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# Differential localization and high expression of SURVIVIN splice variants in human embryonic stem cells but not in differentiated cells implicate a role for SURVIVIN in pluripotency ☆

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**Abstract** The *BIRC5* gene encodes the oncofetal protein SURVIVIN, as well as four additional splice variants (ΔEx3, 2B, 3B and 2α). SURVIVIN, an inhibitor of apoptosis, is also a chromosomal passenger protein (CPP). Previous results have demonstrated that SURVIVIN is expressed at high levels in embryonic stem cells and inhibition of SURVIVIN function results in apoptosis, however these studies have not investigated the other four splice variants. In this study, we demonstrate that all variants are expressed at significantly higher levels in human embryonic stem (hES) cells than in differentiated cells. We examined the subcellular localization of the three most highly expressed variants. SURVIVIN displayed canonical CPP localization in mitotic cells and cytoplasmic localization in interphase cells. In contrast, SURVIVIN–ΔEx3 and SURVIVIN–2B did not localize as a CPP; SURVIVIN–ΔEx3 was found constitutively in the nucleus while SURVIVIN–2B was distributed along the chromosomes during mitosis and also to the mitotic spindle poles. We used inducible shRNA against *SURVIVIN* to inhibit expression in a titratable fashion. Using this system, we reduced the mRNA levels of these three variants to approx. 40%, resulting in a concomitant reduction of *OCT4* and *NANOG* mRNA, suggesting a role for the SURVIVIN variants in pluripotency.
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## 1. Introduction

The oncofetal protein SURVIVIN functions as a requisite member of the chromosomal passenger protein (CPP) complex and is vital in cell proliferation (Carmena et al., 2012). It is also the smallest member of the inhibitor of apoptosis (IAP) family of proteins (Ambrosini et al., 1997). While absent in most adult tissues, SURVIVIN is highly expressed and required during early mammalian development (Adida et al., 1998; Uren et al., 2000), and has similarly been shown to be highly expressed in human (Blum et al., 2009; Filion et al., 2009) and mouse embryonic stem cells (hES and mES respectively) (Coumoul et al., 2004) which recapitulate the early embryo phenotype. In addition to embryonic stem cells, it is expressed and required in several somatic stem cell types (Gheisari et al., 2009; Leung et al., 2007; Marconi et al., 2007; Zhang et al., 2001) suggesting that it may play a broad, still unknown role in stem cell biology.

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SURVIVIN is the product of the *BIRC5* gene and along with canonical *SURVIVIN* the pre-mRNA is alternatively spliced into four variants, *SURVIVIN–ΔEx3* (ΔEx3), *SURVIVIN–2B* (2B) (Mahotka et al., 1999), *SURVIVIN–3B* (3B) and *SURVIVIN–2α* (2α) (Caldas et al., 2005) resulting in transcripts with varied functional domains (Fig. 1A). Although highly investigated in cancer cell lines (for rev. Altieri (2003)), little is known about the normal levels of expression, localization or functional roles of these variants in non-cancerous cells. In this manuscript we demonstrate that all five variants are expressed in hES cells at higher levels in comparison to differentiated cell types. We also demonstrate that SURVIVIN, ΔEx3 and 2B have distinct localization patterns within the cell.

As mentioned above, SURVIVIN is an inhibitor of apoptosis and is necessary for cell proliferation. In somatic cells inhibition of SURVIVIN results in increased apoptosis and reduced cell proliferation. Similarly, studies in human and mouse ES cells have demonstrated that inhibition of SURVIVIN results in increased apoptosis (Blum et al., 2009; Coumoul et al., 2004; Filion et al., 2009) and also blocked the formation of teratomas following transfer of hES cells to immunocompromised mice (Blum et al., 2009). Recent evidence suggests that SURVIVIN and/or its splice variants may have additional roles in cells beyond the classically described CPP and apoptosis inhibition. SURVIVIN has been demonstrated to upregulate human telomerase (hTERT) in colon cancer cells by enhancing activity of MYC and SP1 in an AURORA B dependent fashion (Furuya et al., 2009) and in colonic crypts, inhibition of SURVIVIN is required for the progression of differentiation (Zhang et al., 2001). SURVIVIN has recently been demonstrated to bind to STAT3 and influence the transcription of STAT3 targets (Wang et al., 2010). Homozygous knock-out of SURVIVIN in hematopoietic stem cells (HSCs) results in loss of the hematopoietic compartment, while HSCs heterozygous for SURVIVIN survive and produce all cell lineages excluding erythroid cells (Leung et al., 2007) suggesting that SURVIVIN dosage may be important for its function. To elucidate the effects of SURVIVIN dosage in hES cells, we used an inducible shRNA system to inhibit SURVIVIN in a controlled fashion. We were able to inhibit SURVIVIN expression in a dose dependent manner while still maintaining cell survival and proliferation. Additionally, inhibited cells had decreased expression of the pluripotency regulators OCT4 and NANOG suggesting that SURVIVIN or its splice variants may have a role in maintaining pluripotency.

## 2. Materials and methods

### 2.1. Cell culture

Human ES cells, WA09 (WiCell Research Institute, Madison, WI, http://www.wicell.org) were cultured on dishes coated

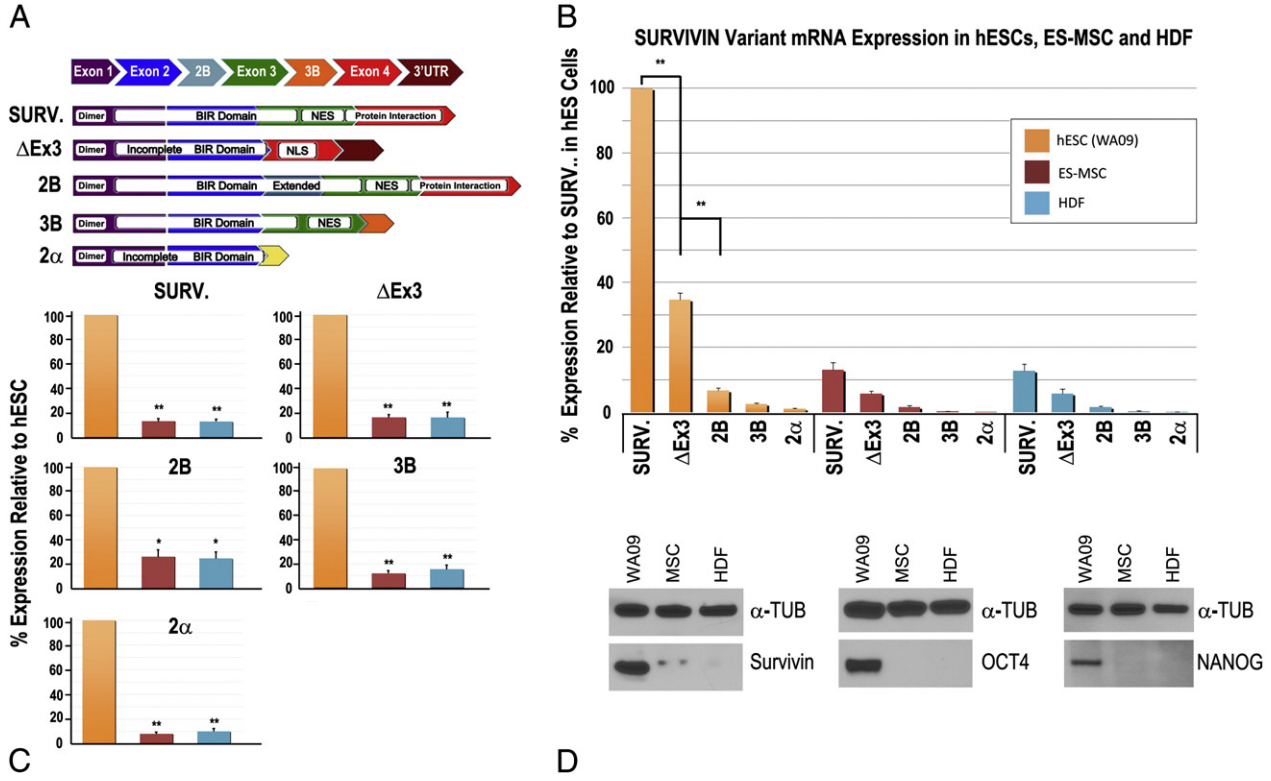


**Figure 1** Characterization of SURVIVIN splice variant expression in human embryonic stem cells. (A) Pictorial representation of SURVIVIN pre-mRNA transcript demonstrating four primary exons (Adida et al., 1998; Altieri, 2003; Ambrosini et al., 1997) and two cryptic exons (2B and 3B) and their corresponding translated protein domains in each variant. (B) qRT-PCR expression levels in 3 cell types. Values are relative to canonical SURVIVIN expression in hES cells. (C) Expression levels of each SURVIVIN splice variant in hES cells and two differentiated cell lines normalized to expression level in hES cells. (D) Western blot detection of canonical SURVIVIN, OCT4 and NANOG in hES cells, ES-MSCs and HDF. α-TUBULIN is shown as a loading control. BIR (*B*aculovirus *I*nhibitor of apoptosis *R*epeat); NES (nuclear export signal); NLS (nuclear localization signal); *$p < .05$, **$p < .001$.

for at least 2 h with Matrigel (BD Biosciences, http://www.bdbiosciences.com). hES cell media containing 80% knockout Dulbecco's modified eagle medium (DMEM), 20% knockout serum replacer, 1% nonessential amino acids, 1% penicillin/streptomycin (100 U per 100 μg/ml), 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and 4 ng/ml basic fibroblast growth factor (bFGF, ReproCELL, San Jose, CA, http://www.reprocell.net) was pre-conditioned by culture with inactivated mouse embryonic fibroblasts (MEF; Millipore, Billerica, MA, http://www.millipore.com) and collected every two days. hES cell lines were cultured with human conditioned media (HuCM) supplemented with 4 ng/ml bFGF and transduced hES cell line media containing 2 ng/ml puromycin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). hES cells were maintained by weekly manual passaging or bulk passaging using 1 mg/ml Collagenase IV (Invitrogen). hES cells were plated for experiments using Accutase (Fisher, Pittsburgh, PA, http://www.fishersci.com) and plated at a density of 26,000 cells/cm$^2$. Retinoic acid (RA) experiments were conducted by culturing hES cells in HuCM supplemented with 10 μm RA (ACROS Organics, New Jersey, http://www.acros.com) and samples were collected every three days using Accutase. hES cell-derived mesenchymal stem cells (MSCs) were generated as previously described (Hwang et al., 2008) and sorted via autoMACS using MSC marker CD 105 magnetic beads (Miltenyi Biotec, Germany, http://www.miltenyibiotec.com). Human dermal fibroblasts were cultured in CF-1 media, 90% DMEM high glucose, 10% Certified FBS (heat inactivated), 1% 2 mM L-glutamine, 1% MEM non-essential amino acids, and 1% pen/strep (100 U per 100 μg/ml) all from Invitrogen.

## 2.2. shRNA constructs

Custom vectors were designed either in a pLKO-puro-IPTG-3xLacO vector containing shRNA sequences (Sigma Aldrich) or the TRIPZ tetracycline inducible vector (Open Biosystems). The DNA sequences targeting SURVIVIN transcripts were as follows: 5′-CCGCATCTCTACATTCAAGAA-3′, (designated as Sh18); 5′-CCTTTCTGTCAAGAAGCAGTT-3′, (Sh20); a non target sequence (ShNT); 5′-TGGCCCAGTGTTTCTTCTGCTT-3′ (Sh1) and 5′-CCCTTAGCAATGTCTTAGGAAA-3′ (Sh4). Lentiviral particles containing these vectors were purchased from the manufacturer and incubated for 2 h with WA09 (WiCell) hES cells in a single cell suspension in standard cell culture incubation conditions (37 °C, 5.0% $CO_2$, in a humidified incubator). A second volume of virus particles was added and the cells were plated onto Matrigel-coated cell culture wells overnight. Fresh human conditioned stem cell media (HuCM) was applied and cells were fed every two days until colonies were observed. Colonies were then hand passaged and maintained with HuCM containing 2 μg/ml puromycin (Sigma-Aldrich) designated as Puro-HuCM. Cells were prepared for experimental treatments using Accutase (Invitrogen) and seeded at 26,000 cells/cm$^2$ in Puro-HuCM with 250 μM IPTG to induce transcription of the shRNA. Control and experimental cells were treated with fresh Puro-HuCM with and without IPTG every 24 h for a total of 72 h. Cells were then collected and analyzed following previously described real time PCR, western blot, or immunocytochemistry protocols.

## 2.3. Flow cytometry of annexin V

Sh18 cells were treated following protocol outlined in Section 2.2. Cells were treated for 72 h with either 250 μM or 2.5 mM IPTG. Negative control cells were not treated and positive control cells were treated for 7 h with 20 μM camptothecin (BioVision, http://www.biovision.com, Milpitas, CA). Live cells were then collected and labeled for Annexin V following the manufacturer's instructions (BioLegend, http://www.biolegend.com, San Diego, CA). Annexin-FITC expression was analyzed using flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) and Accuri C6 (BD Biosciences) and data was processed using FlowJo software (Tree Star Inc., Ashland, OR, http://www.flowjo.com).

## 2.4. Real time polymerase chain reaction

Total cellular RNA was extracted from cells using TRIzol (Invitrogen) and DNAase treated with the DNA-*free* kit (Ambion, Austin, TX, http://www.ambion.com) following manufacturer's directions for stringent conditions. RNA was dialyzed for 30 min against milliQwater, 10 mM Tris and 1 mM EDTA adjusted to pH 7.5 with HCL (all from Fisher). cDNA was synthesized using GeneAmp RNA PCR kit from Applied Biosciences (Fisher) and no-RT reactions were performed without reverse transcriptase and RNAase inhibitor enzymes. One microgram of RNA was used, following the manufacturer's directions. Quantitative real time polymerase chain reaction (qPCR) was performed to determine relative mRNA expression levels. Expression fold changes were calculated using the ΔΔCt method and normalized using the housekeeping gene, β-ACTIN as an internal control. Taqman master mix and gene assays with water only were used as a negative control. mRNA expression assays were analyzed in a triplicate PCR reaction with no-RT negative controls and each experiment was performed at least three independent times. Statistical analyses were performed to identify statistically significant differences ($p < 0.05$) using one way ANOVA and Tukey's post hoc analysis. Primers were selected from predeveloped Applied Biosystems TaqMan Assays, which are pre-designed qPCR primers and TaqMan MGB probes; Oct-4 (Hs01895061_u1) and Nanog (Hs02387400_g1). SURVIVIN splice variant specific primers and probe sequences were used as described in Marconi et al. (2007) and listed in Supplemental Table 1.

## 2.5. Immunocytochemistry

Human ES cells were grown on plastic coverslips pre-treated with Matrigel. Coverslips were rinsed with PBS and fixed for at least 30 min in 2% formaldehyde, and permeabilized in 0.1% Triton X-100 in phosphate-buffered saline (PBST; all reagents from Fisher). Primary and secondary antibody incubations were performed and cover slips were mounted as previously described (Momcilovic et al., 2009). Primary antibodies SURVIVIN, SURVIVIN–2B, and SURVIVIN–ΔEx3 (Abcam, Cambridge, U.K., http://www.abcam.com), NANOG (Cell Signaling, http://www.cellsignal.com) and α-TUBULIN (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) were applied for 40 min. Primary antibodies were detected using species-specific fluorescently labeled secondary antibodies (Invitrogen)

at 37 °C for 40 min and coverslips were mounted onto glass slides using Vectashield mounting medium with DAPI to label DNA (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Images were taken using Deltavision Personal DV microscope. Colocalization analysis was performed using ImageJ Software (http://rsbweb.nih.gov/ij/), with threshold settings of 125 and intensity ratio of 50%.

### 2.6. Western blot analysis

Cells were collected using Accutase (Invitrogen), washed twice with phosphate-buffered saline (PBS, Invitrogen) then lysed on ice using IP lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP–40, 5% glycerol; pH 7.4, with Halt Protease and Phosphatase Inhibitor cocktail (Thermo Scientific, Rockford, IL, http://www.piercenet.com). Six micrograms of total protein was analyzed under reducing conditions on 12% precast polyacrylamide gels (NuPAGE, Invitrogen) followed by transfer to polyvinylidene difluoride membrane (Fisher) then blocked at room temperature for 1 h with 1% BSA (Sigma-Aldrich) in TBS with 0.1% Tween-20 (Fisher). Primary antibodies, Survivin (Abcam) and α-tubulin (Sigma-Aldrich) were applied to membrane and incubated at 4 °C overnight. Horseradish peroxidase-conjugated species-specific secondary antibodies (Invitrogen) were diluted in TBS with 0.1% Tween-20 buffer and incubated at room temperature for 1 h. ECL Advance Western Blotting Detection kit (Fisher) was used according to the manufacturer's directions and chemiluminescence was recorded on ECL film (Fisher).

## 3. Results

### 3.1. Human embryonic stem cells express five *SURVIVIN* splice variants

Global gene expression analysis demonstrated that *SURVIVIN* is highly expressed in hES cells (Blum et al., 2009), however the expression of the remaining splice variants had not previously been examined. *SURVIVIN* pre-mRNA contains four primary exons (Adida et al., 1998; Altieri, 2003; Ambrosini et al., 1997) and two cryptic exons (2B and 3B) (Fig. 1A), resulting in diverse protein domains rendering unique subcellular localization and function (Fig. 1A). Translated canonical SURVIVIN contains a BIR domain (Ambrosini et al., 1997) critical for its anti-apoptotic function, a nuclear export sequence (NES) domain for binding to the nuclear export protein CRM1 (Rodriguez et al., 2002; Stauber et al., 2006), a SURVIVIN dimerization domain (Chantalat et al., 2000; Verdecia et al., 2000) and a c-terminal amphipathic α-helix used for protein–protein interactions (Jeyaprakash et al., 2007). ΔEx3 mRNA contains exons 1,2 and 4, however a frameshift occurs upon removal of exon 3 resulting in a novel translation of exon 4 containing a bipartite nuclear localization signal (NLS) (Mahotka et al., 2002). 2B contains all four primary exons but additionally includes a cryptic exon (2B) between exons 2 and 3. This insertion truncates the BIR domain and as a result 2B has reduced anti-apoptotic ability (Mahotka et al., 1999). Thus, changes in translated domains result in alterations to subcellular localization and function amongst the splice variants.

To determine which alternatively spliced *SURVIVIN* variants hES cells express, we used reverse transcriptase PCR (RT-PCR) with exon specific primers. We detected mRNA expression for canonical *SURVIVIN*, *ΔEx3*, *2B*, *3B*, and *2α* in hES cells (Supplemental Fig. 1). Using quantitative RT-PCR (qRT-PCR) and primer and probe sets specific for each splice variant (Supplemental Table 1, Marconi et al., 2007) we quantified the relative expression of each variant in hES cells (Fig. 1B). Canonical *SURVIVIN* was the highest expressed variant followed by *ΔEx3*, which was expressed at 36% of the level of *SURVIVIN*. The next most expressed variant was *2B*, which was expressed at 6% when compared to *SURVIVIN*. Expressions of *3B* and *2α* were both less than 3% when compared to *SURVIVIN*.

### 3.2. Expression of the *SURVIVIN* splice variants is uniformly high in hES cells when compared to more differentiated cell types

Functions for the alternative splice variants have been described during development (Caldas et al., 2007; Jiang et al., 2005; Zwerts et al., 2007) and relative expression of the variants changes during keratinocyte differentiation (Marconi et al., 2007). To determine if the relative splice variant expression changes during differentiation we differentiated hES cells to mesenchymal stem cells (hESd–MSC) using a published protocol (Hwang et al., 2008). RNA and protein samples from hES cells, hESd–MSCs as well as primary human dermal fibroblasts (HDFs) were collected and analyzed. qRT-PCR revealed significantly higher levels of all *SURVIVIN* splice variants in hES cells when compared to levels detected in hESd–MSCs and HDFs (Figs. 1B, C). Fig. 1B demonstrates levels of each variant detected in all three cell types (normalized to expression levels detected for canonical *SURVIVIN* in hES cells) and demonstrates that in the differentiated cells the splice variants follow the same basic expression pattern with *SURVIVIN* detected at the highest levels, followed by *ΔEx3*, *2B*, *3B* and then *2α*. Very similar expression levels of each variant were detected in hESd–MSCs and HDFs (Fig. 1C) and each was expressed at significantly lower levels than those observed in hES cells. Western blotting detected robust expression of SURVIVIN protein in hES cells (Fig. 1D) but greatly reduced protein expression in hESd–MSCs. SURVIVIN was not detectable in HDFs following loading of equal amounts of total protein. Splice variant specific antibodies suitable for western blotting detection of endogenous protein are not available so we could not compare protein levels for the individual splice variants. In confirmation of both the pluripotent and differentiated phenotypes, the pluripotent transcription factors OCT4 and NANOG were detected by mRNA (not shown) and protein (Fig. 1D) in hES cells but not in hESd–MSCs or HDFs.

To further verify the relative expression of the variants observed following directed differentiation, we evaluated the expression of each variant during retinoic acid induced differentiation. Human ES cells were treated with 10 μM retinoic acid (RA) for 12 days and hES cells were collected every three days. Similar to the previous experiment qRT-PCR revealed significantly higher levels of all *SURVIVIN* splice variants in untreated hES cells when compared to RA treated cells (Supp. Fig. 2B). The expression of all variants rapidly declined during differentiation mirroring the results observed for OCT4 and NANOG. SURVIVIN, *ΔEx3*, and *2B* relative

expression decreased and stayed low while the levels of 3B and 2α fluctuated relative to their expression in undifferentiated hES cells (Supp. Fig. 2B). At each examined day the same basic expression pattern was observed. *SURVIVIN* was detected at the highest levels, followed by *ΔEx3*, *2B*, *3B* and then 2α (Supp. Fig. 2A).

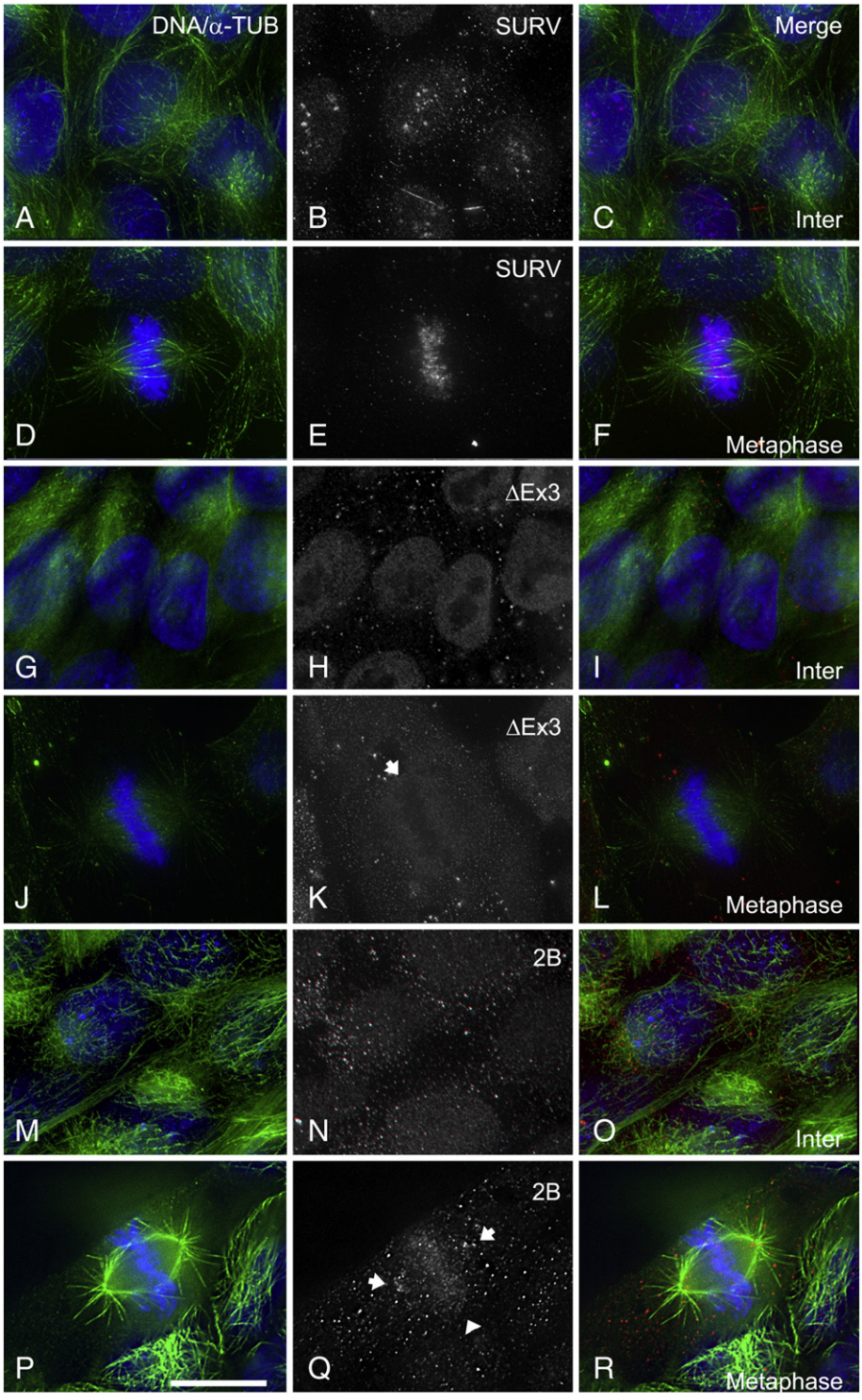


**Figure 2** Subcellular localization of SURVIVIN, ΔEx3 and 2B during interphase and metaphase. (A–F) Canonical SURVIVIN, α-TUBULIN and DNA at interphase (A–C) and metaphase (D–F). (G–L) SURVIVINΔEx3, α-TUBULIN and DNA at interphase (G–I) and metaphase (J–L). (M–R) SURVIVIN–2B, α-TUBULIN and DNA at interphase (M–O) and metaphase (P–R). Arrowhead, 2B localization to the metaphase plate. Arrows, 2B localization to the mitotic spindle poles. Blue = DNA; green = α-TUBULIN; white = SURVIVIN (B, E), ΔEx3 (H, K) and 2B (N, Q); red = SURVIVIN (C, F), ΔEx3 (I, L) and 2B (O, R). Bars = 10 μm.

### 3.3. Human ES cells demonstrate disparate localization amongst individual SURVIVIN splice variants

Previous studies utilizing overexpression of fluorescently labeled proteins have indicated that distinct from canonical SURVIVIN, ΔEx3 and 2B do not localize as chromosomal passenger proteins and instead display differential localization throughout the cell cycle in somatic cells. No previous study has examined the localization of any splice variants in embryonic stem cells. We chose to focus on the subcellular localization of SURVIVIN, ΔEx3 and 2B in hES cells (Fig. 2, Supplemental Figs. 3–5). These three variants represent approximately 98% of the mRNA expression from the *BIRC5* gene in hES cells and antibodies exist that distinguish these three variants from each other. During interphase, SURVIVIN is found in both the cytoplasm and the nucleus (Fig. 2B, also shown in context with more cell cycle stages in Supp. Fig. 3B). It is diffuse in the cytoplasm and primarily localized to bright foci within the nucleus. After nuclear envelope breakdown in prophase, SURVIVIN is no longer diffuse and only found at the centromeres of the aligning chromosomes (Supp. Fig. 3E) particularly those centromeres of unaligned chromosomes (Supp. Fig. 3E, arrow). At metaphase, SURVIVIN is distributed both at the centromeres and the intercentromeric region (Fig. 2E, Supp. Fig. 3H), consistent with its function as a chromosomal passenger protein (CPP). SURVIVIN is found on the interzonal microtubules during anaphase (Supp. Fig. 3K).

By contrast, ΔEx3 is found almost exclusively in the nucleus during interphase (Fig. 2H, Supp. Fig. 4B) and it is excluded from the prominent nucleoli found in hES cells. During prophase, it remains in the nucleus until nuclear envelope breakdown and never localizes to the centromeres as does SURVIVIN (Supp. Fig. 4E). At metaphase, ΔEx3 does not localize as a CPP and is excluded from the condensed chromosomes as evidenced by the negative staining in Fig. 2K (arrow, Supp. Fig. 4H). Finally, ΔEX3 does not show any localization to the interzonal microtubules at telophase (Supp. Fig. 4K) but instead is broadly distributed throughout the cytoplasm.

SURVIVIN–2B was only faintly detected in hES cells. During interphase, 2B clearly delineated the nucleus but also was found in the cytoplasm (Fig. 2N, Supp. Fig. 5B). It was distributed throughout the nucleus with no signs of exclusion from the nucleoli as was the case for ΔEx3. At prophase, it is distributed diffusely throughout the cytoplasm (Supp Fig. 5E). At metaphase, it is found concentrated on the condensed chromosomes (Fig. 2Q, arrowhead, Supp. Fig. 5H). It appears to localize along the length of the chromosomes with no apparent centromeric concentration as would be expected of a CPP. Additionally, it is also found at the mitotic spindle poles (Fig. 2Q, arrows, Supp. Fig. 5H). This is consistent with a report that overexpressed SURVIVIN–2B–GFP fusion proteins localized to the centrosome and impacted microtubule nucleation in HEK293 cells (Ling et al., 2007. However, to the best of our knowledge, this is the first time endogenously expressed 2B has been localized to the centrosome in any cell type.

### 3.4. Inhibition of *SURVIVIN* mRNA results in a concomitant decrease in *OCT4* mRNA expression

Leung et al. (2007) demonstrated that homozygous knock-out of SURVIVIN in the hematopoietic system resulted in widespread loss of hematopoiesis. Surprisingly heterozygous mice were mostly unaffected but a subset of mice had defects in erythropoiesis, concluding that a dosage effect of SURVIVIN expression exists in these cells. To test if a similar dosage effect was detectable in human embryonic stem cells we used a doxycycline (Dox) inducible shRNA system to inhibit *SURVIVIN* expression in a titratable fashion. We transduced hES cells with a lentiviral shRNA construct and selected stable integrants using puromycin. As shown in Fig. 3A when we added Dox for 48 h we were able to inhibit *SURVIVIN* mRNA expression in a dose dependent fashion. Addition of 0.3 μM Dox or 1.0 μM Dox resulted in a statistically significant ($p < 0.05$) decrease in *SURVIVIN* mRNA expression. As noted above, SURVIVIN is highly expressed in the early embryo and in numerous stem cells. This led us to hypothesize that elevated expression may facilitate the pluripotent phenotype in hES cells. To test this we inhibited *SURVIVIN* translation and examined expression of the core pluripotency transcription factor OCT4. A concomitant decrease in expression of *OCT4* mRNA was observed when *SURVIVIN* was inhibited (Fig. 3A). Human ES cells have demonstrated ability to silence exogenous gene expression (Stewart et al., 2008) and we observed a similar result. After transduction, cell lines were continuously maintained under puromycin selection. Experiments were initiated after 6, 14, and 19 passages. At each of these passages cell lines were treated with Dox for 72 h and then collected for mRNA and protein analyses. Despite growth under constant puromycin selection, as passage number increased the cells lost the ability to decrease *SURVIVIN* mRNA expression in response to Dox induction (Fig. 3B). Simultaneously, the cells lost the ability to inhibit *OCT4* expression suggesting that *OCT4* inhibition is a direct result of inhibiting *SURVIVIN* expression and not a nonspecific reaction to Dox treatment.

We did notice a slight depression of *SURVIVIN* and *OCT4* expression in control hES cells treated with 1 μM Dox for 72 h (Supp. Fig. 6A). This decrease was not statistically significant but to remove this minor concern and further evaluate this finding we developed three new cell lines (Sh18, Sh20 and a non-target control ShNT) in which shRNA expression was controlled by the Lac operon. Treatment of control hES cells with 250 μM IPTG did not affect *SURVIVIN* and *OCT4* (Supp. Fig. 6A) mRNA expression. Furthermore, SURVIVIN inhibition did not cause a significant increase in apoptosis (Supp. Figs. 6B, C) even when 2.5 mM IPTG was added (10× the levels used in other experiments). As shown in Fig. 4, IPTG induction resulted in a statistically significant reduction of *SURVIVIN* expression to approx. 40% of control in both Sh18 and Sh20 cell lines. Sh1 (Dox inducible) is shown for comparison. Treatment with IPTG also dramatically reduced SURVIVIN protein expression assayed by western blot (Fig. 4B). IPTG treatment of the parent cell line (WA09) or of ShNT did not result in a change to SURVIVIN protein levels.

### 3.5. All splice variants are inhibited by shRNA expression

Using splice variant specific qRT-PCR we examined the effect of shRNA expression on each variant. *SURVIVIN, ΔEx3 and 2B* showed the greatest inhibition with each variant being inhibited to approx 40% of control levels in Sh18 and Sh20 cell lines. *SURVIVIN–3B* and *SURVIVIN–2α* were also

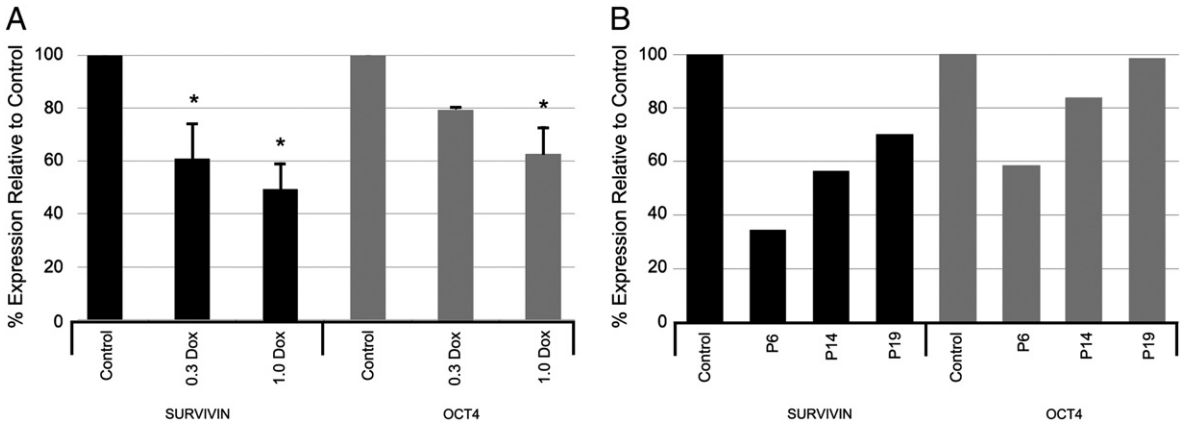


**Figure 3** Doxycycline (Dox) inducible shRNA inhibition of *SURVIVIN*. (A) hES cells stably transduced with the Dox inducible ShRNA, Sh1, were treated with 0.3 μM Dox or 1.0 μM Dox for 48 h. (B) hES cells stably transduced with the Dox inducible Sh4 ShRNA, treated with 1.0 μM Dox for 72 h exhibit silencing over subsequent passaging. *$p < .05$.

inhibited although to a reduced extent. The Sh1 Dox inducible shRNA also inhibited these variants. In all three cell lines *OCT4* expression was reduced significantly. *NANOG* expression was also reduced in all three cell lines but only two lines (Sh18 and Sh20) reached statistical significance. Interestingly, there was some variability between cell lines in the extent of inhibition of these pluripotent factors perhaps due to the extent of inhibition to specific SURVIVIN variants. Despite the decrease in mRNA for *OCT4* and *NANOG*, no significant decrease in protein levels were observed (not shown) after three days of shRNA induction. We have previously noted discordance between *OCT4* and *NANOG* mRNA levels and protein expression (Momcilovic et al., 2009). Longer term IPTG induction (12 days) resulted in a more differentiated morphology, a reduction in NANOG protein levels and an increase in the number of cells which don't express NANOG assayed by immunocytochemistry (Supp. Fig. 7).

## 4. Discussion

SURVIVIN expression is high in the early embryo (Adida et al., 1998; Uren et al., 2000), diminishes during fetal development and it is essentially undetectable in adult tissues with the exception of cancer cells in which SURVIVIN is almost uniformly

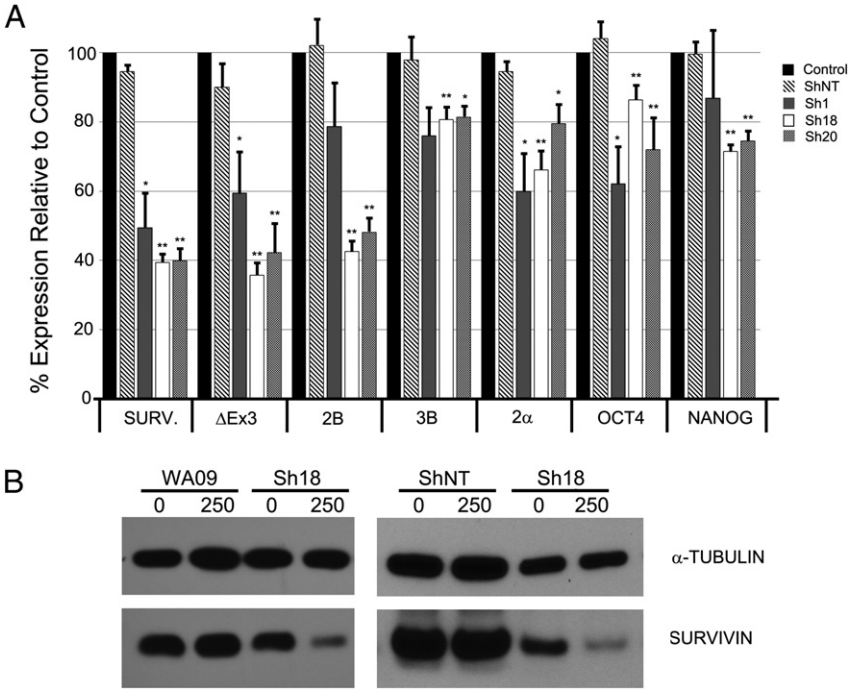


**Figure 4** Inducible shRNA inhibits all *SURVIVIN* splice variants as well as core pluripotency regulators *OCT4* and *NANOG*. (A). Stably transduced hES cell lines were analyzed by qRT-PCR. Cell lines were treated with 1.0 μM Dox (Sh1) or 250 μM IPTG (ShNT, Sh18 and Sh20). (B) Western blot detection of SURVIVIN in Sh18 and control cells. *$p < .05$, **$p < .001$.

expressed (reviewed in Altieri, 2003; F. Li, 2005) and for this reason SURVIVIN is a target for clinical interventions (reviewed by Kelly et al. (2011)). SURVIVIN is also expressed and/or required in several somatic stem/progenitor cell types (Gheisari et al., 2009; Leung et al., 2007; Marconi et al., 2007; Zhang et al., 2001), suggesting that it may also play a broad role in all stem cells. Our results (Fig. 1) and those of others show *SURVIVIN* to be highly expressed in hES cells (Blum et al., 2009). In our study, canonical *SURVIVIN* was expressed approximately nine fold higher than differentiated cell types. This difference is likely not simply attributed to proliferation disparities as all cells were in logarithmic growth and didn't have a significant difference in mitotic index (not shown). SURVIVIN protein was readily detected in hES cells by western blotting at significantly higher levels than observed in hESd–MSCs and dermal fibroblasts.

We extended our observations to include the mRNA expression of the *SURVIVIN* splice variants, *ΔEx3*, *2B*, *3B* and *2α*, which we first confirmed are expressed in hES cells. These variants are also highly expressed in hES cells relative to more differentiated cells. In our differentiated cell types (MSCs, HDFs, and RA induced cells) we observed much lower expression of all variants when compared to hES cells. This change in expression was rapid, occurring within 3 days of RA treatment. In the only other stem cell type in which these variants have been examined, Marconi et al. (2007) demonstrated that keratinocyte stem cells expressed all five variants but that *SURVIVIN*, *ΔEx3* and *2B* were predominant and expressed at similar levels to each other. In contrast, our results demonstrated that canonical *SURVIVIN* was expressed at higher levels than *ΔEx3* which was in turn expressed at higher levels than *2B*. Marconi et al. (2007) demonstrated that during keratinocyte differentiation the expression of these variants changed so that in the transient amplifying and post mitotic cells, *2B* and *2α* were the most predominant forms with the other three variants essentially not expressed. However, we did not observe a similar switch in our differentiated cell lines. All three lines retained the same relative expression between the splice variants while overall expression of all variants diminished in the differentiated cells. None of the cells we examined progressed to a post-mitotic phenotype as did the cells in Marconi et al. (2007) and this may explain the discrepancy between these two studies. Our results are consistent with those observed by Blum et al. (2009). These authors examined canonical *SURVIVIN* expression and showed that cells differentiated in embryoid bodies expressed much less *SURVIVIN* than pluripotent ES cells.

Using specific antibodies for SURVIVIN, ΔEx3 and 2B we identified the subcellular distribution of these three splice variants within hES cells. SURVIVIN has classical CPP localization in hES cells consistent with that observed in other cell types (Noton et al., 2006; Uren et al., 2000). In contrast, ΔEx3 predominantly localized diffusely in the nucleus, but was not observed in the nucleoli. This localization pattern is consistent with previous results of GFP–ΔEx3 fusion proteins in cancer cells (Noton et al., 2006; Song and Wu, 2005). Song and Wu (2005) also demonstrated that the lack of ΔEx3 in the nucleolus is due to ΔEx3 being actively broken down in a proteosome dependent manner at the nucleolus. We did not address this specific question but our data are consistent with a similar mechanism occurring in hES cells. In vitro studies of protein binding and in vivo GFP labeled ΔEx3 demonstrate that ΔEx3 cannot function as a CPP (Noton et al., 2006) and our results during mitosis are consistent with this finding. SURVIVIN–2B localizes to the cytoplasm (Mahotka et al., 2002) but does not localize as a CPP. Overexpression of a 2B–GFP fusion protein demonstrated localization to the mitotic spindle poles (Ling et al., 2007) and we observed a similar localization in hES cells. Our results are the first time this has been observed from endogenous expression of 2B and is likely due to the elevated expression in hES cells. Due to its high expression level in hES cells, SURVIVIN-2B may have a function in these cells that it does not have in differentiated cells.

Strong *SURVIVIN* expression was previously observed in hES cells and the teratomas derived from these cells but not in embryoid bodies (Blum et al., 2009). Expression of a dominant negative SURVIVIN mutant (T34A) which is not able to be phosphorylated by CDK1 resulted in the absence of teratoma formation and increased cell death. Inhibition of *SURVIVIN* by siRNA (Blum et al., 2009) or shRNA (Filion et al., 2009) in these same cells had a similar result. In mouse ES cells shRNA inhibition of *Survivin* resulted in decreased cell survival (Coumoul et al., 2004). Knockout of *Survivin* is early embryonic lethal (Uren et al., 2000) therefore in systems in which SURVIVIN inhibition is not controllable it is difficult to identify other functions that SURVIVIN may have in the cell. In hematopoietic cells, homozygous knockout of *Birc5* in the hematopoietic compartment was lethal however, hematopoietic stem cells (HSCs) heterozygous for *Birc5*, survive and produce all cell lineages excepting deficiencies in erythroid cells (Leung et al., 2007). This suggests that SURVIVIN dosage may be important for its function. For these reasons, we developed a titratable system for knockdown of SURVIVIN expression in hES cells. Inhibition of *SURVIVIN* expression with either a doxycycline or IPTG inducible shRNA resulted in a simultaneous decrease in *OCT4* and *NANOG* expression. IPTG-induced Sh18 targets all five *SURVIVIN* splice variants and resulted in a loss of *OCT4* and *NANOG* mRNA. Interestingly, Sh20 does not target ΔEx3 but only the other four SURVIVIN splice variants. Nevertheless induction of Sh20 resulted in a significant decrease in all variants including ΔEx3, as well as OCT4 and NANOG mRNA. We believe the reduction in ΔEx3 expression observed in the Sh20 cell line is not an off target effect but rather is a secondary consequence of reduction in the other variants. We conclude that the loss of OCT4 and NANOG expression is a downstream consequence of the cellular changes (i.e., differentiation) that occurs due to the inhibition of the four-targeted *SURVIVIN* variants and that this same differentiation results in a decrease in ΔEx3 expression. In support of this hypothesis we demonstrate that RA induced differentiation results in a rapid decline in ΔEx3 expression. These decreases were not observed following expression of a scrambled control and loss of our ability to inhibit *SURVIVIN* expression through silencing also resulted in loss of the ability to inhibit OCT4 expression suggesting the specificity of these results. OCT4, NANOG and SOX2 are the core transcription factors associated with pluripotency. SURVIVIN expression has previously been linked with OCT4 (Guo et al., 2008; C. Li et al., 2012), and SOX2 (Lin et al., 2012) as inhibition of these factors results in a decrease of SURVIVIN expression. This interaction must be downstream of these transcription regulators as they do not bind the *BIRC5* promoter directly (Boyer et al., 2005). Our results are the first time inhibition of SURVIVIN has been linked to a decreased expression of these pluripotency genes.

The mechanism by which SURVIVIN could affect OCT4 and NANOG expression isn't known but at least two possibilities are reasonable. Firstly, hES cells have a unique cell cycle characterized by a very short $G_1$ phase and a lacking or leaky $G_1$/S checkpoint. These cell cycle characteristics are linked to maintenance of pluripotency by shortening the time that hES cells are exposed to differentiation cues during $G_1$ (Becker et al., 2006). Indeed, it has been demonstrated that pharmacological activation of p53 followed by increased expression of p21 leads to accumulation of cells in $G_1$ followed by hES cell differentiation (Maimets et al., 2008). Transition from the $G_1$ to the S phase of the cell cycle is driven by cyclin dependent kinases 4 and 6 (CDK4, CDK6). Their activity is dependent upon the presence of their binding partners, CYCLINS, whose levels oscillate throughout the cell cycle. CDK activity can be blocked by binding of CDK inhibitory proteins belonging to INK4 family (p15, p16, p18, p19) and CIP/KIP family (p21 and p27) that directly inhibit CDK activity. SURVIVIN has been shown to competitively interact with $p16^{Ink4a}$ for CDK4 binding. Survivin is a better binding partner for $p16^{Ink4a}$ than is CDK4. Conversely, while SURVIVIN binds CYCLIN D it does so with less affinity than CYCLIN D has for CDK4. The net result is that SURVIVIN titrates the CDK4 inhibitor ($p16^{Ink4a}$) preferentially from the CDK4 activator (CYCLIN D) (Suzuki et al., 2000) thus driving the cells from $G_1$/S. Ectopic expression of a nuclear localized SURVIVIN in HeLa cells increased CDK4, CYCLIN D, and phosphorylated pRB protein levels as well as increasing $G_1$/S progression (Connell et al., 2008). Inhibition of SURVIVIN may increase the time spent in $G_1$ and therefore increase differentiation resulting in decreases in OCT4 and/or NANOG.

Secondly, recent results have suggested that SURVIVIN may also play a role in gene expression. Two groups have identified a *BIRC5* gene signature by altering *BIRC5* expression in urinary bladder (Salz et al., 2005) or in leukemic cells (Fukuda et al., 2011) and comparing changes in gene expression. Salz et al. (2005) overexpressed cDNA for *SURVIVIN* and identified 290 genes that were either upregulated (188 genes) or downregulated (102 genes). Interestingly, if they overexpressed the T34A *SURVIVIN* mutant they no longer observed these differences in gene expression. Fukuda et al. (2011) deleted *BIRC5* from leukemic cells and identified 1096 genes differentially regulated. These studies do not directly link SURVIVIN to gene regulation. The changes observed could simply be cellular responses to the loss or gain of a required protein. Recently, a more direct role in control of gene expression for SURVIVIN has been demonstrated. SURVIVIN is normally exported out of the nucleus by interaction with the export protein CRM1 (Stauber et al., 2006). Wang et al. (2010) demonstrated that SURVIVIN can be acetylated at K129 by the CREB-binding protein (CBP) and this acetylation promotes SURVIVIN homodimer formation minimizing its interaction with CRM1 thus SURVIVIN remains in the nucleus. They demonstrated that SURVIVIN directly interacts with Signal Transducer and Activator of Transcription 3 (STAT3) and modulates expression of STAT3 downstream targets. SURVIVIN–STAT3 interaction did not affect DNA binding but did differentially regulate expression of downstream targets. This study is the first to show a direct role of SURVIVIN in regulation of gene expression and thus SURVIVIN could be more directly linked to gene expression of pluripotency genes. STAT3 is involved in the maintenance of pluripotency in mouse ES cells (Niwa et al., 1998) but is dispensable in hES cells (Daheron et al., 2004; Humphrey et al., 2004), therefore it is unlikely that this mechanism is responsible for the effects observed in our results but it does suggest a similar mechanism may be involved. Importantly, our results demonstrate that SURVIVIN–ΔEx3 is highly expressed in hES cells. SURVIVIN–ΔEx3 is able to dimerize but does not have the interaction domain for CRM1 that SURVIVIN and 2B have thus it is permanently resident in the nucleus and could affect gene transcription more consistently.

## 5. Conclusions

We have demonstrated that all SURVIVIN variants are expressed at significantly higher levels in human ES cells than in differentiated cells. We examined the subcellular localization of the three most highly expressed variants which displayed differential localization. We also used an inducible shRNA system targeting *SURVIVIN* to inhibit expression in a titratable fashion. Inhibition of SURVIVIN resulted in a concomitant reduction in the expression of OCT4 and NANOG mRNA. These results suggest that SURVIVIN may have a role in the maintenance of pluripotency and further studies will be needed to distinguish that role as well as the specific splice variant(s) involved.

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