Translationaly controlled tumor protein interacts with nucleophosmin during mitosis in ES cells

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**Key words:** Tpt1, Npm1, mitosis, embryonic stem cells, in situ PLA, proliferation

**Abbreviations:** Tpt1, translationally controlled tumor protein; ES, embryonic stem; Npm1, nucleophosmin; Plk1, polo-like kinase 1; RA, retinoic acid; DAPI, 4,6-diamidino-2-phenylidole; PLA, proximity ligation assay; LIF, leukemia inhibitory factor

Somatic cell nuclear transfers and the generation of induced pluripotent stem cells provide potential routes towards non-immunogenic cell replacement therapies. Translationally controlled tumor protein (Tpt1) was recently suggested to regulate cellular pluripotency. Here we explore functions of Tpt1 in mouse embryonic stem (ES) cells. We find that Tpt1 is present in the nucleus and cytoplasm of ES cells, and that specifically nuclear Tpt1 decreases upon cell differentiation. We also find that endogenous Tpt1 forms a complex with endogenous nucleophosmin/nucleoplasm family member 1 (Npm1) in a cell cycle dependent manner. The Tpt1-Npm1 complex peaks sharply during mitosis and is independent of phosphorylation by Polo-like kinase. Differentiation by retinoic acid decreases Tpt1-Npm1 complex levels. Moreover, Tpt1 knock-down or overexpression reduces proliferation whereas Npm1 overexpression increases proliferation in ES cells. Cells depleted for both Tpt1 and Npm1 exhibit significantly reduced proliferation compared to cells depleted for Tpt1 or Npm1 alone, whereas cells overexpressing both Tpt1 and Npm1 show normal proliferation. Our findings reveal a role for the Tpt1-Npm1 complex in cell proliferation and identify the Tpt1-Npm1 complex as a potential biomarker for mitotic ES cells.

**Introduction**

Embryonic stem (ES) cells are pluripotent cells that in theory have a capacity for unlimited self-renewal. Their ability to differentiate into any cell type found in the adult body makes them a potential route toward cell replacement therapies. It has been demonstrated that induced pluripotent stem (iPS) cells, which are similar to ES cells in morphology, proliferation, and capacity to form teratomas, can be generated from mouse fibroblasts by retroviral introduction of merely four transcription factors, OCT4, SOX2, C-MYC and KLF4. In particular, fibroblasts that manage to activate endogenous OCT4 expression grow independently of feeder cells, are epigenetically similar to ES cells, contribute to the germline, and generate viable late-gestation embryos after injection into tetraploid blastocysts. Oct4 is an established stem cell marker and a potent nuclear reprogramming factor. Its endogenous expression is epigenetically controlled and normally tightly restricted to inner cell mass (ICM) and germ cells. It is also expressed in ES cells, which are in vitro propagated cells that originate from ICM. Illegitimate OCT4 activation is also typical for embryonal carcinoma cells. The notion that OCT4 expression is a stringent criterion for pluripotency has now been confirmed in man, and it was recently demonstrated that OCT4 expression is essential for the generation of iPS cells.

Albeit factors responsible for OCT4 activation in ES cells, and for OCT4 reactivation in nuclear transfer experiments, remained elusive until Translationally controlled tumor protein (Tpt1, also referred to as TCTP, Fortilin, Histamine-releasing factor HRF, or P23) was reported to influence OCT4 expression in transplanted somatic cell nuclei. In addition, successful reprogramming of somatic cell nuclei in bovine oocytes was recently demonstrated to depend on phosphorylated Tpt1, and proteome analysis of mouse ES cell lines has revealed that the presence of Tpt1 is a characteristic of undifferentiated ES cells. Tpt1 is well conserved and expressed in all eukaryotes. It was initially identified due to an abundance in ribonucleoprotein particles in mouse tumor cell lines and the cDNA sequences of mouse and human Tpt1 were reported a few years later. The encoded proteins belong to the guanine nucleotide-free family of chaperones whose functions are regulated in a cell cycle dependent manner. Specifically, amino acids 39–65 of the human Tpt1 protein belong to a highly mobile polypeptide segment which contains two serine residues (S46 and S64) that are subject to phosphorylation by polo-like kinase (Plk1), a key regulator of mitosis.

Accumulating evidence suggests that Tpt1 has a role in cell cycle progression. The TPT1 gene is upregulated during entry into the cell cycle. Overexpression of TPT1 results in slow...
that exhibits the strongest differential expression between tumor and tumor-reversed states in human leukemia and breast cancer cells.\textsuperscript{29} This observation agrees with the recent discovery that cancer stem cells and ES cells have similar gene expression patterns.\textsuperscript{30}

In summary, available data suggest that Tpt1 plays a critical role in cell proliferation, cellular pluripotency, and in early embryogenesis. Although how this occurs at the molecular level is not understood. In an attempt to elucidate mechanisms controlling these important processes and to improve our knowledge how they are connected, we sought to delineate Tpt1 functions, and identify Tpt1 interaction partners, in mouse embryonic stem cells.

**Results**

Subcellular localization of Tpt1 in ES and EC cells. Subcellular localization of Tpt1 has so far not been investigated in ES cells. Western blot analyses using fractionated ES cell extracts reveal that endogenous Tpt1 is present in nucleus as well as cytoplasm, and that Tpt1 levels are slightly higher in nucleus compared to cytoplasm in uncommitted ES cells (Fig. 1A, lanes 1 and 2). Cell differentiation induced by withdrawal of leukemia inhibitory factor from the medium results in a preferential decrease in nuclear Tpt1 levels (Fig. 1A, lanes 3 and 4). Lower nuclear Tpt1 levels are also characteristic of embryonic carcinoma cells (Fig. 1A, lanes 5 and 6).

Immunofluorescence confocal microscopy reveals that Tpt1 (green) is present in both nucleus and cytoplasm of ES cells compared to Oct4 (red) which localizes to the nucleus (top row), and that cell differentiation induced by retinoic acid (RA) treatment for 24 h (middle row) or 48 h (bottom row) gives much lower Tpt1 levels while abolishes detectable Oct4 protein. Oct4 was used as marker for undifferentiated cells and nuclear localization. DNA was counterstained with DAPI (blue). Scale bar represents 10 μm.

**Figure 1.** Endogenous Tpt1 is present in nucleus and cytoplasm of ES cells, and cell differentiation preferentially decreases nuclear Tpt1 levels. (A) Western blots reveal that Tpt1 levels are slightly higher in the nucleus (ES Nuc) compared to cytoplasm (ES Cyt) in uncommitted ES cells, that cell differentiation induced by leukemia inhibitory factor (LIF) withdrawal yields much lower nuclear (ES-LIF Nuc) than cytoplasmic (ES-LIF Cyt) Tpt1 levels, and that significantly lower nuclear (EC Nuc) than cytoplasmic (EC Cyt) Tpt1 levels are a characteristic of embryonic carcinoma (EC) cells. Oct4 was used as marker for undifferentiated cells and nuclear localization, and β-actin as a loading control. (B) Immunofluorescence confocal microscopy reveals that the Tpt1 (green) is present in both nucleus and cytoplasm of ES cells compared to Oct4 (red) which localizes to the nucleus (top row), and that cell differentiation induced by retinoic acid (RA) treatment for 24 h (middle row) or 48 h (bottom row) gives much lower Tpt1 levels while abolishes detectable Oct4 protein. Oct4 was used as marker for undifferentiated cells and nuclear localization. DNA was counterstained with DAPI (blue). Scale bar represents 10 μm.

growing cells and a delayed cell cycle progression\textsuperscript{25} whereas overexpression of a Tpt1 double mutant (S46→A and S64→A), in which two Plk1 phosphorylation sites have been substituted for alanines, induces a dramatic increase in multinucleated cells, rounded cells with condensed ball-like nuclei, and cells undergoing cell death.\textsuperscript{33} More recent reports implicate a role for Tpt1 in embryonic development. Knockout mice deficient in both TPT1 alleles are embryonic lethal and depending on if the entire gene\textsuperscript{26} or part of the gene\textsuperscript{27} is deleted, they die around E3.5 and E9.5, respectively. Similarly RNA-interference mediated TPT1 knockdown in Drosophila causes lethality at the late first instar stage and visibly affects cell size, cell number, and organ size.\textsuperscript{28}

The human TPT1 gene has also been demonstrated to make cancer cells adopt more malignant phenotypes. It is the one gene (Fig. 1B and bottom rows), confirming results from the western blot analyses.

Taken together, western blot analyses and immunofluorescence confocal microscopy demonstrate that the subcellular localization of endogenous Tpt1 is both nuclear and cytoplasmic, and that differentiation of ES cells is accompanied by a preferential decrease in nuclear Tpt1 levels.

**Identification of Npm1 as a Tpt1 interaction partner in ES cells.** To identify possible Tpt1 interaction partners in ES cells, recombinant Tpt1 was purified to homogeneity and covalently linked to cyanogen bromide activated sepharose beads. ES cell extracts were incubated together with sepharose-linked Tpt1 and bound proteins were eluted with increasing ionic strength, separated by SDS-PAGE and stained with coomassie brilliant
blue dye. The major band at approximately 38 kDa, was excised from the gel (Fig. 2A, lane 2), and peptide sequencing using nano-LC FT-ICR mass spectrometry uniquely identified the protein as nucleophosmin/nucleoplasmic member 1 (Npm1) (Fig. 2B).

**Tpt1-Npm1 colocalization peaks during mitosis.** In order to examine if endogenous Tpt1 and Npm1 colocalize at different stages of the cell cycle, we employed confocal microscopy. Npm1 which is a known multifunctional phosphoprotein has different cellular localization depending on different posttranslational modifications (Suppl. Fig. S1). Colocalization is detected predominantly in the cytoplasm of interphase ES cells (Fig. 3 top row) and is clearly higher in mitotic ES cells (Fig. 3 bottom row). To quantify the observed Tpt1-Npm1 colocalization differences between mitotic and interphase cells we calculated three different parameters: Pearson's correlation coefficient, percentage overlap of Tpt1, and percentage overlap of Npm1. Tpt1 and Npm1 were confirmed to colocalize in both interphase and mitotic ES cells as indicated by high values of Pearson's correlation coefficient and percentage overlap of both proteins (first part of Table 1). The analyses also revealed a significantly higher degree of Tpt1-Npm1 colocalization in mitotic cells.

In brief, Tpt1 and Npm1 colocalize in ES cells and Tpt1-Npm1 colocalization peaks during mitosis.

**Tpt1-Npm1 colocalization decreases upon differentiation.** To investigate whether Tpt1 and Npm1 colocalize in differentiated cells, ES cells were induced to differentiate by retinoic acid treatment for 24 h. Colocalization analyses of retinoic acid treated cells, using the same approach as for the ES cells, reveal similar colocalization patterns in both interphase and mitotic cells (second part of Table 1). However, there are clearer differences between interphase and mitotic cells, which lead us to compare ES interphase cells and retinoic acid treated interphase cells (third part of Table 1). The analysis reveals a substantial decrease in colocalization of Tpt1 and Npm1 upon retinoic acid induced differentiation, as reflected by a significant reduction in Pearson's Correlation Coefficient. This finding led us to examine whether extended induction of differentiation further decreases Tpt1-Npm1 colocalization (Suppl. Table S1).

In brief, Tpt1 and Npm1 colocalize in ES cells and Tpt1-Npm1 colocalization peaks during mitosis. Tpt1-Npm1 colocalization decreases upon differentiation. To investigate whether Tpt1 and Npm1 colocalize in differentiated cells, ES cells were induced to differentiate by retinoic acid treatment for 24 h. Colocalization analyses of retinoic acid treated cells, using the same approach as for the ES cells, reveal similar colocalization patterns in both interphase and mitotic cells (second part of Table 1). However, there are clearer differences between interphase and mitotic cells, which lead us to compare ES interphase cells and retinoic acid treated interphase cells (third part of Table 1). The analysis reveals a substantial decrease in colocalization of Tpt1 and Npm1 upon retinoic acid induced differentiation, as reflected by a significant reduction in Pearson's Correlation Coefficient. This finding led us to examine whether extended induction of differentiation further decreases Tpt1-Npm1 colocalization. Undifferentiated ES cells were compared to cells treated with retinoic acid for 24, 48 and 72 h, respectively, and Pearson's correlation coefficient was found to decrease almost linearly with the time of retinoic acid induced differentiation (Suppl. Table S1).
Colocalization analyses imply that Tpt1 interacts with Npm1 in ES cells, and that the Tpt1-Npm1 interaction decreases upon differentiation.

Tpt1 physically interacts with Npm1 in ES cells most prominent during mitosis. Colocalization analyses is not an absolute proof of that two proteins indeed interact. To explore the interaction between Tpt1 and Npm1 in further detail we used a state of art method in situ proximity ligation assay (PLA)\(^3\) that enables detection of endogenous protein-protein interactions within three dimensions in a single cell. The distance between anti-Tpt1 and anti-Npm1 needs to be less than 40 nm for the PLA to generate a signal, making the methodology highly specific for physically interacting protein-protein complexes. Confocal micrographs collected at 0.38 µm intervals shows a considerable number of Tpt1-Npm1 complexes in the nucleoplasm of interphase ES cells (Fig. 4A, red dots), and a significantly higher number of Tpt1-Npm1 complexes are observed in mitotic ES cells (Fig. 4A and arrow in Merge). The same pattern of complexes, with more complexes in mitotic cells was observed with two additional sets of antibodies (Suppl. Fig. S2A and B).

To further verify these results extracts prepared from ES cells were subjected to co-immunoprecipitation with anti-Tpt1 followed by western blot. Npm1 was immunoprecipitated with anti-Tpt1 (Fig. 4B and IP Tpt1: 1 M NaCl), no Npm1 was detected in the IgG control. Fractionated ES cell extracts shows that Npm1 can be immunoprecipitated from both nuclear and cytoplasmic extracts with anti-Tpt1 (data not shown), further confirming interaction between Tpt1-Npm1.

The PLA thus confirms the observation made by colocalization analyses, that Tpt1-Npm1 interaction peak during mitosis, and together with co-immunoprecipitation also establishes that endogenous Tpt1 physically interacts with endogenous Npm1 in ES cells (see Suppl. Fig. S3 for in situ PLA positive and negative controls).

The Tpt1-Npm1 interacts independently of Plk1 phosphorylation. Altogether the above results clearly suggest that Tpt1 physically interacts with Npm1 in a cell cycle dependent manner. As Plk1 has been shown to phosphorylate both Tpt1 and Npm1 during mitosis we wanted to investigate whether the physical Tpt1-Npm1 interaction is regulated by Plk1 phosphorylation. To this end we first treated ES cells with the inhibitor wortmannin,\(^3\) that is known to inhibit Plk1 kinase, and analyzed the cells by in situ PLA. Vastly fewer and weaker PLA signals were generated in ES cells following wortmannin treatment (Suppl. Fig. S2C). Moreover, inhibition by wortmannin arrests ES cells at metaphase and causes a substantial cell size increase (arrows Suppl. Fig. S2C) in comparison to non-treated ES cells (arrows Suppl. Fig. S2B). Secondly, another more specific Plk1 kinase inhibitor BI2536,\(^3\) was used in the same set of experiments. No difference was observed in PLA signals in ES cells following BI2536 treatment, which arrests the cells at prophase (Fig. 4C and arrows in Merge). This suggests that the physical Tpt1-Npm1 interaction is not regulated by Plk1 phosphorylation. In addition, treatment with the Cdk2-cyclin E specific inhibitor purvalanol A did not appreciably affect the Tpt1-Npm1 interaction (Suppl. Fig. S2D).

Plk1 inhibition analysis thus demonstrate that the Tpt1-Npm1 interaction do not require phosphorylation by Plk1, although both proteins previously has been shown to be extendedly phosphorylated during mitosis by this kinase.

Tpt1 and Npm1 cooperate to promote proliferation. ES cells possess a unique cell cycle, with a significantly shorter cell proliferation time than differentiated cells, but molecular events responsible for this is unknown. To investigate the effect of increased Tpt1 and Npm1 levels in ES cells on proliferation was determined by overexpression analysis. We made eGFP tagged Tpt1 and Npm1 constructs in a non viral episomal vector and Npm1 or a combination of them, and empty pEPI-eGFP vector was used as control. Using EdU proliferation assay we found that after 48 h of Npm1 overexpression, proliferation increased with 14.7% compared to the control vector (Fig. 5A). Conversely, overexpression of Tpt1, decreased proliferation with 15.9%. Dual Tpt1 and Npm1 overexpression showed no difference in proliferation compared to the control vector suggesting simultaneous overexpression of Tpt1 and Npm1 counteract each other’s effect and result in normal proliferation rate. Moreover, overexpression of Tpt1 gave a significant increase (63% of all transfected cells) in the number of apoptotic like cells with fragmented or condensed ball-like nuclei (Fig. 5B). Interestingly, dual Tpt1 and Npm1

### Table 1. Tpt1-Npm1 colocalization peaks during mitosis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Pearson’s correlation coefficient</th>
<th>% overlap of Tpt1</th>
<th>% overlap of Npm1</th>
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<tr>
<td>ES</td>
<td>Interphase</td>
<td>0.819 ± 0.00376</td>
<td>84.27 ± 0.707</td>
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<tr>
<td>Mitotic</td>
<td>Interphase vs. Mitotic</td>
<td>0.857 ± 0.00292</td>
<td>89.79 ± 0.727</td>
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<td>Interphase</td>
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<td>64.42 ± 3.265</td>
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<tr>
<td>ES vs. +RA 24 h</td>
<td>Interphase vs. Mitotic</td>
<td>0.841 ± 0.00930</td>
<td>75.30 ± 5.260</td>
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<tr>
<td>ES vs. +RA 24 h</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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</table>

Tpt1-Npm1 colocalization is maintained in mitotic cells following induction of differentiation by RA treatment for 24 h whereas colocalization in interphase cells significantly decreases. Data are represented as mean ± SEM. Significant statistical difference with p ≤ 0.01 is indicated by *; n.s. = not significant.
Figure 4. Tpt1 and Npm1 physically interact, and the Tpt1-Npm1 interaction peak during mitosis, in ES cells. (A and C) Tpt1-Npm1 heterodimers were visualized in fixed ES cells with in situ PLA, which is highly specific for physically interacting protein-protein complexes, by antibodies against Tpt1 and Npm1 followed by secondary antibodies fused to PLA probes and ligation of the probes, rolling circle amplification and staining the rolling circle product with complementary oligos labeled with Alexa 613 (red). The PLA probes can only ligate if they are closer than 40 nm. Each red dot correspond to a detected Tpt1-Npm1 complex. DNA was counterstained by Hoechst 33342 (blue). Scale bar represents 10 nm. (A) Immunofluorescence confocal microscopy in combination with in situ PLA detects Tpt1-Npm1 complexes (red) in the nucleoplasm of interphase ES cells, whereas a significantly higher number of Tpt1-Npm1 complexes are seen in mitotic ES cells (arrow in Merge). Complexes are seen throughout the mitotic cells when looking at the different micrographs (0.00–3.40 µm). (B) Co-immunoprecipitation experiments followed by western blot analysis show that Npm1 can be immunoprecipitated using anti-Tpt1 but not with IgG (1 M NaCl). 0.1 M Citrate elutes the antibodies, showing the light chain which has the same molecular weight as Tpt1. (C) The in situ PLA detects Tpt1-Npm1 complexes to the same extent even when ES cells have been treated with the Plk1 inhibitor BI2536. The inhibitor arrests ES cells early during mitosis, in prophase (arrows in Merge, showing the two arrested cells).
overexpression had less apoptotic like cells compared to Tpt1 overexpression alone (19% less). We conclude that both Tpt1 and Npm1 are involved in cellular proliferation in ES cells.

Depletion of Tpt1-Npm1 complex represses ES cell proliferation. The significance of Tpt1-Npm1 complex on proliferation of ES cells was examined by RNA interference experiments. ES cells were transfected with specific short hairpin RNA (shRNA) interference constructs, against either Tpt1 or Npm1 alone or together. To explore long term Tpt1-Npm1 suppression effects, we used shRNA constructs containing antibiotic selection. In preliminary experiments several Tpt1 and Npm1 targeting constructs were tested for their ability to knock down Tpt1 and Npm1 mRNA. Similar effects and no off-target effects were observed for four independent Tpt1 and four independent Npm1 sequences. One Tpt1-shRNA and one Npm1-shRNA construct that caused the greatest knock-down were used in subsequent experiments (data not shown). Proliferation was measured with EdU proliferation assay. Decrease in Tpt1 levels resulted in a minor increase in apoptotic like cells (~4%). Number of proliferative cells decreased significantly with 9% and 13.7% in Tpt1 depleted cells compared to the negative shRNA control at 48 h and 72 h post transfection, respectively. Npm1 depleted cells resulted in 10.2% and 18.5% less proliferative cells relative the negative control plasmid after 48 and 72 h post transfection, respectively. Dual Tpt1 and Npm1 depletion gave a larger decrease with 21.1% less proliferative cells 48 h post transfection and 35.8% less proliferative cells 72 h post transfection compared to the negative shRNA control (Fig. 5C). At 72 h the observed reduction in proliferative cells are greater than the calculated multiplicative effect of the combination of Tpt1 and Npm1. The calculated value are 29.7% reduction and our observed value are 35.8%.

These results clearly show that the Tpt1-Npm1 complex has an important function during cell proliferation.

Discussion

The data presented here reveals that loss of Tpt1 and Npm1 impairs ES cell proliferation, and identifies Npm1 as an endogenous Tpt1 interaction partner. All experiments were performed on two different ES cell lines (R1 and RW4) in parallel. We found no discernible differences between the ES cell lines and thus believe our findings to be generally applicable for ES cells, and not cell line specific. The investigation addressing Tpt1 subcellular localization in ES cells revealed that Tpt1 is present in nucleus as well as cytoplasm, and that specifically nuclear Tpt1 levels decrease upon differentiation of ES cells. This finding is consistent with the report that Tpt1 levels decrease when ES cells differentiate into neurons, and further support the notion that Tpt1 is a global pluripotency regulator, and characteristic for undifferentiated ES cells.

The search for Tpt1 interaction partners in ES cells revealed that Tpt1 forms a complex with Npm1. Npm1 has previously been reported to be expressed at high levels in mouse and human ES cells. It is a multifunctional phosphoprotein that has been implicated in cell proliferation as well as regulation of transcription. Depending on promoter context and interacting partners it appears to either repress or stimulate transcription. A likely explanation for this capacity was recently offered when it was reported that Npm1 functions as a histone chaperone that can remodel local chromatin structure.

We found the Tpt1-Npm1 interaction, to be cell cycle dependent with a prominent peak during mitosis, and to decline during differentiation. On their own, both Tpt1 and Npm1 have been demonstrated to be phosphorylated in a cell cycle dependent manner. Serines 46 and 64 of Tpt1 are confirmed Plk1 phosphorylation sites, and when these are substituted for alanines cells fail to complete mitosis. Similarly, serine 4 of Npm1 is subject to phosphorylation by Plk1, and threonine 199 of Npm1 is phosphorylated by cyclin dependent kinases.

According to the above we reasoned that Tpt1 and Npm1 may be co-regulated by Plk1 in ES cells. To test this hypothesis we treated ES cells with BI2536, which is a specific Plk1 inhibitor. This experiment revealed that the Tpt1-Npm1 interaction is independent of phosphorylation by Plk1. However, we cannot exclude that Plk1 may have some regulatory effect on the Tpt1-Npm1 complex during later stages of mitosis, due to the fact that BI2536 arrest cells early in mitosis. Neither do we rule out the possibility that the Tpt1-Npm1 interaction is co-regulated by other kinases. Especially since Npm1 are reported to be phosphorylated at the nucleoplasm, cytoplasm and during mitosis, all locations where we detect Npm1 in complex with Tpt1. Given that wortmannin was the only drug tested that inhibited the Npm1-Tpt1 complex, and that we have ruled out that it is not due to the Plk1 inhibiting ability of wortmannin, we suggest that phosphoinositide-3-kinase (PI3K) could be involved in the cell cycle regulation of the complex, although further experiments are required to verify this. However, most other drugs inhibiting PI3Ks arrest cell prior to mitosis before the majority of complex formation occurs, and therefore making it hard to design the experiment.
Another interesting finding that points toward a common function for Tpt1 and Npm1 in ES cells is that just like knock-out mice deficient in both TPT1 alleles are embryonic lethal,26,27 knock-out mice that are deficient in both NPM1 alleles are embryonic lethal.46 The phenotypes are similar, with aberrant organogenesis and smaller embryo sizes. This might be connected to that both Npm1 and Tpt1 have been implicated to be involved in cell proliferation. Protein levels of Npm1 have been shown to correlate to the rate of cell proliferation independent of the threonine 199 phosphorylation.47 Npm1 depletion in ES,48 neural stem,49 HeLa cells50 and NPM1 heterozygotic mouse embryonic fibroblasts46 have all previously been shown to have reduced cell proliferation. Consistent with those reports, our experiments showed reduction in cell proliferation when Npm1 is knocked-down.

Figure 5. Tpt1 or Npm1 overexpression influence ES cell proliferation, and proliferation decreases after dual knock-down of Tpt1 and Npm1. (A) Representative image of Npm1 overexpression using a pEPI-eGFp-Npm1 (green) construct in combination with EdU (red) proliferation assay shows that increased levels of Npm1 is accompanied by more proliferative cells (top row) compared to control constructs with empty pEPI-eGFp (green) vector (bottom row). (B) Tpt1 overexpression using pEPI-eGFp-Tpt1 (green) construct gave a huge increase in apoptotic like cells with condensed ball-like nuclei. DNA was counterstained with DAPI (blue). Scale bar represents 10 µm. (C) Knock-down of Tpt1 or Tpt1 together with Npm1. Percentage of EdU positive cells (viewing proliferation) of total GFP positive cells (showing transfection) after 48 hours (white bars) or 72 hours (black bars). Negative control shRNA, Tpt1 shRNA (only 48 hours) and Tpt1 + Npm1 shRNA transfected cells were labeled with EdU for 2 h and subjected to immunofluorescent staining with anti-GFP. GFP positive cells were counted as either EdU positive or negative. Mean values ± standard deviation of three independent experiments are shown.
Remarkably, dual downregulation of Tpt1 and Npm1 resulted in more pronounced decrease in proliferative cells compared to single depletion of either of the two proteins. This further decreases with time indicating that the complex has a role during proliferation and most reasonably during mitosis when the amount of complexes peaks. Overexpression analysis using only Tpt1 gave an enormous increase of cells with condensed ball-like nuclei and some decrease in cell proliferation. This is in accordance with earlier studies in other cell systems where overexpression of TPT1 results in slow growing cells while another study found twice as many cells with condensed ball-like nuclei in Tpt1 overexpressing cells. Increased Npm1 levels gave as expected increased cell proliferation, interestingly dual overexpression of Tpt1 and Npm1 stabilized proliferation to normal ES cell rate, indicating that Tpt1-Npm1 complex promote proliferation.

In summary, our findings identify the Tpt1-Npm1 complex as a novel marker for highly proliferating cells, and offer further insight into the network that controls cellular fate. Misregulation of proliferation leads to pathological conditions such as cancer, our dual knock-down study clearly indicates that Tpt1 together with Npm1 are involved in the mechanism responsible for cell proliferation, whereas increased levels of both proteins in ES cells maintain a balanced proliferation rate.

Materials and Methods

Plasmid construction. Mus musculus Tpt1 cDNA was amplified from IMAGE clone 6824238 and cloned into a modified pEr30a vector (Novagen) containing an N-terminal 6x His-tag/S-tag followed by a Tobacco Etch Virus (TEV) protease cleavage site. The vector (Novagen) containing an N-terminal 6x His-tag/S-tag followed from IMAGE clone 6824238 and cloned into a modified Pet30a Plasmid construction.

In summary, our findings identify the Tpt1-Npm1 complex as a novel marker for highly proliferating cells, and offer further insight into the network that controls cellular fate. Misregulation of proliferation leads to pathological conditions such as cancer, our dual knock-down study clearly indicates that Tpt1 together with Npm1 are involved in the mechanism responsible for cell proliferation, whereas increased levels of both proteins in ES cells maintain a balanced proliferation rate.

Preparation of sepharose-linked Tpt1. Cyanogen bromide activated sepharose beads were washed, expanded and incubated with coupling buffer containing 11.2 mg/ml recombinant Tpt1 overnight at 4°C, washed with coupling buffer, resuspended in 0.2 M glycine pH 8.0, and incubated overnight, 4°C. Following a series of washes the sepharose beads were resuspended in 1.0 M NaCl and stored at 4°C. The amount of sepharose-linked Tpt1 was verified by SDS-PAGE.

Cell cultures. Cell lines were grown at 37°C in humidified atmosphere containing 5% CO₂. Mouse embryonic stem cell lines RW4 and R1 were maintained on mitomycin C inactivated mouse embryonic fibroblasts in Dulbecco’s modified eagle’s medium supplemented with 15% fetal calf serum, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2.0 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U/100 µg/ml penicillin/streptomycin, 20 mM Hepes pH 7.3, and 1,000 U/ml leukemia inhibitory factor (ESGRO, Chemicon). The mouse embryonic carcinoma cell line P19 was maintained on gelatin coated dishes in alpha-MEM medium supplemented with 2.5% fetal calf serum, 7.5% bovine calf serum, and 100 U/100 µg/ml penicillin/streptomycin.

Cell extracts. Whole cell extracts were prepared by harvesting confluent cell cultures containing approximately 3 x 10⁶ cells. Harvested cells were incubated in ice cold extraction buffer (10 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 10 mM KCl, 1.0 mM DTT) containing protease inhibitor cocktail tablet (Complete, Roche Diagnostics) 10 min, 4°C. The addition of NP40 to 1% (v/v) was followed by incubation for 10 min, 4°C. Cell lysates were homogenized and NaCl was added to a final concentration of 420 mM followed by incubation for 1 h, 4°C. The extracts were cleared by centrifugation (19,000 xg, 1 h, 4°C) and the supernatant was frozen and stored in liquid nitrogen.

Cytoplasmic and nuclear cell extracts were prepared using CellLytic NuClear extraction kit (Sigma) according to the manufacturer’s protocol. Protein concentration was estimated using a BSA standard (0.25–2 µg/µl) with Bio-Rad Protein Assay.

Tpt1 interaction partners. Whole cell extract (approximately 8.4 mg) was diluted in cyanogen bromide buffer (CyBr-buffer: 10 mM Hepes pH 7.6, 10% glycerol, 150 mM NaCl, 0.1% NP40). Sepharose-linked Tpt1 was equilibrated in CyBr-buffer and incubated with the whole cell extract on a rotashaker 20 min, 4°C. The mixture was transferred to a column, washed with CyBr-buffer, and potential Tpt1 interaction partners were eluted with an ionic strength gradient (CyBr-buffer containing 150 mM–1.0 M NaCl) and increasing amounts of detergent (CyBr-buffer containing 0.1–5% NP40). Fractions were collected, analyzed on 10% SDS-PAGE gels, stained with coomassie brilliant blue dye and bands were identified by nano-LC FT-ICR mass spectrometry.

Western blot. Proteins were separated using SDS-PAGE, followed by semi-dry electrophoresis onto polyvinylidene difluoride membranes for 1 h, 100 mA/gel in transfer buffer (48 mM Tris, 39 mM Glycin, 1.3 mM SDS, 10% methanol) and immunologically detected. Membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 1 h and incubated with primary antibody (anti-β-actin (C4): sc-47778; anti-HRF (FL-172): sc-30124 [Santa Cruz]; anti-Oct4 [611203, Clone 40, BD Biosciences]; anti-TCCTP [ab37506];
anti-Npm1 [ab10530, Abcam]) in blocking solution overnight at 4°C. After washing with PBS-Tween, blots were incubated with secondary antibody (AP conjugated goat anti-mouse IgM + IgG + IgA (H + L); AP conjugated goat anti-rabbit IgM + IgG (H + L chain specific) [Southern Biotechnology Associates]) in blocking solution for 1 h at room temperature. Visualization of proteins was done with BCIP/NBT kit (Invitrogen).

**Immunofluorescence analyses and confocal microscopy.**

5–8 x 10^4 cells were grown on glass coverslips overnight. Differentiation was achieved by incubation with 2.0 mM retinoic acid for up to 72 h. Cells were fixed in 4% paraformaldehyde/PBS for 20 min, permeabilized with 0.25% Triton X-100/PBS for 5 min, and blocked in 5% normal goat serum or 10% fetal calf serum in 0.1% Triton X-100/PBS for 20 min. Primary (anti-HRF/Tpt1 [M099-3, Clone 6E9, Nordic Biosite or ab37506, Abcam]; anti-Npm1 [ab15440 or ab10530, Abcam]; anti-Oct4 [611203, Clone 40, BD Biosciences]; anti-GFP [A-11122, Invitrogen]) and secondary (Alexa Fluor 488 or 555 labeled goat anti-mouse IgG; Alexa Fluor 488 or 555 labeled goat anti-rabbit IgG [Molecular Probes]) antibodies diluted in 0.1% Triton X-100/PBS-0.5% normal goat serum or 1% fetal calf serum were sequentially added for 2 and 1 h, respectively, each followed by washes in 0.1% Triton X-100/PBS. Nuclei were counterstained with 4,6-diamidino-2-phenylidole (DAPI). Coverslips were air dried, mounted, and analyzed using an inverted Zeiss LSM 510 META confocal microscope equipped with a Zeiss image processing system. An 63x/1.4 oil objective and sequential scanning with narrow band-pass filters was used (420–480 nm for DAPI, 505–530 nm for Alexa 488 and 560–615 nm for Alexa 555).

**Colocalization analyses.** Confocal micrographs were collected at 0.38 μm intervals to create Z axis stacks, and images rendered from the Z stacks were analyzed with the BioPix IQ 2.0 software. Mitotic and interphase cells were selected manually. A minimum of five different z-stacks, containing at least 10 cells each, were taken for each analysis. Colocalization was visualized using ImageJ (http://rsb.info.nih.gov/ij). SPSS 13.0 was used for statistical evaluation of colocalization results generated by BioPix IQ 2.0. Two sample t-test, or one-way analyses of variance followed by post hoc and Turkey’s test, were done to yield mean ± SEM (standard error of mean). Values are considered significant if p ≤ 0.01 for the differences between analyzed groups.

**Generic in situ proximity ligation assay.** 3–4 x 10^4 cells were grown on chamber slides overnight. Fixation, permeabilization, blockage and primary antibody (anti-Tpt1 [ab37506, Abcam]; anti-Npm1 [ab10530, Abcam]) incubation were performed as described for immunofluorescence analyses. Duolink (Olink Biosciences) in situ PLA was performed according to the manufacturer’s protocol. PLA probes were diluted in 0.1% Triton X-100/PBS-1% fetal calf serum and incubated in a pre-heated humidity chamber for 1 h at 37°C, followed by hybridization, ligation, amplification and detection. Slides were analyzed by immunofluorescence confocal microscopy.

**Co-immunoprecipitation.** Co-immunoprecipitation was performed with Dynabeads Protein G (Invitrogen) according to the manufacturer’s protocol by addition and crosslinking with dithiobispropionimidate-2HCl of 10 μg polyclonal anti-TCTP [ab37506, Abcam] or normal rabbit IgG antibody [sc-2027, Santa Cruz]. Approximately 0.2 mg of whole cell extracts was incubated with the antibody-beads overnight, 4°C. Proteins were eluted in 1 M NaCl (50 mM Tris pH 7.5, 1.0 M NaCl, 0.1% NP40, 1.0 mM DTT) with the use of the magnet. Extended elution, where antibodies were eluted, was done with 0.1 M Citrate with the use of the magnet. Elutes were mixed with 2x Laemli buffer and heated to 95°C for 5 min and analyzed by western blot.

**Inhibition of polo-like kinase.** 3–4 x 10^4 cells were grown on chamber slides overnight and the medium was supplemented with the Plk1 inhibitor BI2536 (Axon Medchem) at a final concentration of 100 nM for 14 h prior to in situ PLA analyses.

**Overexpression analysis and RNA interference.** Mus musculus Tpt1 and Npm1 cDNA was amplified from IMAGE clones 6824238 and 5710278, respectively, and cloned into pEPI-eGFP.3 inserts were verified by sequencing.

Short hairpin RNA (shRNA) interfering constructs were obtained from SABIbioscences. Preparation of shRNA was done according to the manufacturer’s protocol. Four different constructs for Npm1 and Tpt1, respectively, were tested and constructs with best downregulation outcome were chosen for further analysis. Npm1 clone 3: GCC AGA AGC AAT GAA CTA T, with puromycin selection and Tpt1 clone 1: GAG CTG CAG AGC AGA TTA, with GFP tag was used at concentration of 0.4–1 μg plasmid. Equal amounts of negative shRNA control: GGA ATC TCA TTC GAT GCA TAC with either puromycin selection or GFP tag was used.

4–5 x 10^4 cells were transfected with Lipofectamine LTX (Invitrogen) 4 h post seeding according to the manufacturer’s standard protocol. 24 h after transfection puromycin (1.5 μg/μl) selection was started and 48–72 h post transfection proliferation status was analyzed using immunofluorescence confocal microscopy.

**Proliferation assay.** Proliferation was examined using Click-iTTM EdU Imaging Kit (Invitrogen). EdU at final concentration of 10 μM was added and incubated in 37°C for 2 h followed by fixation in 4% paraformaldehyde/PBS for 20 min and permeabilized with 0.25% Triton X-100/PBS for 10 min. EdU incorporation into DNA was detected according to the manufacturer’s protocol with the optional antibody detection step included. GFP positive cells were manually counted as either proliferating (EdU positive) or none proliferating (EdU negative) and compared to empty vector (pEPI-GFP) or negative shRNA controls. Fisher’s exact test was used to assess significance regarding reduction in the proportion EdU positive cells at 48 and 72 h post transfection. Values are considered significant if p ≤ 0.05.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/JohanssonCC9-11-Sup. pdf