl have individually been shown to be involved in cell proliferation in other cell systems. Concerning these aspects, further analysis using shRNA and overexpression in combination with EdU proliferation assay could be used to investigate these theories.

Since Tpt1 now has been proven to interact with both Npm1 (paper I) and Ncl-P (paper II) in a cell cycle dependent manner with a peak during mitosis and it previously has been shown that Npm1 and Ncl interact\(^{174}\), one could speculate if all three proteins occur in a single complex. However, since Ncl/Npm1 has been shown not to interact during prometaphase and metaphase\(^{174}\) and we observed the individual interactions of Ncl-P/Tpt1 and Tpt1/Npm1 during these phases, it suggests that they are not part of a single complex.

**Ncl-P interacts with Oct4 during interphase in both murine and human ES cells**

The staining pattern of Ncl-P in interphase cells resembles the staining pattern of Oct4, and since both proteins have been shown to be involved in transcriptional regulation, we investigated if they interact in ES cells. We found that Ncl-P/Oct4
colocalize during interphase in ES cells and this observation was quantified using BioPix iQ 2.0. *In situ* PLA also verified the interaction between Ncl-P and Oct4 during interphase. Although similar as with Ncl-P/Tpt1, three antibody combinations were tested but only two combinations detected complexes, and the anti-Ncl not resulting in complexes was different compared to the Ncl-P/Tpt1 interaction. This further strengthens our theory that the reason of lacking complexes is due to that the epitope recognized by the antibody is the part where the interaction occurs and therefore is blocked for antibody detection. We used *in situ* PLA to investigate if the interaction is specific to murine ES cells or also exist in human ES cells. The experiments showed that the interaction also exists in human ES cells at the same time as human ES cells that spontaneously had started to differentiate showed a large increase in Ncl-P/Oct4 complexes. No such observation was found with murine ES cells.

When comparing literature one find interesting similarities (underlined) between the Oct4 binding motif (AGTCAAAT) and the binding site for the transcription factor complex LR1 (CCTCCTGGTCAAGGCTGAA), which Ncl is a component of\textsuperscript{175}. It is therefore tempting to speculate that Ncl-P and Oct4 are components of a transcription factor complex, which mostly is active during the initiation of differentiation, due to the increased amount of complexes observed in those cells. Unfortunately none of our anti-Ncl can immunoprecipitate Ncl. Otherwise ChIP experiments at Oct4 binding regions would be interesting to perform, to see if our speculation is correct or not.

**SAF-A binds to the Oct4 proximal promoter (Paper III)**

Oct4 is essential for ES cell maintenance as well as nuclear reprogramming in both SCNT and iPS cell creation. Specific regulatory mechanisms for Oct4 are still partly unsolved and a better understanding of Oct4 regulation could improve the reprogramming efficiencies to create patient specific ES cells.

**SAF-A binds the Oct4 proximal promoter and shows involvement in Oct4 expression**

In an attempt to find novel proteins involved in the regulatory mechanisms of Oct4 expression, we screened for factors only binding to CR1 in the Oct4 proximal promoter, when ES cells were sustained in an undifferentiated state. Using DNase footprinting, DNA affinity chromatography followed by identification with nano-LC FT-ICR mass spectrometry and ChIP followed by qPCR, we showed that SAF-A binds preferentially to the unmethylated 1A-like region in the Oct4 proximal promoter in undifferentiated ES cells. Initiation of differentiation by LIF withdrawal resulted in substantially less SAF-A bound to the Oct4 proximal promoter. SAF-A was also found to bind to the Nanog promoter in a similar manner, mostly occurring in undifferentiated ES cells. We tested if SAF-A is involved in general transcriptional
regulation, using shRNA against SAF-A in combination with incorporation of 5-ethylidyne uridine, and found that there was little correlation between SAF-A expression and global transcription in ES cells.

To explore the involvement of SAF-A on Oct4 expression, RNA-interference was performed in combination with both immunofluorescence and qPCR. Depletion of SAF-A was accompanied by an approximate 50% decrease in Oct4 levels (both mRNA and protein), together with a 50% increase in the mesodermal marker Brachyury, 48 and 96 hours post transfection, respectively, suggesting a loss of pluripotency and directed differentiation towards mesodermal lineages. We also showed that ectopic expression of human SAF-A could rescue the knockdown phenotype. To further explore the involvement of SAF-A in Oct4 expression, eGFP tagged SAF-A constructs were prepared and transfected into ES cells. Immunofluorescence analysis was used to visualize effects on Oct4 in respect to elevated SAF-A levels. Increased levels of a truncated version of SAF-A, lacking the C-terminal aa. 553-806, gave decreased levels of Oct4. Less transfected cells were also observed with the truncated SAF-A construct implying lethality of this particular construct. Increased levels of full length SAF-A resulted in some cells showing increased levels of Oct4, although high endogenous expression of SAF-A and Oct4 makes it difficult to interpret.

**SAF-A interacts with RNA pol II, Oct4, Sox2 and STAT3**

Previous reports have suggested that SAF-A interacts with RNA pol II in other cell systems. To investigate if that is the case also in ES cells we performed in situ PLA and co-immunoprecipitation. We found that SAF-A and RNA pol II interact in ES cells and that the interaction is independent of mRNA and CTD phosphorylations. All the above indicate a possibility of SAF-A being involved in the transcriptional initiation of Oct4. Oct4 is known to self-regulate by binding to the distal enhancer as a heterodimer with Sox2. To see if SAF-A also exists in close proximity to Oct4 and Sox2, in situ PLA was again employed, and showed that SAF-A binds close to both Oct4 and Sox2 in ES cells. Induced differentiation by LIF withdrawal resulted in less complexes for both Oct4/SAF-A and Sox2/SAF-A.

To our great surprise differentiation induced by LIF withdrawal does not decrease SAF-A mRNA and protein levels. In contrary, SAF-A initially increases and then returns to original levels at day three. Thus, Oct4 downregulation cannot be credited simply to a decrease in SAF-A levels. Since murine ES cells are dependent on added LIF working through the JAK/STAT pathway with STAT3, we explored the possible interaction between SAF-A and STAT3 with in situ PLA, which were shown to interact, providing an important link in a chain of interactions ranging from extrinsic signaling of LIF, to intrinsic response in the nucleus, resulting in Oct4 expression.
If SAF-A’s sole task in the chain of transcriptional regulation was to control Oct4 expression, it would only generate two complexes in each *in situ* PLA experiment together with RNA pol II, provided that Oct4 is a subject to biallelic expression in ES cells. Since that is not the case, it is tempting to speculate that SAF-A controls a larger set of genes in ES cells. We have found that SAF-A also binds to the Nanog promoter and one report shows the binding of SAF-A to the promoter of the pluripotency gene *Klf2*\textsuperscript{163}. Further investigations about this is required to explore SAF-A’s true role in ES cell maintenance.

**Additional Npm1 and Tpt1 interactions in ES cells (Paper IV)**

To further explore the role of Npm1 and Tpt1 in ES cells, we wanted to investigate if proteins important for ES cell maintenance and iPS cell creation have any connection to Tpt1 and Npm1. Npm1 has previously been found to interact with c-Myc and that the interaction is involved in cell proliferation\textsuperscript{141}.

*Npm1 interacts with all three core transcription factors*

In order to investigate if Npm1 interacts with any of the proteins characterized as important for maintenance of ES cells and iPS cell creation, we used *in situ* PLA to screen for novel protein-protein interactions. We found Npm1 to individually interact with all three core transcription factors: Oct4, Sox2 and Nanog. All three interactions occur in the nucleoplasm of interphase cells, so Npm1 should be phosphorylated, since it is outside of the nucleolus. Using an antibody recognizing phosphorylated Npm1 (Npm1-P), we showed that Oct4 and Npm1-P create protein complexes also during mitosis, although in a lesser extent compared to the amount of complexes found during interphase. Npm1 was co-immunoprecipitated together with anti-Sox2 and induced differentiation for 24 hours, by either LIF withdrawal or addition of either RA or DMSO, resulting in that the number and intensity of Npm1/Sox2 complexes were decreased with LIF withdrawal and DMSO addition, while sustained or even slightly increased with RA addition. This implies that Npm1/Sox2 has a function not only in ES cells but also in ectodermal cells, at least during the initial stages of differentiation. Given that Npm1 shows individual interactions with all three core transcription factors, it might have something to do with transcriptional regulation. Previously it has been shown that Npm1 functions as a histone chaperone that remodels local chromatin structures\textsuperscript{139}. The most logical explanation for these three interactions would be that Npm1 remodels the chromatin structure so that the different transcription factors can bind and activate the specific genes. Several other possible functions include cell cycle regulation and directed differentiation, but additional analyzes are needed before any conclusions can be drawn.
Tpt1 interacts with Oct4 but Tpt1 depletion does not affect Oct4 expression

Previously it has been shown that Tpt1 influences Oct4 expression in transplanted somatic cell nuclei\textsuperscript{117}. Using \textit{in situ} PLA, Tpt1 and Oct4 were found to interact in ES cells independently of cell cycle stage. No interaction was observed between Tpt1 and Sox2 or with Nanog. To examine if depletion of Tpt1 affected Oct4 expression we used shRNA constructs to deplete Tpt1 from ES cells in combination with qPCR. Forty eight hours post transfection we had a decrease of Tpt1 to approximately 50%, but found no obvious effect on Oct4. If any effect, it was a slight increase in Oct4, contradictory to the results of Koziol\textsuperscript{117}. We did not have any possibility to select for cells transfected with Tpt1 shRNA and only obtained a 50% decrease in the Tpt1 levels. This could explain why we got contradictory results, given the high expression of Tpt1 in ES cells\textsuperscript{114-115}, so that the 50% Tpt1 remaining could be enough to keep the Oct4 expression at a normal level.

Tpt1 and Npm1 depletion induce different differentiation markers

To explore if Tpt1 and Npm1 are important for ES cell maintenance, shRNA against Tpt1 and Npm1 were used in combination with qPCR to analyze the effect of their depletion on the levels of Oct4, Sox2, Nestin (ectoderm), Brachyury (mesoderm) and GATA4 (endoderm). We found that decreasing Tpt1 with approximately 50% gave an increase in Sox2 (approximately 100%) and in Nestin (approximately 50%), implying that the downregulation of Tpt1 is connected to differentiation towards ectodermal lineages. This is in accordance with a previous finding, where Tpt1 levels were shown to decrease during neural cell differentiation\textsuperscript{116}. When depleting Npm1, we observed a minor increase in Oct4 and Sox2 levels (approximately 10%), together with a larger increase in the mesodermal marker Brachyury (approximately 40%). This support our earlier finding that Npm1 is needed for differentiation toward ectodermal lineages, since depletion of Npm1 did not affect the ectodermal marker Nestin.

Even though we have found several new characteristics for both Tpt1 and Npm1 in ES cells, there are certainly still more fascinating aspects to consider to get the full picture about these interesting proteins. Would the ectopic expression of this pair of proteins enhance the efficiency of iPS cell creation? One recent report added Tpt1 together with Oct4, c-Myc, Klf4 and Sox2, but found no increased efficiency in iPS cell creation\textsuperscript{97}. Although the combination of Tpt1 and Npm1 have not been tested, and given the results we present here about their involvement in cell proliferation and interaction partners, it would be really interesting to explore. Also the aspect if they are a potential therapeutic target for effective elimination of cancer cells would be intriguing to study further.
**Npm1 and SAF-A show a large interaction in ES cells**

Not included in any of the papers in this thesis is the finding that Npm1 and SAF-A show a large interaction in the nucleoplasm of interphase cells (Figure 9A). The Npm1/SAF-A interaction also exists during cell-division, with a peak during the last phases of mitosis, i.e. anaphase and telophase (Figure 9B, indicated by arrows). The complex pattern of Npm1/SAF-A resembles a previously known interaction between Npm1 and histone H3\(^{131}\) (Figure 9C). We tested a possible function in both cell proliferation and transcription with shRNA depletion, but did not see any obvious involvement. A possible decrease in cell proliferation with less EdU incorporated is observed as well as some correlation with transcription, but this needs to be further investigated in detail before any conclusions can be drawn. Another interesting result, except the found interaction, is that dual depletion of SAF-A and Npm1 results in a high increase (approximately 300%) of the mesodermal marker Brachyury 96 hours post transfection. This also needs to be further analyzed.

![Figure 9. In situ PLA experiments showing the interaction between Npm1/SAF-A (A-B) and Npm1/H3 (C) in ES cells (red dots). Arrows in (B) indicate a cell being in the last phases of cell-division. DNA was counterstained with Hoechst 33342 (blue) and scale bar represents 10 µm.](image-url)
Concluding remarks

- In this thesis, we have in total found twelve novel protein-protein interactions in ES cells, summarized in Figure 10.

- Tpt1 was found to interact with Npm1 in a cell cycle dependent manner, with a significant peak during mitosis and decrease during differentiation. Tpt1/Npm1 interaction occurs independently of phosphorylations by Plk1 and was found to collaborate in ES cell proliferation. Dual depletion gave a larger decrease in cell proliferation than their combined individual decrease.

- Ncl was found to interact individually with Tpt1 and Oct4 in a cell cycle dependent manner, where Ncl needs to be phosphorylated for both interactions to occur. Ncl-P/Tpt1 interaction occurs mostly during mitosis and decreases with differentiation, proposing that it may have a function in cell proliferation. Ncl-P/Oct4 interaction occurs during interphase both in human and murine ES cells, and might be part of a transcription factor complex.

- SAF-A was found to bind preferentially to the unmethylated Oct4 proximal promoter, and alteration of SAF-A protein levels affected the mRNA and protein levels of Oct4. SAF-A was found to interact with RNA pol II, STAT3, Oct4 and Sox2.

- Npm1 forms complexes with all three core transcription factors: Oct4, Sox2 and Nanog. Npm1/Sox2 interaction is sustained or slightly increased when ES cells are induced to differentiate along the ectodermal pathway, while decreasing in the other two differentiation pathways. Tpt1 forms complexes with Oct4, but neither with Sox2 nor Nanog. Depletion of Tpt1 and Npm1 increases specific differentiation markers, indicating their importance for ES cell maintenance.

Figure 10. Novel ES cell protein-protein interactions presented in this thesis. Blue circles represent the four proteins we came to focus upon. Purple circles represent the three core transcription factors. Pink circles represent additional proteins.
Acknowledgements

I would like to take the opportunity to express my gratitude to all the people who have contributed and helped me, in one way or another, with this thesis. Special thanks to:

Stina Simonsson, for that you stood by me and gave me the opportunity to start a PhD in your group. I am very grateful for all you have done for me, both within the PhD project and on a personal level. I wish you good luck in the future and hope you get a fantastic lab at the new cancer center!

Dzeneta Vizlin Hodzic, for that you have been a research friend throughout my PhD period. Thank you for all your help and support during our five years together: help and ideas, encouragement, roommate on different campuses, fun dinners, cozy walks and beautiful lunches. Good luck for your dissertation, it will go great, I am fully convinced! And I look forward to meeting little Alvin that you carry around, you and Suad will be wonderful parents!

Yalda Rahpeymai Bogestål, for that you fortified two years of my PhD period when you did your postdoc in our group. Thank you for all your help during the time you were with us and afterwards! You had a talent of being able to read off how I was feeling and I am extra grateful for that. Always looking forward to our trip to Cold Spring Harbor Laboratories and NYC!

Frida Svensson, who started as BMA in our group at the same time I came here. Thank you for all your support with different projects (without your first results it would not look like this dissertation does today), your positive energy and fun memories from everything! Thank you also to all who have spent time in Stina’s group as examiners or summer projects, it has been great to have you all in our lab!

Per Elias, for that you stood by me and var my supervisor. It has been very nice and secure to know that you always were there.

Tomas Simonsson, for all your help, both in terms of experiments and work on manuscripts. And Tomas group members Jessica Ryme and Rikard Runnberg, who have been a great help and have many interesting discussions. Most of all, I am grateful for that we have come to the best for both of you!!!

Anne Uv, for your lovely Finnish sometimes positive attitude, you are an inspiration! Thank you to all of you + Iris Härds group members who have included and in a good way gone through the years, as you have been a great help and support. Sincerely thank you to Erika Hallbäck Tång and Tina Chavoshi. You are both wonderful girls and I hope you both enjoy the time to yourself, you are both wonderful girls.


Simin Rymo, för din alltid glada och snälla personlighet och den omtanke du sprider runt omkring dig. Lycka till med din avhandling och disputation i december! Louise Andersson, för alla tips och all hjälp inför avhandlingsskrivandet och disputationen

Gunnar Hanssons, Susanne Tenebergs och övriga grupper som fyller suturängplan med liv.

Tack till alla doktorander för gemensamt slit och trevligt sällskap på kurslab.

Maria Smedh and Julia Fernandez-Rodrigues at CCI for help and exceptional knowledge when it comes to confocal microscopy. You have both given me a lot of knowledge during the time I have spent at CCI.

Och så alla underbara människor utanför labbet…

Tack till Andrea och alla team Surprise medlemmar 2004-2007, för er förståelse och stödning, och för alla roliga träningssläger och tävlingar jag fick tillsammans med er under den här tiden, det är minnen jag alltid kommer bära med mig.


Thanks also to all the nice people that I have gotten to know at different international competitions when I have operated as technical specialist: Anna, Inka, Wieland, Dann, Philippe, Noémi, Michael and everyone else that I got the opportunity to work with during the last season.

Liselott, för att du alltid bryt dig om hur jag mår och hjälpt mig på många olika sätt genom åren vi känt varandra. Tack för de mysiga fikastunderna vi haft det senaste. Ditt stöd de sista månaderna har varit guld värt!

Maggie, för att du är en underbar vän, alltid stöttande och positiv! Lycka till med din polisutbildning, du kommer bli en utmärkt polis!

Klara, tack för att du hela doktorandtiden stöttat mig och hjälpt mig se saker från den positiva sidan! Är så glad att ni är tillbaka i Göteborgsregionen igen så vi har möjligheten att ses lite oftare.

Johanna, för all stödning samt allt trevligt vi hittat på genom åren vi känt varandra.

Pirjo, för att du alltid bryt dig om mig, parkerat och hängt mig se saker från den positiva sidan. Har haft roliga minnen och mysigt sällskap på diverse utflykter och resor.

Övriga medlemmar i Ericson familjen, Sune, Jennica & Dan, Jens & Josephine för många roliga minnen och mysigt sällskap på diverse utflykter och resor.
Hela kusinskaran, Camilla, Lisette, Urban, Svante & Rickard med familjer, för att ni alla är så härliga personer och gör att släktkalasen blir fyllda med liv.


Tack också till mormor Aina för att du är världens häftigaste mormor!!! För ditt engagemang i mina studier från det att du lärde mig läsa när jag var 5 år till alla artiklar du gett mig som handlat om forskning de senaste åren!

♥ Min storasyster Marina för att du alltid stöttat och uppmuntrat mig till att inga mål är för stora för att uppnå! Vetskapen om att du alltid funnits där betyder jättemycket för mig! Tack även för all din hjälp med att förbättra avhandlingen. ♥

Min lillasyster Carina för att du är den du är och varit under hela vår uppväxt! Du är den som alltid kunnat få mig på bra humör bara genom att jag fått vara i din närvaro. Tack för alla roliga minnen vi skapat ihop under alla resor vi gjort ihop! Tillsammans klarar vi allt…

Tack till mina underbara föräldrar Laila & Lars-Rune, för att ni alltid stöttat och trott på mig genom hela min uppväxt och alltid ställer upp oavsett vad det gäller. Tack för att jag alltid är välkommen både hem till Tjörn och till Mallorca! Det har varit som en rogvande oas ibland under den här tiden.

Sist av allt vill jag tacka personen som betytt mig allra mest för mig under min doktorandtid nämligen min älskade Björn. Utan dig hade jag inte klarat av det här, du har fått ner mig på jorden och fått mig att inse vad som betyder något här i livet och att nästan allt i alla fall löser sig till slut. Du har fått mig att fortsätta kämpa och ge allt den sista tiden. Utan ditt stöd hade inte det här varit möjligt för mig att genomföra. Har gjort många underbara resor med dig genom åren och hoppas på många fler! Älskar dig mest av allt!!!
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