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Cyclin D2 and the CDK Substrate p220^{NPAT} are Required for Self-Renewal of Human Embryonic Stem Cells

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Abstract

Self-renewal of pluripotent human embryonic stem (hES) cells utilizes an abbreviated cell cycle that bypasses E2F/pRB dependent growth control. We investigated whether self-renewal is alternatively regulated by cyclin/CDK phosphorylation of the p220^{NPAT}/HiNF-P complex to activate *histone* gene expression at the G1/S phase transition. We show that Cyclin D2 is prominently expressed in pluripotent hES cells, but cyclin D1 eclipses cyclin D2 during differentiation. Depletion of cyclin D2 or p220^{NPAT} causes a cell cycle defect in G1 reflected by diminished phosphorylation of p220^{NPAT}, decreased cell cycle dependent *histone H4* expression and reduced S phase progression. Thus, cyclin D2 and p220^{NPAT} are principal cell cycle regulators that determine competency for self-renewal in pluripotent hES cells. While pRB/E2F checkpoint control is relinquished in human ES cells, fidelity of physiological regulation is secured by cyclin D2 dependent activation of the p220^{NPAT}/HiNF-P mechanism that may explain perpetual proliferation of hES cells without transformation or tumorigenesis.

Keywords

WA09; H9 human embryonic stem cells; cyclin D2; p220^{NPAT}; cell cycle regulation; S phase; pluripotency; self-renewal

INTRODUCTION

Human embryonic stem (hES) cells and induced pluripotent stem cells provide the necessary versatility for regenerative medicine by virtue of their ability to differentiate into multiple distinct cell types. Retention of pluripotency and nuclear re-programming of somatic cells are mediated by intricate molecular circuits. Physiologically responsive execution of regulatory signals reflects combinatorial integration of information contained within cellular networks and is achieved by a limited number of transcription factors, microRNAs, as well as epigenetic modifications involving CpG methylation of DNA, variant histones and post-translational modifications of histones (Jaenisch and Young, 2008; Meissner et al, 2008; Creyghton et al, 2008; Marson et al, 2008; Dejosez et al, 2008; Rao and Orkin, 2006). This increased knowledge of mechanisms controlling lineage-commitment provides the conceptual basis for experimental manipulation and indefinite propagation of ES cells (Xu et al, 2008; Yu and Thomson, 2008; Fujita et al, 2008; Xu et al, 2005). To understand proliferative expansion of human ES cells, it is necessary to define cell cycle factors and functional relationships between components of regulatory circuits that control hES self-

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renewal (Becker et al, 2006; Becker et al, 2007; Lako et al, 2009; Neganova and Lako, 2008; White and Dalton, 2005). Pluripotent embryonic stem cells have a unique cell division cycle (<16 h) with a characteristic abbreviated G1 phase (<3 h) based on studies with mouse, monkey and human ES cells (Savatier et al, 1994; Fluckiger et al, 2006; Becker et al, 2006). Recent studies indicate that human ES cells have the intrinsic capacity to progress from mitosis to S phase through this abbreviated G1 for at least two division cycles (Becker et al, 2009). Early differentiation of human ES cells results in a lengthened G1 (Becker et al, 2009) indicating that the abbreviated self-renewal cell cycle is biologically linked to pluripotency.

Molecular mechanisms controlling cell cycle progression during self renewal have been examined in mouse (Fujii-Yamamoto et al, 2005; Bechard and Dalton, 2009; White et al, 2005; Cartwright et al, 2005; Faast et al, 2004; Stead et al, 2002) and human ES cells (Becker et al, 2007; Ghule et al, 2007; Ghule et al, 2008; Ghule et al, 2009; Filion et al, 2009; Zhang et al, 2009; Neganova et al, 2009; Yang et al, 2008; Saretzki et al, 2008). Studies with mouse ES cells in culture have revealed that both pRB hyper-phosphorylation and activation of E2F responsive genes are constitutive (White et al, 2005; Stead et al, 2002; Savatier et al, 1994). This inactivation of pRB by phosphorylation also appears to occur during development of pre-implantation embryos in vivo (Xie et al, 2005). Thus, the normal regulation of ES cell cycle progression is independent of the CDK-dependent pRB/E2F switch at the Restriction (R) point that controls proliferative commitment at the G1/S phase transition in somatic cells (Blagosklonny and Pardee, 2002; Pardee et al, 1978; Pardee, 1974). Rather, we have proposed that one of the major CDK substrates that regulate the G1/S phase transition in hES is $p220^{NPAT}$ (Becker et al, 2007; Ghule et al, 2007; Ghule et al, 2008).

The $p220^{NPAT}$ regulatory protein represents a global transcriptional co-activator of the *histone* multi-gene family and interacts with histone gene promoter factor HiNF-P (Miele et al, 2005; Mitra et al, 2009; DeRan et al, 2008; Zhao et al, 2000; Ye et al, 2003; Ma et al, 2000). Control of histone gene expression in hES cells is facilitated by targeting of $p220^{NPAT}$ to specialized subnuclear organelles termed Histone Locus Bodies [HLBs], where the regulatory machinery for *histone* gene transcription is assembled and organized (Ghule et al, 2007; Ghule et al, 2008; Ghule et al, 2009). Taken together, current data support the concept that CDK phosphorylation of $p220^{NPAT}$ localized at HLBs is required for induction of histone gene promoter activity and obligatory to support the S phase dependent de novo biosynthesis of histone proteins for packaging newly replicated DNA. However, which cyclin-CDK combination stimulates this 'S-point'-related Cyclin/CDK/$p220^{NPAT}$/HiNF-P mechanism at HLBs remains to be defined.

We have previously shown that Cyclin D2 and CDK4 proteins are the most prominently expressed cyclin-CDK pair in human embryonic stem cells (i.e., WA01/H1 and WA09/H9) (Becker et al, 2006). Because cyclin D2 is a regulatory subunit of CDK4, and because $p220^{NPAT}$ is the postulated target of a putative Cyclin D2/CDK4 complex in hES cells, we investigated whether siRNA depletion of cyclin D2 or $p220^{NPAT}$ is rate-limiting for cell cycle progression beyond the G1/S phase transition. The principal finding of this study is that deficiency in either cyclin D2 or $p220^{NPAT}$ blocks cell cycle progression in G1 with a concomitant reduction in the number of Histone Locus Bodies, the CDK dependent phosphorylation of $p220^{NPAT}$ and the fraction of cells that actively synthesize DNA. Hence, competency for proliferation and the architectural organization of cell cycle regulatory machinery for histone gene expression are both impaired. We propose that Cyclin D2 and $p220^{NPAT}$ are rate-limiting for the induction of the Cyclin/CDK/$p220^{NPAT}$/HiNF-P/histone axis in human embryonic stem cells, and thus our study defines a major cell cycle pathway during self-renewal of pluripotent hES cells.

MATERIALS AND METHODS

Cell lines and tissue culture

The human ES cell line H9 (WA09) was acquired from WiCell Research Institute (Madison, WI). Human ES cells were grown on a monolayer of inactivated mouse embryonic fibroblasts (MEFs) that were isolated from 13.5 day embryos of CF-1 mice (Charles River Laboratories, Wilmington, MA). MEFs were cultured until passage 3 in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1% L-glutamine and 1% penicillin/streptomycin (both Invitrogen) and mitotically inactivated using irradiation at 40 Gy before seeding on a 6-well plate at 1.125×10^6 cells/plate.

Human ES cells were expanded as recommended by the supplier. Media were changed daily and cells were replated every 6–7 days. Adherent hES cell colonies were mobilized by incubation with collagenase Type IV (1 mg/ml; Invitrogen) for 15–20 min at 37°C and mild mechanical disruption. Cells were maintained in media consisting of 80% DMEM/F12, 20% KnockOut-Serum Replacer (KO-SR), 2 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and 4ng/ml basic fibroblast growth factor (bFGF/FGF-2) (all from Invitrogen).

Transient transfection

Cell cultures with well established human ES cell colonies on day 3 after propagation were transiently transfected using Oligofectamine (Invitrogen) with small interfering RNA (siRNA) duplexes specific for *cyclin D2* mRNA (siGENOME SMART pool), $p220^{NPAT}$ mRNA (Gao et al, 2003) and universal controls (all from Dharmacon RNA Technologies, Chicago, IL). Cell cultures were maintained without media change for 48 h to allow gene knockdown at 37°C. To determine the effects of gene knockdown, mRNA expression was analyzed by RT-PCR and protein levels were examined by western blotting and immunofluorescence microscopy (see below).

Reverse transcription-quantitative PCR (RT-qPCR)

Total mRNA was extracted using TRIzol reagent according to the protocol of the manufacturer (Invitrogen). Purified total RNA was subjected DNase I digestion, followed by column purification using the DNA Free RNA Kit (Zymo Research, Orange, CA). RNA was eluted and quantitated by spectrophotometry, and 1 μg was added to a reverse transcription reaction using the iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA) with a mixture of random hexamers and oligo-dT primers. Relative quantitation was determined using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) with real-time SYBR Green supermix (Applied Biosystems) to measure fluorescence. Levels of each mRNA were calculated using the ΔΔCT method with 28S rRNA as an endogenous reference (Livak and Schmittgen, 2001). The primers used in this study were described and validated for RT-qPCR in earlier publications (Becker et al, 2006; Becker et al, 2007).

In Situ immunofluorescence microscopy and BrdU labeling

Human ES cells grown on MEF coated coverslips were examined by *in situ* immunofluorescence microscopy to visualize $p220^{NPAT}$, Ki67 and DAPI. In brief, cells were rinsed twice with PBS and fixed in 3.7% formaldehyde in PBS for 10 min. After rinsing once with PBS, the cells were permeabilized in 0.1% Triton-X-100 in PBS, and rinsed twice with PBSA (0.5% bovine serum albumin [BSA] in PBS) followed by antibody staining. The antibodies used were Ki67 (H-300)(1:100, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), $p220^{NPAT}$ (1:1,000; mouse monoclonal, BD Biosciences, San Jose, CA), $p220^{NPAT}$ (1:1,000; rabbit polyclonal (Ma et al, 2000; Zhao et al, 2000), and

phospho-epitope specific $p220^{NPAT}$ (CDK dependent T1270 phospho-epitope; 1:500; rabbit polyclonal, generous gift from Wade Harper, Harvard Medical School). Antigen-antibody complexes were visualized using goat anti-mouse and goat anti-rabbit AlexaFluor secondary antibodies emitting at 488 and 594 nm, respectively (1:800; Molecular Probes/Invitrogen). DNA was visualized by DAPI (4′, 6-diamidino-2-phenylindole) staining. Fluorescence signals were recorded with a CCD camera attached to an epifluorescence microscope (Zeiss Axioplan 2, Zeiss Inc., Thorwood, NY). Images of $p220^{NPAT}$ detection were deconvolved using MetaMorph Imaging Software (Molecular Devices, Downington, PA).

Incorporation of 5-bromo-2′-deoxyuridine (BrdU, Roche, Indianapolis, IN) was used to determine the S phase population of cells. Cells were grown on MEF coated coverslips for 3 days, followed by transfection with siRNA for another 48 h. Cells were allowed to incorporate BrdU for 20 min at 37°C and then fixed with ethanol and 50 mM glycine (pH 2.0) for 20 min at −20°C. Subsequent detection of BrdU was accomplished with antibodies for BrdU (1:10 dilution) according to the instructions of the manufacturer (Roche). Incorporation of BrdU into nuclei was visualized at 488 nm using immunofluorescence microscopy.

Western blot analysis

Proteins were separated in 6% or 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) Immobilon-P membrane (Millipore, Billerica, MA) for 30 min at 10V in a semi-dry transfer apparatus (model HEP-1, Owl Separation Systems, Portsmouth, NH) for proteins with low molecular mass (e.g., cyclins), and for 2.5 h at constant current (200mA) in a wet transfer apparatus (Bio-Rad Laboratories, Hercules, CA) for $p220^{NPAT}$. Immunodetection was performed using an appropriate dilution of specific antibodies with the Western Lightning Chemiluminescence Reagent Plus assay (Perkin-Elmer Life Sciences, Waltham, MA). The following primary antibodies and dilutions were used: cyclin D1 (1:1,000; mouse monoclonal, Santa Cruz Biotechnology), cyclin D2 (1:1,000; rabbit polyclonal, Cell Signaling Technology, Danvers, MA), $p220^{NPAT}$ (1:1,000; mouse monoclonal, BD Biosciences), α-tubulin (1:10,000; mouse monoclonal, Sigma Aldrich, St Louis, MO).

RESULTS

Cyclin D2 protein is prominently expressed in pluripotent human ES cells

Self-renewal of pluripotent human ES cells proceeds through a unique and rapid cell cycle because of an abbreviated G1 phase (Becker et al, 2006). Differences in cell cycle kinetics between pluripotent and somatic cells are reflected by distinctions in expression of multiple cell cycle markers (Becker et al, 2006). G1 phase progression in normal somatic cells requires the activity of D-type cyclins that mediate the initial stages of proliferative commitment prior to the onset of S phase. In hES cells (WA09/H9), expression of *cyclin D2* is highly elevated relative to *cyclin D1* at both mRNA (Fig. 1A) and protein (Fig. 1B) levels, while *cyclin D3* mRNA expression is minimally detectable (Fig. 1A). These data are consistent with our previous observations with WA01/H1 and WA09/H9 hES cells (Becker et al, 2006), as well as with results in which we showed that cyclin D2 protein levels are higher in hES cells compared to several somatic cell types (e.g., WI38, IMR90, HeLa, TG98G and SaOS2)(data not shown).

Cyclin D2 mRNA levels are maximal at 2 to 3 h after release from a nocodazole-imposed mitotic block, coincident with the precocious G1/S phase transition of pluripotent of hES cells (Fig. 1A). Cyclin D2 protein levels continue to accumulate into S phase (i.e., 4 h and 5 h). The selective expression of *cyclin D2* mRNA during the G1 phase of the cell cycle (Figs.

1A and 1B) may be characteristic for cell types with multi-lineage potential, as is illustrated by elevated expression of *cyclin D2* mRNA in multi-potent NT-2 cells (Fig. 1C) and in WA01/H1 hES cells (Becker et al, 2006). Importantly, programming of pluripotent human ES cells, which results in early stages of lineage-commitment and loss of pluripotency, reduces expression of *Cyclin D2* and increases *Cyclin D1* protein levels (Fig. 1C). These results suggest that Cyclin D2 protein expression is functionally linked to pluripotency in hES cells.

Depletion of cyclin D2 or the CDK-substrate p220^{NPAT} inhibits cell cycle progression in human embryonic stem cells

Previous data suggest that cyclin D2 and CDK4 proteins represent the most prominently expressed cyclin/cyclin-dependent kinase pair in pluripotent hES cells (Becker et al, 2006). Cell cycle progression at the G1/S phase transition in hES cells does not depend on CDK phosphorylation of pRB that releases E2F. However, the CDK substrate p220^{NPAT}, a co-activator that stimulates histone gene transcription together with transcription factor HiNF-P (Miele et al, 2005), may be a major cell cycle regulator. Therefore, we used RNA interference to investigate whether cyclin D2 and/or p220^{NPAT} are rate-limiting for cell cycle progression. Using siRNAs specific for cyclin D2 and p220^{NPAT}, respectively, we achieved a knockdown of ~60% for cyclin D2 and ~75% for p220^{NPAT} at the transcript level (Fig. 2A). Neither siRNA had major effects on *HiNF-P* mRNA levels or *CDK4* expression (Fig. 2B). Remarkably, we observed reciprocal cross-regulation upon siRNA depletion of cyclin D2 or p220^{NPAT}. Knockdown of cyclin D2 reduces mRNA and protein levels of p220^{NPAT} (Figs. 2A and 2C), while knockdown of p220^{NPAT} diminishes *cyclin D2* mRNA and protein levels (Figs. 2A and 2C). These reciprocal changes in mRNA levels of *cyclin D2* and *p220^{NPAT}* are consistent with these two proteins participating in shared regulatory functions during self-renewal in hES cells.

We investigated the biological effects of cyclin D2 or p220^{NPAT} knockdown in hES cells by examining several in situ markers for cell cycle progression using immunofluorescence microscopy. Asynchronously growing human ES cells have an S-phase population of 60–65% which directly reflects the length of S phase relative to the overall duration of the cell cycle (Becker et al, 2006). Asynchronous control hES cells treated with non-silencing RNA (siNS) in our experiments exhibit a similar S-phase fraction as measured by BrdU incorporation (Fig. 3A). Importantly, treatments with cyclin D2 or p220^{NPAT} siRNA resulted in a 50% reduction of BrdU incorporation (Fig. 3A) indicating a reduced number of cells in S phase.

The CDK responsive co-activator p220^{NPAT} normally accumulates during the G1 phase in two nuclear foci (‘Histone Locus Bodies’ or HLBs) that correspond with the major histone gene super cluster on chromosome 6p. Four foci become visible around the G1/S transition due to enlargement of a second, less prominent pair of foci that coincides with a second histone gene cluster at chromosome 1q. The change in detection of the two distinct pairs of foci in hES cells is presumably due to p220^{NPAT} recruitment to *histone* gene promoters that increases immunofluorescence signals above a threshold for detection (Miele et al, 2005; Ghule et al, 2007). Depletion of cyclin D2 or p220^{NPAT} levels dramatically increases the fraction of nuclei containing two p220^{NPAT} foci and reduces the number of cells with four HLBs (Fig. 3B). The reduction of cells with four p220^{NPAT} foci mimics the changes we observed in the fraction of BrdU positive cells reflecting S phase. Ki67 is a third nuclear marker that monitors cell cycle progression through stage-specific nuclear patterns (Becker et al, 2006). In control hES cells treated with non-silencing RNA (siNS), ~75% of nuclei exhibit a staining pattern characteristic of late G1 and S-phase (Fig. 3C, siNS). However, this fraction is significantly reduced after treatment with siRNAs for cyclin D2 or NPAT, and cells display staining patterns reflecting accumulation in an earlier stage of G1 (Fig.

3C). The convergence of in situ results obtained for BrdU, HLBs and Ki67 indicates that depletion of either cyclin D2 or p220^{NPAT} inhibits cell cycle progression through G1 into S phase.

Alterations in the subnuclear organization and phosphorylation of p220^{NPAT} upon cyclin D2 or p220^{NPAT} depletion

In somatic cells, phosphorylation of p220^{NPAT} is mediated by Cyclin E/CDK2 activity (Miele et al, 2005; Zhao et al, 2000; Ma et al, 2000) as part of a cell cycle regulatory mechanism that induces histone gene expression and supports chromatin packaging of newly replicated DNA during S phase. The prevalence of Cyclin D2 and CDK4 in human ES cells suggests that cyclin D2/CDK4 complexes may phosphorylate p220^{NPAT} in G1. Knockdown of cyclin D2 is therefore expected to reduce CDK phosphorylation of p220^{NPAT} on one or more known phospho-epitopes prior to the G1/S phase transition. To test this prediction, we examined the in situ nuclear organization and phosphorylation of p220^{NPAT} in HLBs (Figs. 4 and 5) in cells treated with cyclin D2 siRNA or non-silencing RNA. Approximate cell cycle position was monitored by co-staining with Ki67. For comparison, we also investigated the same parameters in cells treated with siRNA for p220^{NPAT} (Figs. 4 and 5).

Control cells treated with non-silencing RNAs exhibit the expected two to four p220^{NPAT} foci that coincide with HLBs depending on the cell cycle stage (Fig. 4). As expected, there are no p220^{NPAT} foci or phospho-p220^{NPAT} foci in early G1, and these foci are first detected in late G1 (Figs. 4 and 5). When control cells progress through S and G2 phases (based on Ki67 staining), p220^{NPAT} and its CDK phosphorylated form are consistently detected as four foci (Figs. 4 and 5). However, detection of both p220^{NPAT} and phospho-p220^{NPAT} is reduced in cells treated with siRNAs for cyclin D2 or NPAT (Figs. 4 and 5). Importantly, the two foci of p220^{NPAT} present in late G1 remain detectable as a single pair until G2 phase (with cell cycle staging based on Ki67 staining) (Fig. 4). In the presence of siRNAs for cyclin D2 or p220^{NPAT}, phospho-p220^{NPAT} foci are not detected during late G1 phase and just two phospho-p220^{NPAT} foci are detected in S and G2 phases (Fig. 5). Thus, in cells escaping a G1 arrest, the number of HLBs and phospho-p220^{NPAT} foci is uncoupled from cell cycle progression through S and G2 phases (as reflected by Ki67 staining). Although a limited number of phospho-p220^{NPAT} foci is detected in individual cells, the overall detection of phospho-p220^{NPAT} foci in the population is reduced by ~80% in cells treated with siRNAs for either cyclin D2 or p220^{NPAT} (Fig. 5B). These data indicate that cyclin D2 and p220^{NPAT} are both required for self-renewal and are generally consistent with the model that cyclin D2 may support phosphorylation of p220^{NPAT} in hES cells.

Effects of cyclin D2 and p220^{NPAT} knockdown on expression of histone genes and cell cycle regulators

To study the mechanistic consequences of either cyclin D2 or p220^{NPAT} deficiency, we analyzed expression of a panel of cell cycle regulatory genes in siRNA treated hES cells using RT-qPCR (Figs. 6 and 7). Reduction of cyclin D2 or p220^{NPAT} by siRNA treatment dramatically diminishes total histone H4 gene expression (Fig. 6A) and generates a proportional decrease in the expression of human *histone H4* genes that are functionally expressed in human ES cells (Fig. 6B). The reduction in *histone H4* gene expression can be directly attributed to a block in the putative cyclin D2/CDK4/p220^{NPAT}/HiNF-P pathway that we postulate controls histone gene transcription in hES cells (Becker et al., 2006), as well as to indirect effects that block cells at the G1/S phase transition (see Fig. 3). Examination of the mRNA levels for the various cyclins upon siRNA knockdown for either cyclin D2 or p220^{NPAT} reveals modest decreases (< 2 fold) in the expression of A- and B-type cyclins, but no appreciable changes in expression of other D-type or E-type cyclins (Fig. 7A). These data indicate that loss of cyclin D2 is not compensated by other cyclins

(i.e., D-, E-, A- or B-types). The decrease of *cyclin A* and *B* mRNA levels upon knockdown of cyclin D2 or p220^{NPAT} occurs concomitant with an increase in the expression of two CDK inhibitors (i.e., *p27/KIP1/CDKN1B* and *p57/KIP2/CDKN1C*) (Fig. 7B). We also observed selective down-regulation in the expression of several *E2F* gene members (e.g., diminished *E2F1* expression upon cyclin D2 knock-down) (Fig. 7C) and general reductions in the expression of the three *pRB*-related pocket proteins (i.e., *p105/RB1*, *p107/RBL1* and *p130/RBL2*) (Fig. 7D). The concerted decrease in the mRNA levels for *cyclins A* and *B* and the upregulation of mRNAs for CDK inhibitors is consistent with a cell cycle delay or arrest in G1 upon cyclin D2 or p220^{NPAT} reduction.

DISCUSSION

In this study, we have investigated the regulatory significance of the cyclin/CDK/p220^{NPAT}/HiNF-P/histone pathway ('S-point pathway') during self-renewal of hES cells. This S point pathway operates in parallel to pRB/E2F mediated control at the R point in somatic cells, but while the pRB/E2F switch is not operative in hES cells, these cells remain dependent on the CDK mediated activation of the p220^{NPAT}/HiNF-P complex to initiate histone gene expression at the G1/S phase cell cycle transition. Our studies reveal that Cyclin D2 protein is prominently expressed in pluripotent human ES cells (WA09/H9) and that lineage programming results in a switch from cyclin D2 to cyclin D1 protein as cells initiate early stages of differentiation. Knockdown of cyclin D2 reduces in situ phosphorylation of the CDK substrate p220^{NPAT} at HLBs. Furthermore, depletion of either cyclin D2 or p220^{NPAT} causes a cell cycle delay or arrest during the brief G1 phase that characterizes pluripotent human embryonic stem cells. Thus, our data demonstrate that both cyclin D2 and p220^{NPAT} are rate-limiting for cell cycle progression, consistent with the cell cycle regulatory importance of the S-point pathway that controls induction of histone gene expression in hES cells.

Several studies emphasize the importance of distinct cyclins and CDKs in conjunction with pluripotency markers in controlling self-renewal in naïve ES cells (Zhang et al, 2009; Neganova et al, 2009; Lee et al, 2008; Card et al, 2008; Becker et al, 2007; White et al, 2005; Faast et al, 2004; Stead et al, 2002). However, there are differences in the observations and interpretations regarding which of these proteins are key cell cycle regulators. These differences could perhaps be attributed to the extent to which embryonic stem cells are pristinely pluripotent and/or in part to the biological derivation of mouse and human ES cells (Zwaka and Thomson, 2005). For example, previous studies with mouse ES cells have shown that CDK2 activity is constitutive (White et al, 2005), consistent with hyper-phosphorylation of pRB throughout the cell cycle (Savatier et al, 1994). The dependence of cell cycle progression on CDK2 activity may be invoked when mouse ES cells lose pluripotency during differentiation by modulations of cyclin A2 and E1 protein levels that may establish a growth factor dependent CDK2-pRB-E2F-cyclin E feedback loop (White et al, 2005). Pluripotent human ES cells are refractory to external growth factor deprivation at least in short term cultures (Becker et al, 2009) and may become dependent on the pRB-E2F switch only during lineage programming. Our present studies show that loss of pluripotency coincides with decreased cyclin D2 and increased cyclin D1protein expression, suggesting that this reciprocal modulation may contribute to establishment of the pRB/E2F mechanism in lineage-committed cells.

Studies with mouse ES cells have also revealed that cyclins A2 and B1 protein are constitutive and more abundant in mouse ES cells than in somatic mouse embryonic fibroblasts (Fujii-Yamamoto et al, 2005), and that CDK1-cyclin B1 is an important regulator of mitotic division (Stead et al, 2002). Furthermore, CDK6-cyclin D3 is a prominently and selectively expressed cyclin-CDK pair in mouse ES cells (Faast et al, 2004). Consistent with

these findings, we observe similarly robust expression of A and B-type cyclins in human ES cells. We attribute these high levels to the increased proportion of hES cells in S, G2 and M phases due to the abbreviated self-renewal cell cycle. However, this work and previous studies with human ES cells (Becker et al, 2007) show that cyclin D2 expression is more pronounced than cyclin D1 or cyclin D3 and that CDK4 is more abundant than CDK6. Therefore, we propose that self-renewal of human ES cells (H9/WA09) may at least in part rely on CDK4-cyclin D2 activity.

While our studies were in progress, Card and colleagues reported that the levels of cyclin D1 are attenuated by miR-302a in human ES cells (Card et al, 2008). The pluripotency factor Oct4, which interacts with the cell cycle controlled promoters of *histone H2B* genes (Boyer et al, 2005), regulates the levels of miR302 (Card et al, 2008) as a component of a cluster of microRNAs which includes miR302a and miR-302b. Thus, the expression of cyclin D1 protein may be suppressed during self-renewal by Oct4 dependent, microRNA-mediated mechanisms. This concept is consistent with our data indicating that cyclin D2 is preferentially expressed (compared to cyclin D1) in hES cells.

Early studies by Savatier and colleagues revealed that during G1 in mouse ES cells most if not all pRB is hyper-phosphorylated and thus incapable of inhibiting E2F, while pRB protein levels decrease when cells progress from mitosis to S phase (Savatier et al, 1994). Hence, orderly progression of the cell cycle and S phase entry in human ES cells may also not be dictated by modulations in E2F activity during self renewal. We have previously observed that mechanisms of *histone* gene regulation, which are E2F independent, are firmly established in hES cells and that *cyclin D2* and *CDK4* mRNAs are the most prominently expressed cyclin-CDK pair (Becker et al, 2007). In this study, we find that cyclin D2 inhibition blocks cell cycle progression and prevents the stimulatory phosphorylation of the essential *histone* gene co-activator $p220^{NPAT}$ to induce *histone* gene expression. Thus, our data suggest a functional relationship between selective expression and activity of cyclin D2 and cell cycle control in primitive pluripotent cells that exhibit an abbreviated G1 period. We propose that the cyclin D2/CDK4/$p220^{NPAT}$/HiNF-P/*histone* axis represents a key 'S-point' pathway that controls the G1/S phase transition during self-renewal of human embryonic stem cells. While the pRB/E2F component of cell cycle checkpoint control may be relinquished in human ES cells, fidelity of physiological regulation is secured by retention of the $p220^{NPAT}$ mechanism, which may explain why perpetual proliferation of hES cells is not accompanied by transformation or tumorigenesis.

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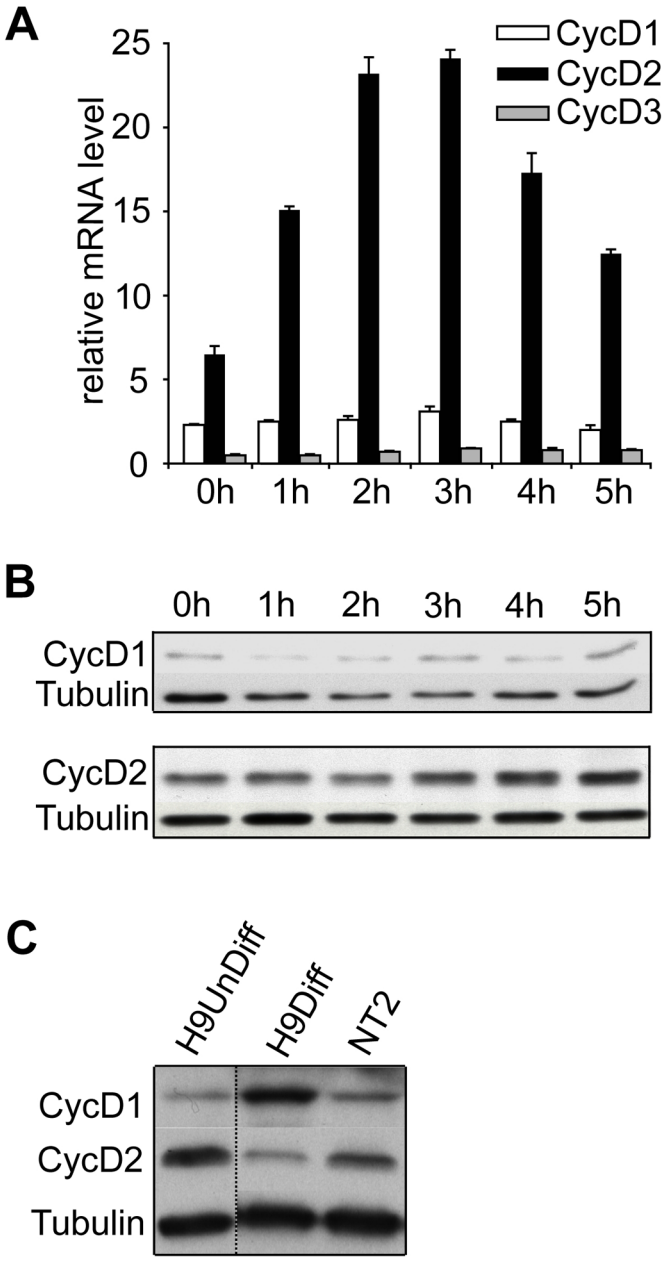

Figure 1. Cyclin D2 is prominently expressed during G1 and S phase in human ES cells

A. The relative mRNA levels of *cyclin D1, D2*, and *D3* (respectively, *CycD1, CycD2 and CycD3*) were analyzed by RT-qPCR for the first five hours after release of WA09/H9 hES cells from nocodazole induced mitotic synchronization, based on previous data (Becker et al, 2006). Data were normalized relative to 28S rRNA. **B**. The corresponding protein levels of cyclins D1 and D2 were determined by western blotting using α tubulin as loading control. **C.** Western blot analysis reveals cyclin D1 and D2 protein levels in asynchronous pluripotent H9 human ES cells (H9UnDiff), H9 human ES cells cultured for 72 h under differentiating conditions (H9Diff), and NT2 teratocarcinoma cells (NT2).

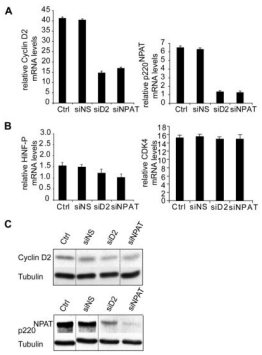

Figure 2. Efficacy of siRNA mediated depletion of cyclin D2 and p220^{NPAT} levels

A. The relative mRNA levels of *cyclin D2* (left) and *p220^{NPAT}* (right) were monitored by RT-qPCR in control WA09/H9 hES cells (Ctrl), as well as cells treated with non-silencing RNA (siNS) or siRNAs targeting *cyclin D2* (siD2) and *p220^{NPAT}* (siNPAT). Data were normalized relative to *28S rRNA*. **B.** Same as in Panel A, but showing the relative mRNA levels for HiNF-P protien (left), which is the heterodimeric partner of p220^{NPAT}, and CDK4 (right), which complexes with Cyclin D2. **C.** Western blot analysis shows protein levels for cyclin D2 and p220^{NPAT} in cells treated with silencing and non-silencing RNAs as in Panels A and B. Levels of α tubulin were monitored as loading control. The dotted lines indicate rearrangements of the lanes (from the same blot and exposure) to permit vertical alignment of treatment groups.

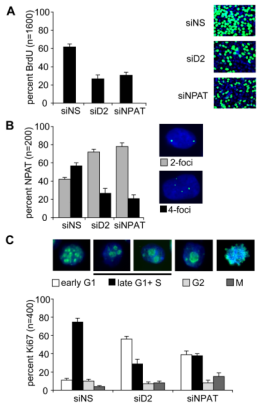

Figure 3. Depletion of cyclin D2 and p220^{NPAT} by siRNA treatment inhibits cell cycle progression

A. Quantification of the percentage of cells that incorporate BrdU (left) and representative micrographs of BrdU incorporating cells obtained by immunofluorescence microscopy (right) in hES colonies treated with non-silencing RNA (siNS) or specific siRNAs for *cyclin D2* (siD2) and *p220^{NPAT}* (siNPAT). The data in the graph are based on n=400 cells per treatment. **B.** As in Panel A, but the graph shows quantification of the percentage of cells (n=200) with either two or four p220^{NPAT} foci (left) and micrographs of representative nuclei (right). **C.** As in Panel A, but micrographs show representative cells in specific cell cycle stages based on Ki67 staining (top) and the graph shows quantification of the percentage of cells in the indicated cell cycle stages (bottom)(n=400). All error bars in Panels A, B and C represent SEM.

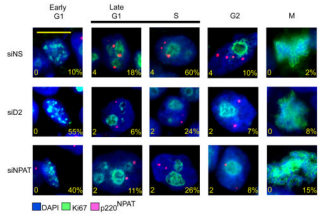

Figure 4. SiRNA mediated knockdown of p220^{NPAT} and cyclin D2 alters the subnuclear organization of p220^{NPAT} containing Histone Locus Bodies

The micrographs show fluorescence signals for p220^{NPAT} (red) in representative hES nuclei as visualized by DAPI staining (blue) at specific cell cycle stages based on Ki67 staining (green) in cells treated with non-silencing RNA (siNS) or specific siRNAs for *cyclin D2* (siD2) and *p220^{NPAT}* (siNPAT). For each treatment group (rows), we determined the fraction of cells in a specific cell cycle stage (% in bottom right corner of each micrograph) and the typical number of p220^{NPAT} foci per nucleus (bottom left corner). Percentages are based on n=200 cells per treatment group. The scale bar represents 20μm.

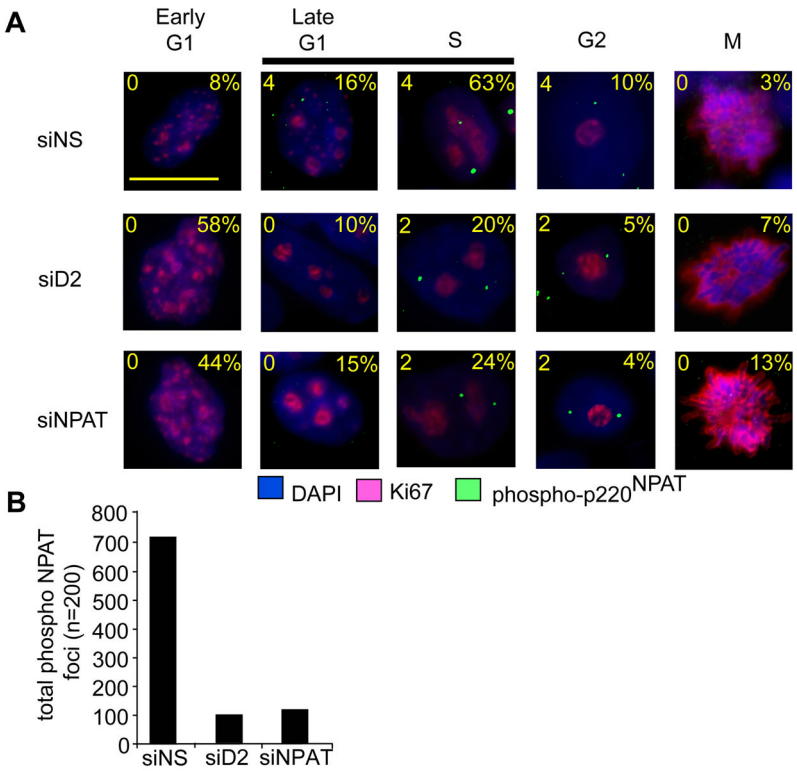

Figure 5. Reduced CDK phosphorylation of p220^{NPAT} after siRNA mediated depletion of cyclin D2 and p220^{NPAT} levels

A. Immunofluorescence microscopy was used to detect fluorescence signals for phosho-p220^{NPAT} (green; T1270 phospho-epitope) in representative hES nuclei as visualized by DAPI staining (blue) at specific cell cycle stages based on Ki67 staining (red). Cells were treated with non-silencing RNA (siNS) or specific siRNAs for *cyclin D2* (siD2) and *p220^{NPAT}* (siNPAT) (rows). The fraction of cells in a specific cell cycle stage (% in top right corner) and the typical number of phospho-p220^{NPAT} foci per nucleus (top left corner) are indicated in each micrograph. Percentages are based on n=200 cells per treatment group. The scale bar represents 20μm. **B.** The bar graph shows the total number of phospho-p220^{NPAT} foci in each of the three treatment groups for n=200 cells.

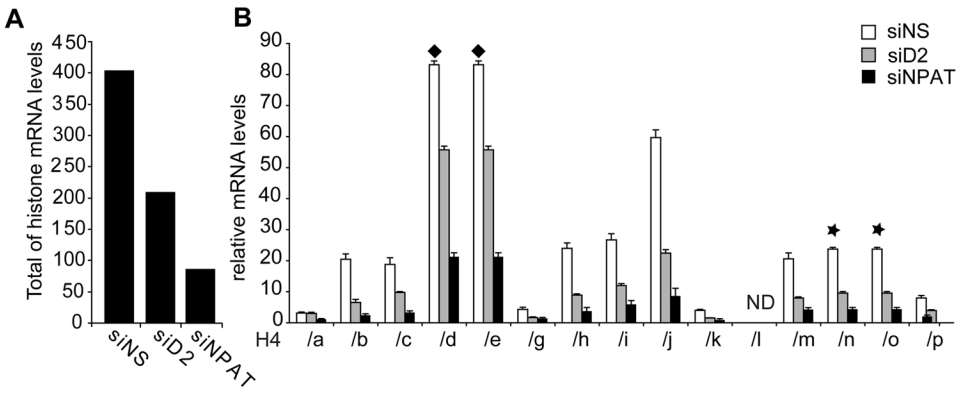

Figure 6. Diminished cyclin D2 or p220^{NPAT} levels upon siRNA treatment inhibit *histone* gene expression

RT-qPCR was used to quantify the *histone H4* mRNA levels for fourteen of the fifteen known human *histone H4* genes in cells treated with non-silencing RNA (siNS) or specific siRNAs for *cyclin D2* (siD2) and *p220^{NPAT}* (siNPAT). **A.** Bar graph showing the cumulative expression values of the fourteen *H4* genes in each of the three treatment groups. **B.** Individual expression values for each of the *histone H4* gene copies. The *H4/d* and *H4/e* genes (indicated by closed diamonds), as well the *H4/n* and *H4/o* genes (closed stars) are recent duplicates, and PCR primers cannot discriminate between these duplicate gene copies; the values shown are divided by two to compensate for copy number. All mRNA levels were normalized using *28S rRNA* as internal control. Error bars represent SEM.

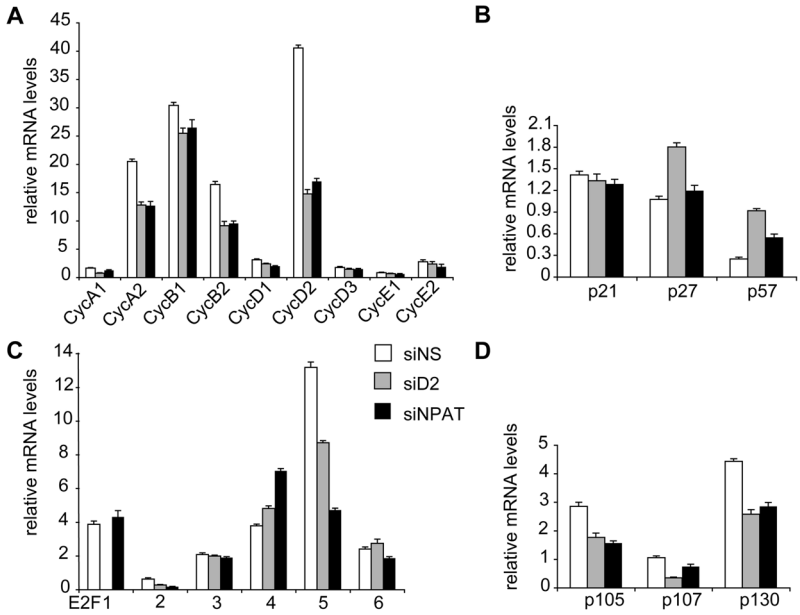

Figure 7. Alterations in the expression of cell cycle regulatory factors in hES cells treated with siRNAs for *cyclin D2* or p*220*^{NPAT}

Each of the four panels shows the relative mRNA levels of the indicated genes as measured by RT-qPCR in cells treated with non-silencing (siNS) or siRNAs against *cyclin D2* (siD2) and *p220*^{NPAT} (siNPAT). The graphs show expression values for distinct *cyclin* subtypes (**A**), the CDK inhibitors *p21*, *p27*, and *p57* (**B**), the E2F gene transcription factor family (*E2F-1 to -6*) (**C**), and the pocket proteins *p105*, *p107*, and *p130* (**D**). All mRNA levels measured were normalized using *28S rRNA* as internal control. Error bars represent SEM.