ARTICLE
Journal of Cellular Biochemistry 113:1333–1347 (2012)

Journal of Cellular Biochemistry

Comparative Analysis of Pre-Replication Complex Proteins in Transformed and Normal Cells

Domenic Di Paola[1,2] and Maria Zannis-Hadjopoulos[1,2]*

[1]Goodman Cancer Research Center, McGill University, Montreal, Quebec, Canada H3G 1Y6

[2]Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1Y6

ABSTRACT

This study examines the abundance of the major protein constituents of the pre-replication complex (pre-RC), both genome-wide and in association with specific replication origins, namely the *lamin B2*, *c-myc*, 20mer1, and 20mer2 origins. Several pre-RC protein components, namely ORC1-6, Cdc6, Cdt1, MCM4, MCM7, as well as additional replication proteins, such as Ku70/86, 14-3-3, Cdc45, and PCNA, were comparatively and quantitatively analyzed in both transformed and normal cells. The results show that these proteins are overexpressed and more abundantly bound to chromatin in the transformed compared to normal cells. Interestingly, the 20mer1, 20mer2, and *c-myc* origins exhibited a two- to threefold greater origin activity and a two- to threefold greater in vivo association of the pre-RC proteins with these origins in the transformed cells, whereas the origin associated with the housekeeping *lamin B2* gene exhibited both similar levels of activity and in vivo association of these pre-RC proteins in both cell types. Overall, the results indicate that cellular transformation is associated with an overexpression and increased chromatin association of the pre-RC proteins. This study is significant, because it represents the most systematic comprehensive analysis done to date, using multiple replication proteins and different replication origins in both normal and transformed cell lines. J. Cell. Biochem. 113: 1333–1347, 2012.

KEY WORDS: DNA REPLICATION; NASCENT DNA ABUNDANCE; PRE-REPLICATION COMPLEX; CHROMATIN IMMUNOPRECIPITATION; TRANSFORMED VS. NORMAL CELLS

Origins of DNA replication are specific sites within initiation zones in the genome at which replication is initiated due to the coordinated binding and highly controlled activation of the pre-replication complex (pre-RC) proteins [Bell and Dutta, 2002]. It is estimated that there are approximately 10^4 to 10^6 replicons per mammalian cell, each containing one functional origin of DNA replication with the replicon size varying from 10 to 300 kb, depending on the stage of development, growth conditions, or cell transformation status [Edenberg and Huberman, 1975; Hand, 1978; Anglana et al., 2003]. Chromosomal DNA replication is a highly regulated process both spatially and temporally, ensuring that the genome is replicated only once per cell cycle [Machida et al., 2005]. In any given S phase, DNA replication initiates at multiple origins across the genome, whose activation depends on the assembly and activation of pre-RCs [reviewed in Sclafani and Holzen, 2007]. Interference with the control mechanisms that maintain this process results in detrimental gains and losses of genomic DNA frequently seen in cancer [Lau et al., 2007]. In order to understand how DNA replication is regulated in normal cells and how it may become deregulated in transformed cells, the distinguishing features between replication origins and the proteins that interact with them in normal and transformed cells must be determined.

Cellular transformation alters the regulation of origin activation, resulting in replicons being approximately half the size of those observed in normal cells [Martin and Oppenheim, 1977; Oppenheim and Martin, 1978], suggesting that there are dormant (inactive) origins which are activated in transformed cells. Consistent with more origins being activated, transformed cells displayed a 2- to 10-fold increase of single strand nuclease sensitive regions [Collins et al., 1982]. Furthermore, transformed cells exhibited asynchronous replication timing of homologous loci relative to their normal counterparts [Amiel et al., 1998], in correlation with the observation that throughout S phase progression, there is a differential chromatin organization and rearrangements among normal and transformed cells [de Campos Vidal et al., 1998; Mello et al., 2007].

There are some discrepancies regarding the organization of DNA replication sites, with some studies reporting fundamental differences between normal and transformed cells [Kennedy et al., 2000; Frum et al., 2009] and others not [Dimitrova and Berezney, 2002; Panning and Gilbert, 2005]. In either case, the parameters governing

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Dr. Maria Zannis-Hadjopoulos, 3655 Promenade Sir William Osler, Rm. 707, Montreal, QC, Canada H3G 1Y6. E-mail: maria.zannis@mcgill.ca

Received 5 August 2011; Accepted 18 November 2011 • DOI 10.1002/jcb.24006 • © 2011 Wiley Periodicals, Inc.
Published online 1 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

replication kinetics are conserved between normal and transformed cells, as both cell types exhibit a heterogeneity in replication fork velocity, with a mean fork velocity of 1–2 kb/min [Conti et al., 2007; Frum et al., 2009]. Interestingly, in early S phase, replication forks of normal cells pause at 9–35 kb from newly initiated origins before resuming DNA synthesis, but no such pausing was observed in transformed cells [Frum et al., 2008]. Transformed cells have been found to produce replication signals 2.5 times greater than normal cells, consistent with low-level re-replication in otherwise unperturbed cell cycles [Dorn et al., 2009], in agreement with previous studies, describing an approximate twofold increase of initiation sites in transformed compared to normal cells [Martin and Oppenheim, 1977; Oppenheim and Martin, 1978]. The low-level re-replication observed in transformed cells may account for the differential origin usage observed between normal and transformed cells, such as the twofold increase of origin activity in transformed compared to normal cells, at the NOA3 [Tao et al., 1997], *c-myc* [Tao et al., 2000; Tao et al., 2001], 20mer1-6 [Di Paola et al., 2006], and the clone 3, 13, and 32 chromosomal origins [Di Paola et al., 2010]. Transformed cells also exhibited a polarity or position change of replication initiation compared to normal cells [Itoh-Lindstrom and Leffak, 1994], as was detected in a 5 kb region encompassing the ARSH1 locus; normal skin fibroblast (NSF) cells exhibited two prominent replication initiation sites, whereas HeLa cells contained multiple replication initiation sites within the same region [Hu et al., 2004a]. Similar findings were detected in close proximity to the putative ribulose-5-phosphate 3-epimerase gene, with NSF and normal breast epithelial cells (MCF10) exhibiting one prominent initiation site, unlike HeLa cells, which exhibited a diffuse pattern of initiation within the same region [Hu et al., 2004b]. It was recently reported that normal cells exhibited a site-specific pattern of origin activity, in contrast to a broader zonal pattern observed in cancer cells along a 78 kb region of human chromosome 2q34 [Valenzuela et al., 2011]; the same study also revealed that the number of activated origins in cancer cells is statistically larger than that obtained in normal cells, suggesting that the flexibility of origin usage is significantly increased in cancer cells compared to their normal counterparts [Valenzuela et al., 2011]. However, some origins exhibit similar initiation activity in both normal and transformed cells, such as the *lamin* B2 origin [Kumar et al., 1996], the β-globin origin, the 343 origin and the S14 origin [Tao et al., 1997], as well as the origin within the promoter region of the human *FMR1* gene [Gray et al., 2007]. Overall, three patterns of origin activity emerge, when comparing transformed and normal cells: (1) equal activity in transformed and normal cells, (2) increased activity in transformed cells, or (3) activity solely in transformed cells.

Few studies have analyzed comparatively the origin activity profiles of normal and transformed cells and even fewer have examined the in vivo association profiles of pre-RC proteins with origins in the two cell types. An overexpression of ORC subunits and increased ORC-chromatin association has been shown in transformed mammalian cells [McNairn and Gilbert, 2005]. Furthermore, chromosomal loci containing the clone 3, 13, and 32 origins were observed to have a two- to threefold higher origin activity as well as in vivo association of ORC2, Cdc6, and Cdt1 in transformed compared to normal cells [Di Paola et al., 2010]. These findings indicated that transformed cells have an increased expression and chromatin bound level of pre-RC proteins, extending the apparent connection between cellular transformation and deregulation of pre-RC proteins, in addition to all the downstream pathways they affect [Lau and Jiang, 2006; Dutta, 2007; Lau et al., 2007; Lau et al., 2009].

This is the first study that examines in detail, the fundamental differences regarding the expression, global-chromatin-bound (genome-wide) and local-chromatin-bound (on specific replication origins) levels of the major members of the pre-RC between normal and transformed cells. We have comparatively and quantitatively analyzed several replication proteins involved in the initiation and elongation of DNA replication in both transformed and normal cells and found them to be overexpressed and have an increased chromatin association in the transformed cells. Four origin-containing regions were examined by in situ chromosomal DNA replication assays, three of which exhibited increased activity in transformed cells and one exhibited equal activity in both transformed and normal cells. No example of a transformation specific origin was used, because, although postulated on a global scale [Martin and Oppenheim, 1977; Oppenheim and Martin, 1978], no such origin has been locally mapped and, therefore, due to lack of sequence information, no primer sets could be designed for quantification purposes in this study. Furthermore, chromatin immunoprecipitation (ChIP) assays showed that these proteins are differentially bound to replication origins in the two cell types, correlating with their level of activity.

MATERIALS AND METHODS

CELL CULTURE AND FACS ANALYSIS

HeLa, NSF (primary normal skin fibroblasts), WI38 (human lung embryo fibroblasts), and WI38 VA13 2RA (WI38 transformed with SV40 virus) cells were acquired from American Type Culture Collection (Manassas, VA) and cultured in α-minimal essential medium supplemented with penicillin (100 g/ml), streptomycin (100 g/ml), 1 mM L-glutamine, tylosin (8 g/ml), and 10% (v/v) fetal bovine serum. When the cells reached 30–50% confluence, they were harvested for the isolation of nascent DNA, while upon reaching 60–80% confluence, they were harvested for isolation of chromatin immunoprecipitated DNA, and upon reaching 100% confluence, they were serum-starved for 48–72 h and harvested for the isolation of genomic DNA. For flow cytometry analysis, cells were washed twice in ice-cold phosphate-buffered saline (PBS), and resuspended in Vindelov's solution (3.4 mM Tris, 75 M propidium iodide, 0.1% NP-40, 0.01 M NaCl, 700 U/L Rnase A), overnight at 4°C, then analyzed using a Beckman flow cytometer and the CellQuest program.

ISOLATION OF GENOMIC DNA

Genomic DNA was isolated using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Oakville, ON, Canada), as per instructions of the manufacturer.

ISOLATION OF NASCENT DNA

Nascent DNA was prepared using the λ-exonuclease method, as previously described [Tao et al., 2000, 2001], with the following modifications: The λ-exonuclease digested samples were heated at 100°C for 3 min, then immediately subjected to electrophoresis on a 2% agarose gel. DNA was visualized by staining with 0.02% (w/v) methylene blue (Sigma) and the origin-containing nascent DNA, ranging between 350 and 1,000 bp in size was excised from the gel, purified with the Sephaglas BandPrep Kit (GE Healthcare, Piscataway, NJ), as per instructions of the manufacturer, and resuspended in TE.

PREPARATION OF WHOLE CELL EXTRACTS (WCEs)

For the preparation of WCEs, the cells were harvested, washed twice with ice-cold PBS and resuspended in 2× packed cell volume (pcv) with high salt buffer (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 20 mM EDTA, 50 mM DTT and complete protease inhibitor tablet [Roche Molecular Biochemicals, Indianapolis, IN]). Following a 1-h incubation at 4°C in constant agitation, the cells were centrifuged at 14,000*g*, the supernatant harvested and its protein concentration determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA).

CHROMATIN LOADING

Cell fractionation and preparation of the chromatin-enriched fractions was performed as described in Tatsumi et al. [2000]. Cells were harvested from 10-cm dishes into ice-cold PBS, centrifuged, resuspended in 1 ml of lysis buffer A (10 mM HEPES–KOH, pH 7.9, 100 mM NaCl, 300 mM sucrose, 0.1% Triton X-100 and complete protease inhibitor tablet [Roche Molecular Biochemicals]), and lysed on ice for 10 min. After centrifugation at 2,000*g* for 3 min at 4°C, pellets were washed once more with ice-cold lysis buffer A and resuspended in lysis buffer B (10 mM HEPES–KOH, pH 7.9, 200 mM NaCl, 300 mM sucrose, 0.1% Triton X-100, 5 mM $MgCl_2$ and complete protease inhibitor tablet [Roche Molecular Biochemicals]) containing 1,000 U of DNase I (Invitrogen). Following incubation at 25°C for 30 min, the chromatin-enriched fraction was isolated in the supernatant after centrifugation at 2,500*g* for 5 min at 4°C.

IN VIVO CROSS-LINKING AND CHROMATIN FRAGMENTATION

Cells cultured in complete media were washed with pre-warmed PBS and treated with 1% formaldehyde for 10 min to crosslink proteins and DNA in vivo [Sibani et al., 2005a]; they were then washed and scraped into ice-cold PBS and resuspended in lysis buffer (50 mM HEPES–KOH pH 7.5, 140 mM NaCl, 1% Triton X-100, 2 mM EDTA) supplemented with a complete protease inhibitor tablet (Roche Molecular Biochemicals). Following passage through a 21 G needle three times, the nuclei were harvested, resuspended in one packed nuclear volume of lysis buffer, and sonicated until DNA fragments of less than 1 kb were obtained. Chromatin size was monitored by electrophoresis. For cell counting, one untreated plate was scraped into PBS and resuspended. The cells were then counted with a hematocytometer, and this number was used to derive the total number of treated cells. Also the concentrations of the extracts were determined using the Bradford protein assay (BioRad, Hercules, CA) in order to normalize samples across cell lines.

IMMUNOPRECIPITATION AND DNA ISOLATION

Immunoprecipitation (IP) was carried out as previously described [Sibani et al., 2005a], with the following modifications: Briefly, sheared chromatin lysates (500 μg) were pre-cleared by incubation with 50μl of protein A or protein G agarose (Roche Molecular Biochemicals) to reduce background caused by non-specific adsorption to the beads, incubated for 6 h with either 20 μg of anti-ORC1 (Santa Cruz, sc-28741), anti-ORC2 (Santa Cruz, sc-32734)/(gift from Dr. Anindya Dutta), anti-ORC3 (Santa Cruz, sc-23888)/(gift from Dr. Anindya Dutta), anti-ORC4 (Santa Cruz, sc-20634)/(gift from Dr. Anindya Dutta), anti-ORC5 (Santa Cruz, sc-20635), anti-ORC6 (Santa Cruz, sc-32735)/(gift from Dr. Anindya Dutta), anti-Cdc6 (Santa Cruz, sc-8341), anti-Cdt1 (Santa Cruz, sc-28262), anti-MCM4 (Santa Cruz, sc-9841), anti-MCM7 (Santa Cruz, sc-9966), anti-Cdc45 (Santa Cruz, sc-20685), anti-PCNA (Santa Cruz, sc-56), anti-Ku70 (Santa Cruz, sc-17789), anti-Ku86 (Santa Cruz, sc-9034), anti-14-3-3 (Santa Cruz, sc-629), or normal rabbit serum (NRS) at 4°C with constant rotation. Protein A/G agarose (50 μl) was added and incubated overnight at 4°C. The pelleted beads were washed successively twice with 1 ml of lysis buffer for 15 min each at 4°C, followed by 1 ml of WB1 (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate, complete protease inhibitor tablet), 1 ml of WB2 (50 mM Tris–HCl pH 7.5, 0.1% NP-40, 0.05% sodium deoxycholate, complete protease inhibitor tablet) and 1 ml of sterile TE. The beads were resuspended in 200 μl TE/1% SDS, incubated at room temperature (rt) for 15 min and centrifuged at 3,000 rpm for 1 min at rt. Half of the supernatant was then incubated overnight at 65°C to reverse the crosslinks, followed by 100 μg of proteinase K at 55°C for 2 h. The DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and eluted in 100 μl TE. The remaining half of the supernatant was boiled for 10 min in SDS–PAGE loading buffer and subjected to electrophoresis on a 5% stacking/8% separating SDS–PAGE gel for Western blot analysis.

WESTERN BLOT ANALYSIS

Western blot analysis was carried out according to standard protocols [Sambrook et al., 1989]. Briefly, the indicated amounts of WCEs, chromatin-enriched fractions, and crosslinked immunoprecipitated samples mentioned above were resuspended in SDS loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 min and loaded onto a 5% stacking/8% separating SDS–PAGE gel. Following electrophoresis and transfer onto a PVDF membrane, the membrane was immunoblotted with the indicated primary and corresponding HRP-conjugated secondary antibodies. The following antibodies were used: anti-ORC1 (Santa Cruz, sc-23887)/(gift from Dr. Bruce Stillman), anti-ORC2 (Santa Cruz, sc-32734), anti-ORC3 (Santa Cruz, sc-23888), anti-ORC4 (Santa Cruz, sc-20634), anti-ORC5 (Santa Cruz, sc-20635), anti-ORC6 (Santa Cruz, sc-32735), anti-Cdc6 (Santa Cruz, sc-8341), anti-Cdt1 (Santa Cruz, sc-28262)/(gift from Dr. Hideo Nishitani), anti-MCM4 (Santa Cruz, sc-9841), anti-MCM7 (Santa Cruz, sc-9966), anti-Cdc45 (Santa Cruz, sc-20685), anti-PCNA (Santa Cruz, sc-56), anti-Ku70 (Santa Cruz, sc-17789), anti-Ku86 (Santa Cruz, sc-9034), anti-14-3-3 (Santa Cruz, sc-629), anti-histone H3 (Millipore, 05-499), anti-tubulin

(Sigma, T9026), and anti-actin (Sigma, A2066). Proteins were visualized using the enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL).

REAL-TIME PCR QUANTIFICATION ANALYSES

PCRs were carried out in a total volume of 20 μl with 5 μl of genomic, nascent, or immunoprecipitated DNA, using the Light-Cycler (Roche Diagnostics), as previously described [Di Paola et al., 2006, 2010]. The sequences and amplification conditions for all primer sets are shown in Supplementary Table I. Genomic DNA (1, 2, 3, and 4 ng) from NSF cells was used to generate the standard curves needed for quantification of the PCR products. A negative control without template DNA was included with each set of reactions. PCR products were also resolved on 2% agarose gels, visualized with ethidium bromide, and photographed with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000). No extraneous bands were generated with any of the primer sets.

RESULTS

EXPRESSION AND CHROMATIN-BOUND LEVELS OF PRE-RC AND REPLICATION PROTEINS IN TRANSFORMED AND NORMAL CELLS

FACS analysis was performed on asynchronously growing HeLa, NSF, WI38, and WI38(SV40) cells in order to control for the comparison of both expression and chromatin-bound levels of pre-RC and replication proteins between normal (NSF and WI38) and transformed (HeLa and WI38(SV40)) cell lines. The results show that for both cell types, the cell distribution was the same in each phase of the cell cycle (Fig. 1A,B).

To assess the expression levels of the various pre-RC proteins, WCEs were prepared from asynchronously growing transformed and normal cells, and were subjected to SDS–PAGE and immunoblotted for ORC1-6, Cdc6, Cdt1, MCM4, MCM7, Cdc45, PCNA, Ku70, Ku86, and 14-3-3. With the exception of 14-3-3, which exhibited similar expression levels in both cell types, all the other proteins examined were overexpressed in the transformed cells (Fig. 1C).

To examine whether the overexpression of the pre-RC proteins in the transformed cells affected their association with chromatin, chromatin-bound extracts were prepared from asynchronously growing transformed and normal cells, and were subjected to SDS–PAGE and immunoblotted for ORC1-6, Cdc6, Cdt1, MCM4, MCM7, Cdc45, PCNA, Ku70, Ku86, and 14-3-3. Again, all these proteins exhibited elevated chromatin association in the transformed cells, mirroring their expression profile, with the exception of 14-3-3, which exhibited similar levels of chromatin association in both cell types (Fig. 1D).

The nuclear marker, histone H3, is present in both the WCEs and the chromatin-bound extracts, whereas the cytoplasmic marker, tubulin, is present in the WCEs but undetectable in chromatin-bound extracts (Fig. 1C,D), as expected, indicating the fidelity of our fractionation procedure.

Importantly, the changes in expression and chromatin bound levels observed between HeLa and NSF cells were not due to cell type but due to transformation, as the same results were obtained using the isogenic pair of WI38 and WI38(SV40). Overall, these results indicate that overexpression and increased chromatin association of replication proteins is a feature of cellular transformation, in agreement to what has been previously reported [McNairn and Gilbert, 2005; Lau et al., 2007].

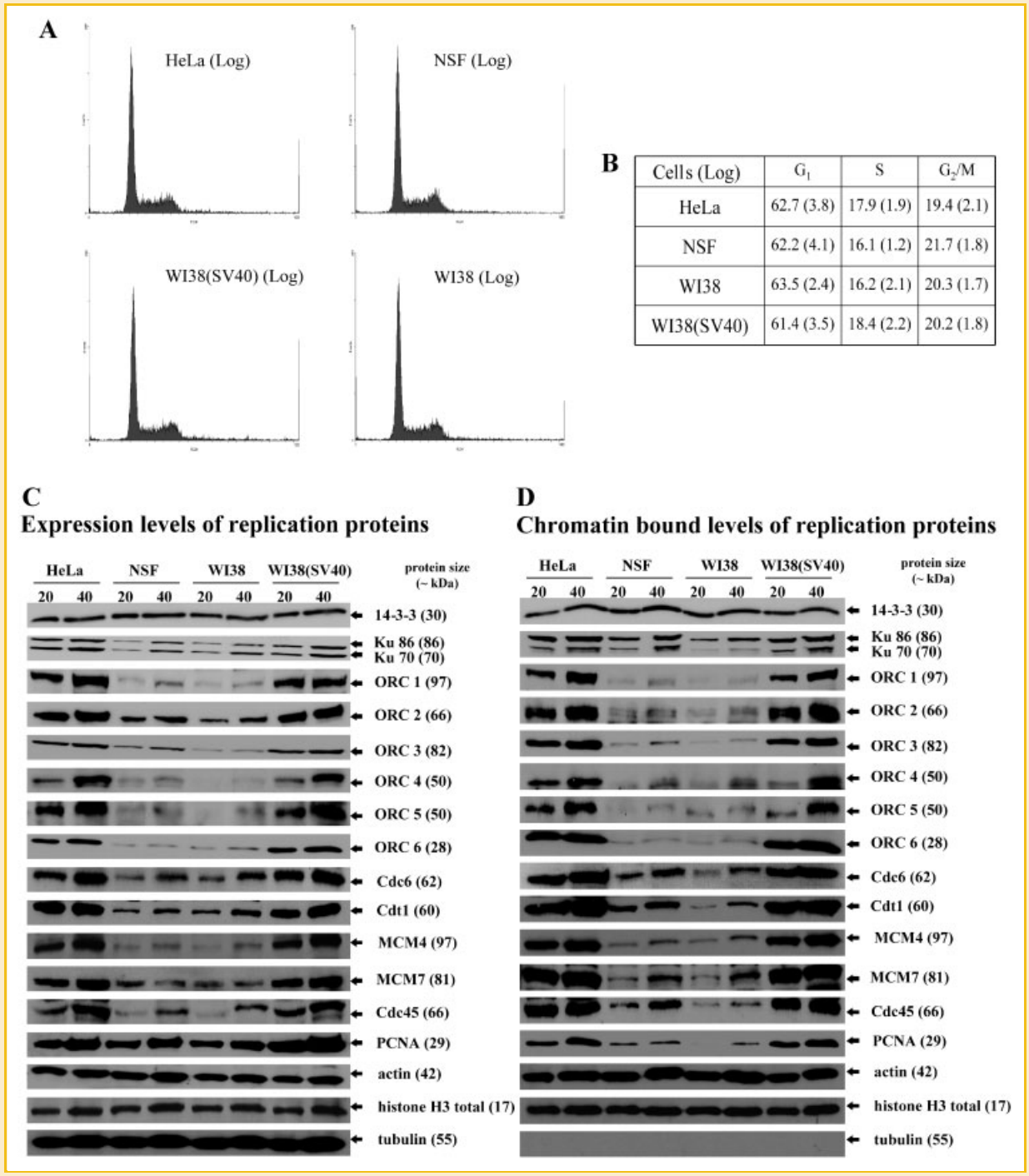
COPY NUMBER OF THE 20mer1, 20mer2, *lamin B2*, AND *c-myc* CHROMOSOMAL LOCI IN TRANSFORMED AND NORMAL CELLS

In order to perform quantitative comparisons of replication origin activity between the transformed and normal cells, the copy number per haploid genome of all regions examined in this study was assessed. For this, equal amounts of genomic DNA from both transformed and normal cells was amplified by real-time PCR, using primer sets denoted in Supplementary Table I, which target the 20mer1, 20mer2, *lamin B2*, and *c-myc* chromosomal loci. The copy number per haploid genome for each chromosomal locus was produced after normalization of the results by making NSF equal to one copy per haploid genome. The 20mer1 (Supplementary Fig. 1A), 20mer2 (Supplementary Fig. 1B), *lamin B2* (Supplementary Fig. 1C), and *c-myc* (Supplementary Fig. 1D) chromosomal loci, encompassing their respective replication origins, were present at one copy per haploid genome in all cell lines.

ORIGIN ACTIVITIES AT THE CHROMOSOMAL LOCI OF 20mer1, 20mer2, *lamin B2*, AND *c-myc* IN TRANSFORMED AND NORMAL CELLS

Confirmation of the presence of a single copy per haploid genome of all chromosomal loci in all cell lines permitted measurement of the in vivo origin activity by quantification of the nascent DNA abundance at the chromosomal loci of 20mer1 (Fig. 2A), 20mer2 (Fig. 2B), *lamin B2* (Fig. 2C), and *c-myc* (Fig. 2D) in the transformed and normal cells. Nascent DNA abundance was measured at two reference points for each region, an origin-containing sequence (peak activity) and a non-origin-containing sequence (negative control with background activity) located at least 4 kb away from any of the origin regions. For the 20mer1 region, amplification of primer set M20mer1, containing the origin sequence, ranged between 10.4- and 29.1-fold more than amplification of primer set 20merC1 located ~6 kb away, containing non-origin sequence; for the 20mer2 region, amplification of primer set M20mer2, containing the origin sequence, ranged between 10.2- and 29.9-fold more than amplification of primer set 20merC2 located ~6 kb away, containing non-origin sequence; for the *lamin B2* region, amplification of primer set LB2, containing the origin sequence, ranged between 24.7- and 29.3-fold more than amplification of primer set LB2C1 located ~4 kb away, containing non-origin sequence; and finally, for the *c-myc* region, amplification of primer set Myc11, containing the origin sequence, ranged between 10.7- and 24.3-fold more than amplification of primer set Myc1 located ~6 kb away, containing non-origin sequence.

The nascent strand abundance across the chromosomal loci of 20mer1, 20mer2, *lamin B2*, and *c-myc* was determined in the same preparation of short nascent DNA and normalized to that of an internal reference, the *lamin B2* locus, to control for the possibility of a greater recovery of nascent DNA from the transformed cells compared to the normal cells. Specifically, amplification of nascent



Cells (Log)	G_1	S	G_2/M
HeLa	62.7 (3.8)	17.9 (1.9)	19.4 (2.1)
NSF	62.2 (4.1)	16.1 (1.2)	21.7 (1.8)
WI38	63.5 (2.4)	16.2 (2.1)	20.3 (1.7)
WI38(SV40)	61.4 (3.5)	18.4 (2.2)	20.2 (1.8)

Fig. 1. FACS and comparative analysis of the expression and chromatin-bound levels of ORC1-6, Cdc6, Cdt1, MCM4, MCM7, Cdc45, PCNA, 14-3-3, Ku70, and Ku86 in transformed and normal cells. A: Asynchronous cultures of logarithmically growing cells (log) were harvested and stained with propidium iodide to monitor their cell cycle distribution; a representative flow cytometric analysis collected from three individual experiments is shown for each of the following cell lines (HeLa, NSF, WI38, and WI38(SV40)). B: Quantification of the percentage of cells present in each phase of the cell cycle (G_1, S, and G_2/M). Averages of three experiments performed in triplicate and their standard deviations (in brackets) are shown. C: Expression levels and D: Chromatin bound levels of ORC1-6, Cdc6, Cdt1, MCM4, MCM7, Cdc45, PCNA, 14-3-3, Ku70, and Ku86 in the two transformed (HeLa and WI38(SV40)) and two normal (NSF and WI38) cell lines. Twenty and forty μg of WCEs or chromatin-bound extracts were subjected to SDS-PAGE, transferred onto PVDF membrane and probed with anti-ORC1-6, anti-Cdc6, anti-Cdt1, anti-MCM4, anti-MCM7, anti-Cdc45, anti-PCNA, anti-14-3-3, anti-Ku70, and anti-Ku86 antibodies. Anti-actin was used as a loading control. Anti-histone H3, a nuclear marker, and anti-tubulin, a cytoplasmic marker, were used as quality control for the chromatin-bound fraction to indicate the reliability of the fractionation procedure. The size (in kDa) of each protein detected by Western blot is indicated next to its name.

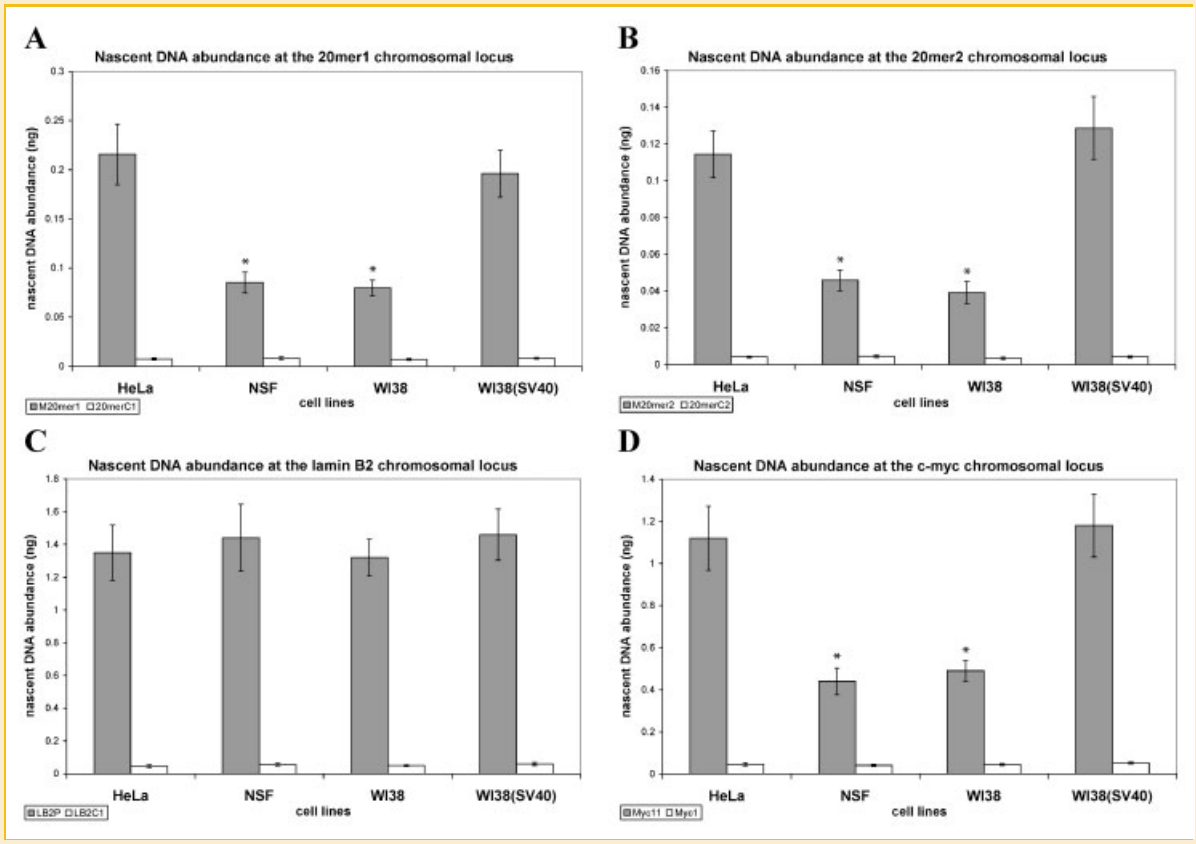


Fig. 2. Comparative analysis of the nascent DNA abundance at the 20mer1, 20mer2, *lamin B2*, and *c-myc* chromosomal loci in transformed and normal cells. Histogram plots of the quantification by real-time PCR of nascent DNA abundance (ng) at the 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) chromosomal loci in the two transformed (HeLa and WI38(SV40)) and two normal (NSF and WI38) cell lines. The location and sequence information of the primers used for the amplification of the origin-containing regions (gray bars) and the non-origin-containing control regions (white bars) are as described in Supplementary Figure 1 and Supplementary Table I. The error bars represent the average of at least two experiments performed in triplicate and 1 SD. Statistically significant differences ($P < 0.05$) between the transformed and normal cells in the abundance of nascent DNA at the specified DNA regions are indicated with an asterisk.

DNA with primer set LB2C1 (background activity compared to *lamin B2* peak region, primer set LB2) gave baseline values that were used to normalize the results from all the nascent DNA preparations of all cell lines, permitting comparison of data between different preparations and different cell lines.

Histogram plots of the nascent DNA abundance measured at the chromosomal loci of 20mer1 (Fig. 2A), 20mer2 (Fig. 2B), *lamin B2* (Fig. 2C), and *c-myc* (Fig. 2D) in all the cell lines examined revealed a greater than 10-fold increase of all origin containing compared to non-origin containing regions (which exhibited background levels, with less than 10% origin activity), indicating that all the nascent DNA preparations were of good quality and confirmed that the origin regions of each locus examined in all cell lines are located at true sites of initiation of DNA replication [DePamphilis, 1997].

The results also show a two- to threefold higher origin activity associated with the chromosomal loci of 20mer1 (Fig. 2A), 20mer2 (Fig. 2B), and *c-myc* (Fig. 2D) in the transformed versus the normal cells, suggesting a transformation-related activation of these origins. Use of the isogenic pair of WI38 and WI38(SV40) cells again ruled out the possibility that the observed increased frequency of initiation in the transformed cells might be due to cell type. In contrast, the origin activity associated with the chromosomal locus of *lamin B2* was similar in both the transformed and normal cells (Fig. 2C), suggesting that this origin, which lies within the constitutively active housekeeping region of the genome that codes for *lamin B2*, may adopt an open chromatin configuration, allowing for similar origin activity in all cell lines examined regardless of cell type or transformation status, as previously observed [Biamonti et al., 1992; Kumar et al., 1996].

ABUNDANCE OF PRE-RC AND REPLICATION PROTEINS AT THE CHROMOSOMAL LOCI OF THE 20mer1, 20mer2, *lamin B2*, AND *c-myc* REPLICATION ORIGINS IN TRANSFORMED AND NORMAL CELLS

The in vivo association of the pre-RC proteins ORC1-6, Cdc6, Cdt1, MCM4, MCM7, and other replication proteins, such as Cdc45, PCNA, Ku70, Ku86, and 14-3-3 with the chromosomal loci of the 20mer1, 20mer2, *lamin B2*, and *c-myc* replication origins was measured in transformed and normal cells by quantification of chromatin immunoprecipitated DNA corresponding to the origin regions (M20mer1, M20mer2, LB2, and Myc11) compared to their respective non-origin containing (control) regions (20merC1, 20merC2, LB2C1, and Myc1) (Figs. 3–6A-D). The cell cycle distribution of

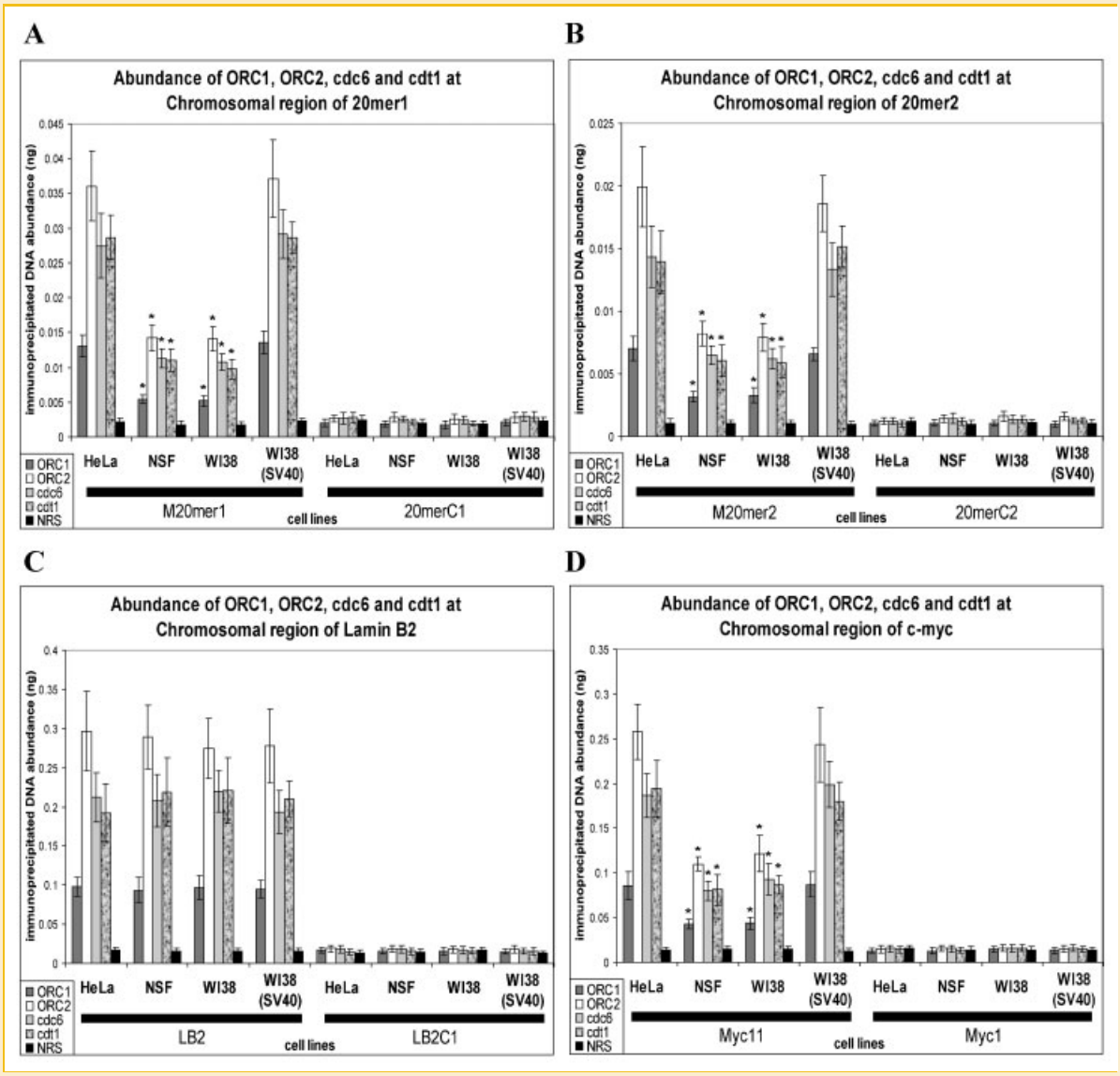


Fig. 3. In vivo association of ORC1, ORC2, Cdc6, and Cdt1 at the 20mer1, 20mer2, *lamin B2*, and *c-myc* chromosomal loci in transformed and normal cells. Histogram plots of the quantification by real-time PCR of immunoprecipitated DNA abundance (ng) at the 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) chromosomal loci in the two transformed (HeLa and WI38(SV40)) and two normal (NSF and WI38) cell lines. ChIP was performed with antibodies directed against ORC1 (dark gray bars), ORC2 (white bars), Cdc6 (light gray) and Cdt1 (spotted gray bars); normal rabbit serum (NRS) (black bars) was used as a negative control. The primer sets of 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) are denoted in Supplementary Table I. The error bars represent the average of at least two experiments performed in triplicate and 1 SD. Statistically significant differences ($P < 0.05$) between the transformed and normal cells in the association of the indicated protein to the specified DNA regions are indicated with an asterisk.

asynchronously growing cells from all cell lines was found to be the same by FACS analysis (Fig. 1A,B), permitting the comparisons between the transformed and normal cells. Equal amounts of cross-linked chromatin extracts were immunoprecipitated with antibodies against the above-mentioned proteins and an equivalent amount of NRS as a negative control to give baseline values that were used to normalize results from all ChIP preparations of all cell lines, permitting comparison of data between different preparations and different cell lines.

Histogram plots of the immunoprecipitated DNA abundance of ORC1, ORC2, Cdc6, and Cdt1, measured at the chromosomal loci of 20mer1 (Fig. 3A), 20mer2 (Fig. 3B), *lamin B2* (Fig. 3C), and *c-myc* (Fig. 3D) in all the cell lines used showed an increased in vivo association of these proteins with the origin compared to non-origin sequences. The enrichment of ORC1, ORC2, Cdc6, and Cdt1 at the 20mer1 origin varied from 6.1- to 17.1-fold over NRS for the transformed cells and from 3.0- to 8.1-fold over NRS for the normal cells, while it was 6.4- to 13.7-fold greater than the non-origin containing sequence for the transformed cells and 2.9- to 5.5-fold greater than the non-origin containing sequence for the normal cells (Fig. 3A). Their enrichment at the 20mer2 origin varied from 6.5- to 19.6-fold over NRS for the transformed cells and from 3.1- to 8.1-fold over NRS for the normal cells, while it was 6.7- to 16.2-fold greater than the non-origin containing sequence for the transformed

cells and 2.9- to 5.8-fold greater than the non-origin containing sequence for the normal cells (Fig. 3B). Their association with the *lamin B2* origin varied from 6.1- to 18.9-fold enrichment over NRS for both the transformed and normal cells, while it was 6.0- to 15.8-fold greater than the non-origin containing sequence for both the transformed and normal cells (Fig. 3C). Finally, their enrichment at the *c-myc* origin varied from 6.1- to 19.9-fold over NRS for the transformed cells and from 2.9- to 8.2-fold over NRS for the normal cells, while it was 6.6- to 17.2-fold greater than the non-origin containing sequence for the transformed cells and 3.0- to 7.6-fold greater than the non-origin containing sequence for the normal cells (Fig. 3D).

Histogram plots of the immunoprecipitated DNA abundance of ORC3, ORC4, ORC5, and ORC6 measured at the chromosomal loci of 20mer1 (Fig. 4A), 20mer2 (Fig. 4B), *lamin B2* (Fig. 4C), and *c-myc* (Fig. 4D) in all the cell lines used showed an increased in vivo association of these proteins with each of the origin compared to non-origin sequences. The enrichment of ORC3, ORC4, ORC5, and ORC6 at the 20mer1 origin varied from 8.1- to 13.4-fold over NRS for the transformed cells and from 3.8- to 6.5-fold over NRS for the normal cells, while it was 8.3- to 13.5-fold greater than the non-origin containing sequence for the transformed cells and 3.9- to 5.9-fold greater than the non-origin containing sequence for the normal cells (Fig. 4A). Their enrichment at the 20mer2 origin varied

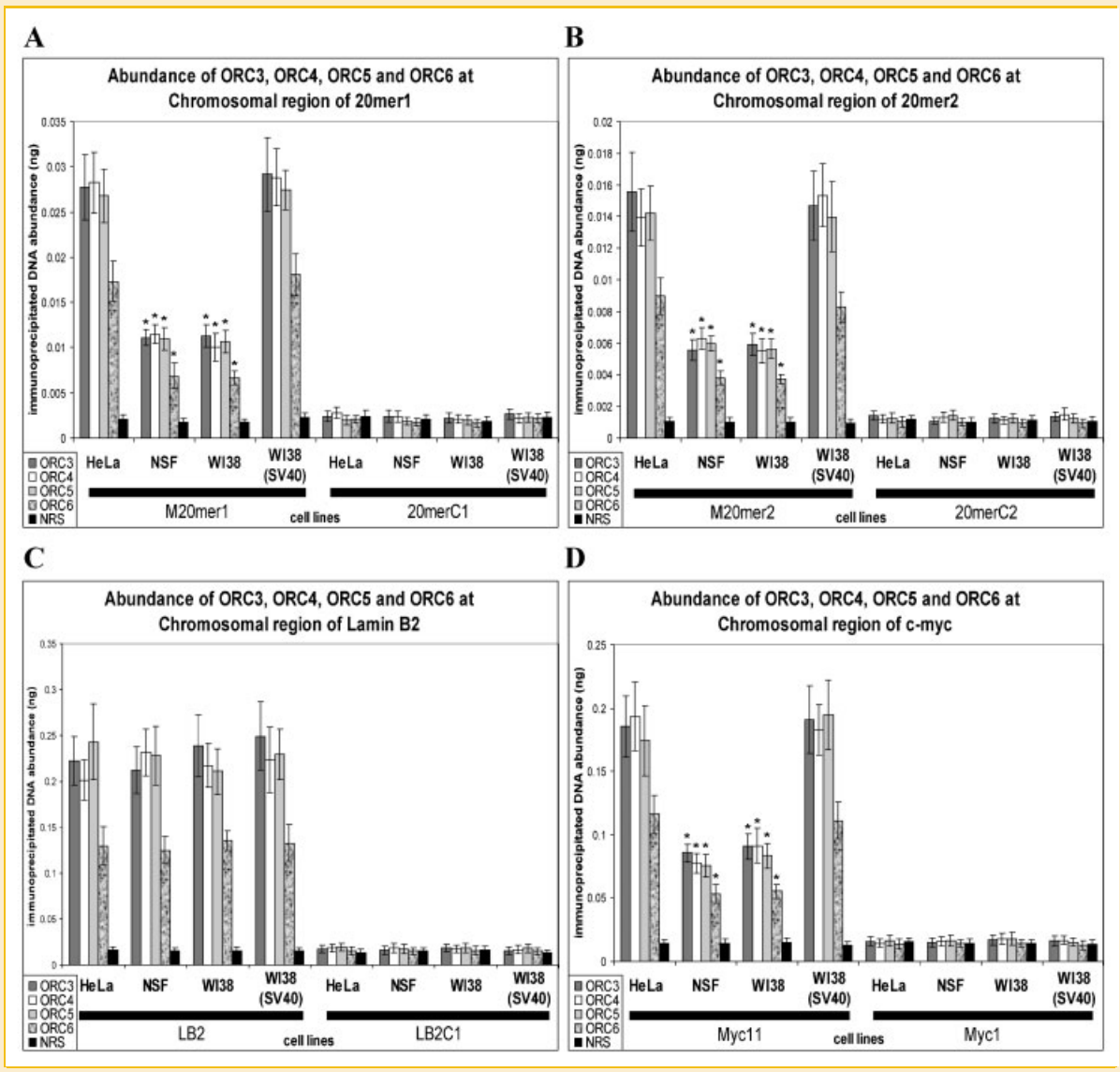


Fig. 4. In vivo association of ORC3, ORC4, ORC5, and ORC6 at the 20mer1, 20mer2, *lamin B2*, and *c-myc* chromosomal loci in transformed and normal cells. Histogram plots of the quantification by real-time PCR of immunoprecipitated DNA abundance (ng) at the 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) chromosomal loci in the two transformed (HeLa and WI38(SV40)) and two normal (NSF and WI38) cell lines. ChIP was performed with antibodies directed against ORC3 (dark gray bars), ORC4 (white bars), ORC5 (light gray), and ORC6 (spotted gray bars); normal rabbit serum (NRS) (black bars) was used as a negative control. The primer sets of 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) are denoted in Supplementary Table I. The error bars represent the average of at least two experiments performed in triplicate and 1 SD. Statistically significant differences ($P < 0.05$) between the transformed and normal cells in the association of the indicated protein to the specified DNA regions are indicated with an asterisk.

from 8.3- to 16.2-fold over NRS for the transformed cells and from 3.7- to 6.2-fold over NRS for the normal cells, while it was 8.6- to 11.5-fold greater than the non-origin containing sequence for the transformed cells and 3.8- to 5.1-fold greater than the non-origin containing sequence for the normal cells (Fig. 4B). Their association with the *lamin B2* origin varied from 8.0- to 16.6-fold enrichment over NRS for both the transformed and normal cells, while it was 8.2- to 16.2-fold greater than the non-origin containing sequence for both the transformed and normal cells (Fig. 4C). Finally, their enrichment at the *c-myc* origin varied from 8.3- to 15.9-fold over NRS for the transformed cells and from 3.7- to 6.2-fold over NRS for the normal cells, while it was 8.5- to 13.5-fold greater than the non-origin containing sequence for the transformed cells and 3.8- to 5.7-fold greater than the non-origin containing sequence for the normal cells (Fig. 4D).

In similar analyses, histogram plots of the immunoprecipitated DNA abundance of MCM4, MCM7, Cdc45, and PCNA measured at the chromosomal loci of 20mer1 (Fig. 5A), 20mer2 (Fig. 5B), *lamin B2* (Fig. 5C), and *c-myc* (Fig. 5D) in all the cell lines used showed a modest twofold increased in vivo association of MCM4 and MCM7 with each of the origin compared to non-origin sequences, but showed a similar in vivo association of Cdc45 and

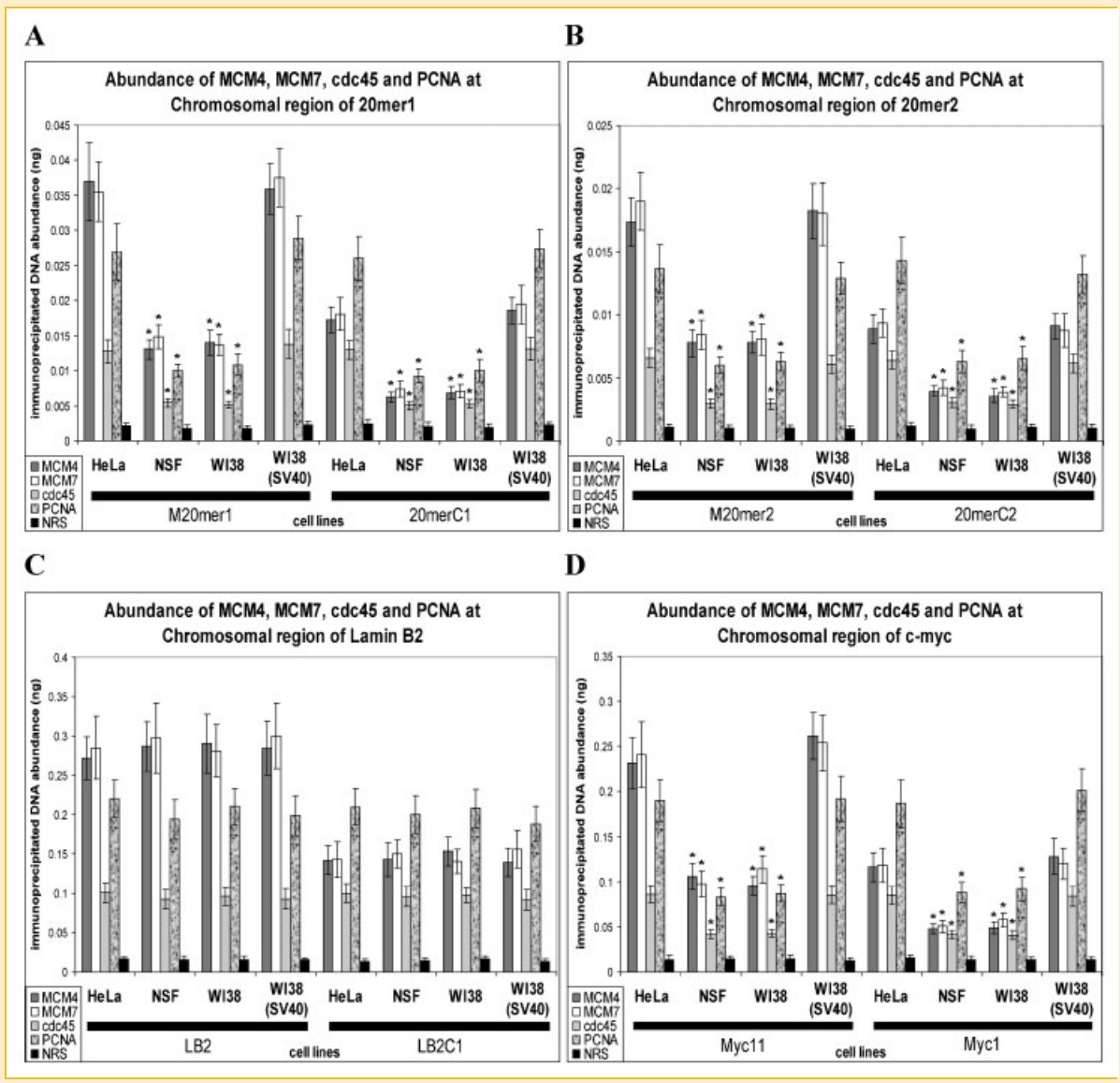


Fig. 5. In vivo association of MCM4, MCM7, Cdc45, and PCNA at the 20mer1, 20mer2, *lamin B2*, and *c-myc* chromosomal loci in transformed and normal cells. Histogram plots of the quantification by real-time PCR of immunoprecipitated DNA abundance (ng) at the 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) chromosomal loci in the two transformed (HeLa and WI38(SV40)) and two normal (NSF and WI38) cell lines. ChIP was performed with antibodies directed against MCM4 (dark gray bars), MCM7 (white bars), Cdc45 (light gray bars), and PCNA (spotted gray bars); normal rabbit serum (NRS) (black bars) was used as a negative control. The primer sets of 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) are denoted in Supplementary Table I. The error bars represent the average of at least two experiments performed in triplicate and 1 SD. Statistically significant differences ($P < 0.05$) between the transformed and normal cells in the association of the indicated protein to the specified DNA regions are indicated with an asterisk.

PCNA at both the origin and non-origin sequences. The enrichment of MCM4, MCM7, Cdc45, and PCNA at the 20mer1 origin varied from 6.1- to 17.5-fold over NRS for the transformed cells and from 2.9- to 8.3-fold over NRS for the normal cells, while it was 1.0- to 2.1-fold greater than the non-origin containing sequence for both cell types (Fig. 5A). Their enrichment at the 20mer2 origin varied from 6.1- to 19.2-fold over NRS for the transformed cells and from 2.9- to 8.3-fold over NRS for the normal cells, while it was 1.0- to 2.2-fold greater than the non-origin containing sequence for both cell types (Fig. 5B). Their association with the *lamin B2* origin varied from 6.0- to 20.0-fold enrichment over NRS for both the transformed and normal cells, while it was 1.0- to 2.0-fold greater than the non-origin containing sequence for both cell types (Fig. 5C). Finally, their enrichment at the *c-myc* origin varied from 6.2- to 21.4-fold over NRS for the transformed cells and from 2.9- to 7.7-fold over NRS for the normal cells, while it was 1.0- to 2.2-fold greater than the non-origin containing sequence for both cell types (Fig. 5D).

Lastly, histogram plots of the immunoprecipitated DNA abundance of Ku70, Ku86, and 14-3-3 measured at the chromosomal loci of 20mer1 (Fig. 6A), 20mer2 (Fig. 6B), *lamin B2* (Fig. 6C), and *c-myc* (Fig. 6D) in all the cell lines used showed an increased in vivo association of these proteins with each of the origin compared to non-origin sequences, corroborating previous results [Novac et al.,

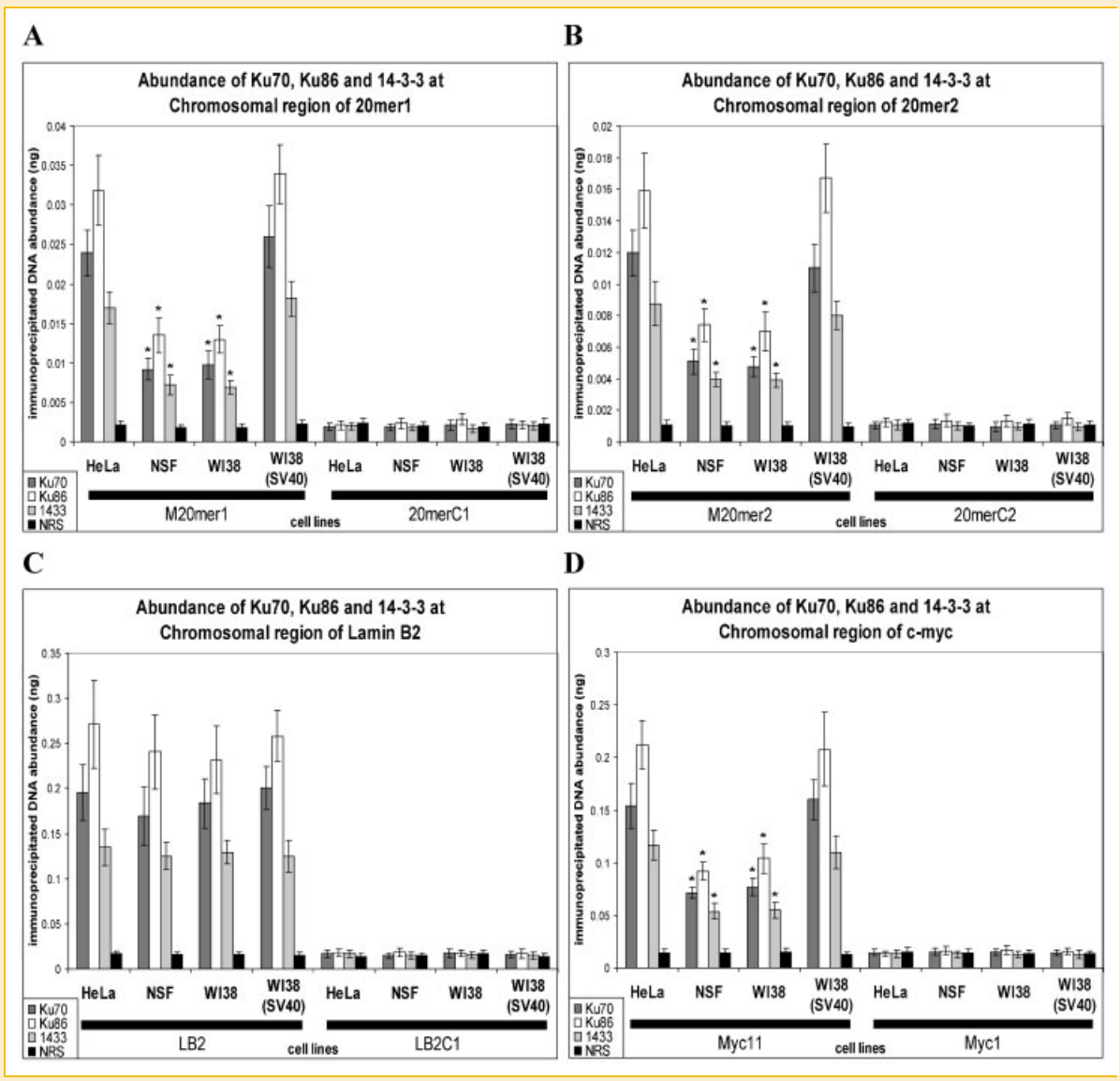


Fig. 6. In vivo association of Ku70, Ku86, and 14–3–3 at the 20mer1, 20mer2, *lamin B2*, and *c-myc* chromosomal loci in transformed and normal cells. Histogram plots of the quantification by real-time PCR of immunoprecipitated DNA abundance (ng) at the 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) chromosomal loci in the two transformed (HeLa and WI38(SV40)) and two normal (NSF and WI38) cell lines. ChIP was performed with antibodies directed against Ku70 (dark gray bars), Ku86 (white bars), and 14–3–3 (light gray bars); normal rabbit serum (NRS) (black bars) was used as a negative control. The primer sets of 20mer1 (A), 20mer2 (B), *lamin B2* (C), and *c-myc* (D) are denoted in Supplementary Table I. The error bars represent the average of at least two experiments performed in triplicate and 1 SD. Statistically significant differences ($P < 0.05$) between the transformed and normal cells in the association of the indicated protein to the specified DNA regions are indicated with an asterisk.

2001, 2002; Alvarez et al., 2002; Callejo et al., 2002, 2006; Sibani et al., 2005ab; Yahyaoui et al., 2007; Rampakakis et al., 2008; Yahyaoui and Zannis-Hadjopoulos, 2009]. The enrichment of Ku70, Ku86, and 14-3-3 at the 20mer1 origin varied from 8.1- to 15.2-fold over NRS for the transformed cells and from 4.0- to 7.5-fold over NRS for the normal cells, while it was 8.5- to 15.7-fold greater than the non-origin containing sequence for the transformed cells and 4.1- to 5.8-fold greater than the non-origin containing sequence for the normal cells (Fig. 6A). Their enrichment at the 20mer2 origin varied from 8.1- to 17.6-fold over NRS for the transformed cells and from 3.9- to 7.3-fold over NRS for the normal cells, while it was 8.3- to 12.9-fold greater than the non-origin containing sequence for the transformed cells and 3.9- to 5.6-fold greater than the non-origin containing sequence for the normal cells (Fig. 6B). Their association with the *lamin B2* origin varied from 8.2- to 17.2-fold enrichment over NRS for both the transformed and normal cells, while it was 8.3- to 15.4-fold greater than the non-origin containing sequence for both the transformed and normal cells (Fig. 6C). Finally, their enrichment at the *c-myc* origin varied from 8.3- to 17.0-fold over NRS for the transformed cells and from 3.7- to 7.0-fold over NRS for the normal cells, while it was 8.6- to 15.9-fold greater than the non-origin containing sequence for the transformed cells and 4.1- to 6.2-fold greater than the non-origin containing sequence for the normal cells (Fig. 6D).

Importantly, the low abundance of DNA detected in the NRS immunoprecipitates in all ChIP experiments indicated that the conditions were stringent enough to prevent substantial non-specific association of DNA with the immunoglobulins or agarose beads, further confirming the specificity of the ChIPs.

DISCUSSION

PRE-RC PROTEINS ARE OVEREXPRESSED AND BOUND MORE ABUNDANTLY TO CHROMATIN IN TRANSFORMED COMPARED TO NORMAL CELLS

A broad range of recent evidence suggests that pre-RC proteins play a role during oncogenesis [Lau et al., 2007]. In light of our previous studies, indicating a differential origin activity between normal and transformed cells, we examined whether similar differences existed in pre-RC protein expression and chromatin association that were associated with cellular transformation.

The results show that all six ORC subunits were overexpressed and had an elevated association with chromatin in the transformed compared to normal cells, as was previously shown for ORC1, ORC2, and ORC4 [McNairn and Gilbert, 2005], as well as ORC6 in colorectal cancer [Gavin et al., 2008]. Similarly, increased protein levels and chromatin association were observed in the transformed cells for Cdc6 and Cdt1, in agreement with previous studies using normal and tumor lung cell specimens, where the overexpression of Cdc6 and Cdt1 promoted malignant behavior [Karakaidos et al., 2004; Liontos et al., 2007]. As previously shown, overexpression of Cdc6 resulted in the specific methylation and silencing of tumor suppressors in pre-malignant lesions [Gonzalez et al., 2006], suggesting that deregulation of pre-RC proteins might alter transcription of cancer-promoting genes.

Furthermore, the results show that MCM4, MCM7, Cdc45, PCNA, Ku70, and Ku86 were overexpressed and bound to chromatin at higher levels in the transformed compared to normal cells. In agreement, increased expression of MCM4 was observed in cervical, esophageal, and non-small cell lung cancers [Huang et al., 2007; Gan et al., 2010; Kikuchi et al., 2011]. Also, deregulated MCM7 was previously observed to contribute to oncogene driven tumorigenesis [Honeycutt et al., 2006] and was an adequate marker of proliferation in prostate cancer [Padmanabhan et al., 2004]. Moreover, the levels of Cdc45 have been shown to be consistently higher in human cancer-derived cells compared to primary human cells [Pollok et al., 2007], while a novel form of PCNA has been discovered in malignant breast cells [Bechtel et al., 1998] that has implications as a potential biomarker in breast and esophageal cancer [Malkas et al., 2006; Hammoud et al., 2007]. Finally, Ku70 and Ku86 have been shown to be overexpressed in non-melanoma skin cancer and colorectal cancer [Mazzarelli et al., 2005; Parrella et al., 2006], as well as to be involved in metastasis of breast cancer [Lagadec et al., 2010].

Interestingly, 14-3-3 was the only protein that displayed similar expression and chromatin bound levels in the transformed and normal cells. This might be partly because the pan antibody used in this study recognizes all isoforms of 14-3-3 and some of the isoforms encode proteins that either promote or suppress tumor activity, implying that a cellular balance between the various 14-3-3 isoforms is important for the proper functioning of the cell. For example, the overexpression of the ε isoform is involved in renal carcinoma [Liang et al., 2009], the β and γ isoforms induce oncogenic transformation when overexpressed [Takihara et al., 2000; Radhakrishnan and Martinez, 2010], while the σ isoform is a putative tumor suppressor which counteracts the effects of the γ isoform, reversing oncogenic transformation [Radhakrishnan and Martinez, 2010; Zurita et al., 2010]. This cellular equilibrium between the oncogenic and tumor suppressor properties of various 14-3-3 isoforms may explain the similar expression and chromatin bound levels observed here in the transformed and normal cells.

Overall, the observed differences in the expression and chromatin-bound levels of these replication proteins between the transformed and normal cells corroborates previous studies, showing that overexpression and increased chromatin association of pre-RC proteins correlated with increasing tumor grade and poor prognosis [Gonzalez et al., 2005; Lau et al., 2007], pointing to a potential link between deregulation of pre-RC proteins and cancer [Lau et al., 2007].

THE ASSOCIATION OF PRE-RC PROTEINS WITH REPLICATION ORIGINS IN BOTH TRANSFORMED AND NORMAL CELLS CORRELATES WITH THE ACTIVITY OF THE ORIGIN

These observed differences between transformed and normal cells prompted us to examine the origin activity at the 20mer1, 20mer2, *c-myc*, and *lamin B2* chromosomal loci (Fig. 2A–D) and compare it to the association of pre-RC proteins with these same chromosomal loci (Figs. 3–6A–D) in both cell types.

The nascent DNA data showed that the 20mer1, 20mer2, *c-myc*, and *lamin B2* chromosomal loci each contain replication origins in both transformed and normal cells, but the 20mer1, 20mer2, and

c-myc origins exhibited two- to threefold more activity in the transformed cells (Fig. 2A,B,D), whereas the *lamin B2* origin exhibited similar activity in both cell types (Fig. 2C), indicating that cellular transformation does not equally affect the activity of all replication origins, in agreement with previous studies [Kumar et al., 1996; Tao et al., 1997, 2000, 2001; Di Paola et al., 2006, 2010].

The ChIP data showed that ORC1-6, Cdc6, Cdt1, Ku70, Ku86, and 14-3-3 associate in vivo with the 20mer1, 20mer2, *lamin B2*, and *c-myc* origins with a relative enrichment of ~2.9- to 17.2-fold over their respective non-origin containing control regions, in agreement with their involvement in the initiation phase of DNA replication, concurring with the handful of previous ChIP studies, examining the binding of various members of the pre-RC to human origins of replication [reviewed in Schepers and Papior, 2009].

A lower enrichment (~2-fold) of MCM4 and MCM7 at the origins over their respective non-origin containing control regions was observed by comparison to the aforementioned proteins, in agreement with its dual role in both the initiation and elongation phases of DNA replication, as the MCM2-7 complex is slightly enriched at the origin region, but then travels with the replication fork across the replicon as well as its ability to move along DNA once loaded, correlating with previous observations [Gonzalez et al., 2006; Evrin et al., 2009; Remus et al., 2009]. This slight enrichment of MCMs at origin regions may be due to excess MCM2-7 complexes playing an important role in maintaining genomic integrity to protect human cells from replicative stress by licensing backup origins of replication [Ge et al., 2007; Ibarra et al., 2008].

No enrichment at the origins compared to their respective non-origin containing control regions was observed for Cdc45 and PCNA, consistent with their role in the elongation phase of DNA replication, traveling with the replication fork across the replicon [Sporbert et al., 2005; Gambus et al., 2006; Pacek et al., 2006; Moldovan et al., 2007; Gorisch et al., 2008; Aparicio et al., 2009; Cohen et al., 2009].

Overall, the results revealed a two- to threefold higher abundance of all the pre-RC proteins examined at the 20mer1, 20mer2, and *c-myc* origins of the transformed cells by comparison to the normal ones, but an equal abundance at the *lamin B2* origin in both cell types. Taken together, the nascent DNA (Fig. 2A–D) and ChIP (Figs. 3–6A–D) data indicate that the association of replication proteins with origins correlates with the replication profile of the latter, suggesting a direct effect on origin activity.

Several studies have described the deregulation of pre-RC proteins during oncogenesis [reviewed in Lau et al., 2007], as subtle pre-RC deregulation produces low levels of re-replication that may evade the surveillance of the replication checkpoint machinery, resulting in genomic instability and tumorigenesis, leading to the suggestion that there is a pre-RC checkpoint lacking in cancer cells [Lau and Jiang, 2006]. Depletion of the pre-RC protein Cdc6 caused normal cells to arrest in G1-G1/S and S phase in a non-lethal state, but multiple cancer cell lines underwent G1-G1/S arrest and cell death, due to a defective ATR-dependent S phase checkpoint activation [Lau et al., 2009]. Similarly, transformed cells underwent cell death by depletion of ORC2, Cdc6 and Cdt1 proteins or by Cdc7 kinase [Wohlschlegel et al., 2000; Shreeram et al., 2002; Feng et al., 2003; Montagnoli et al., 2004; Prasanth et al., 2004], whereas normal cells were able to mount protective cellular responses that prevented inappropriate replication [Feng et al., 2003; Montagnoli et al., 2004; Lau and Jiang, 2006].

The elevated in vivo association of pre-RC proteins with the 20mer1, 20mer2, and *c-myc* origins in the transformed cells may be the result of deregulation of the pre-RC checkpoint, causing certain origins to fire more than once per cell cycle, due to their improper licensing, leading to re-replication and thereby causing genetic instability and then cancer [Dutta, 2007; Blow and Gillespie, 2008]. Alternatively, the differential origin activities might be due to the 20mer1, 20mer2, and *c-myc* origins being activated at a lower frequency per cell cycle in normal cells, but at a higher frequency in transformed cells; in this scenario, it is conceivable that in normal cells, at least during some S phases, the DNA at the 20mer1, 20mer2, and *c-myc* origins might be replicated by upstream or downstream origins flanking this region, resulting in origin interference [Lebofsky et al., 2006]. In contrast, the equal in vivo association of pre-RC proteins with the *lamin B2* origin in both transformed and normal cells indicates that this origin remains unaffected by the aberrant deregulation of the pre-RC checkpoint. As previously reported, only a subset of origins was sensitive to an origin licensing perturbation, as re-replication was unevenly distributed across chromosomes in tumor cell lines [Vaziri et al., 2003]; the *lamin B2* origin may reside in an unaffected area of the genome. Alternatively, the *lamin B2* origin is activated at similar frequencies in transformed and normal cells due to its constitutively expressed gene domain [Biamonti et al., 1992], resulting in an open chromatin configuration that encompasses the *lamin B2* origin, hence allowing equal accessibility for pre-RC protein binding in both cell types. Overall, the results indicate that the in vivo association of pre-RC proteins with origins in transformed and normal cells correlates with the activity of the origin.

CELLULAR TRANSFORMATION AND PRE-RC PROTEINS

Previous studies have shown that pre-RC deregulation directly promotes pro-oncogenic events, such as decreased DNA damage sensitivity, consistent with the fact that pre-RC proteins are frequently deregulated in a multitude of tumor types [Gonzalez et al., 2006; Honeycutt et al., 2006; Lau et al., 2007, 2009; Blow and Gillespie, 2008]. This study's findings indicate that overexpression and increased chromatin-association of pre-RC proteins are common features of cellular transformation, both of which have an important role in maintaining proper control of S phase. Further investigation is required to determine whether the overexpression and elevated chromatin-association of pre-RC proteins might cause cellular transformation or vice versa. A better understanding of the mechanisms involved in the process of cellular transformation will elucidate early events in the step-wise progression towards the development of cancer and will lead to improved measures of prevention and therapy.

ACKNOWLEDGMENTS

This work is dedicated to the memory of Dr. Gerald B. Price, whose expert intellectual and technical advice and most of all friendship is greatly missed. This research was supported by grants from the

Canadian Prostate Cancer Research Initiative and NCIC—Canadian Cancer Society. D.D.P. is a past recipient of a Canadian Institutes of Health Research (CIHR) Cancer Consortium Training Grant Award, a McGill Faculty of Medicine internal studentship award, and present recipient of a Fonds de la Recherche en Santé du Québec (FRSQ) studentship award.

REFERENCES

Alvarez D, Novac O, Callejo M, Ruiz MT, Price GB, Zannis-Hadjopoulos M. 2002. 14-3-3 sigma is a cruciform DNA binding protein and associates in vivo with origins of DNA replication. J Cell Biochem 87:194–207.

Amiel A, Litmanovitch T, Lishner M, Mor A, Gaber E, Tangi I, Fejgin M, Avivi L. 1998. Temporal differences in replication timing of homologous loci in malignant cells derived from CML and lymphoma patients. Genes Chromosomes Cancer 22:225–231.

Anglana M, Apiou F, Bensimon A, Debatisse M. 2003. Dynamics of DNA replication in mammalian somatic cells: Nucleotide pool modulates origin choice and interorigin spacing. Cell 114:385–394.

Aparicio T, Guillou E, Coloma J, Montoya G, Mendez J. 2009. The human GINS complex associates with Cdc45 and MCM and is essential for DNA replication. Nucleic Acids Res 37:2087–2095.

Bechtel PE, Hickey RJ, Schnaper L, Sekowski JW, Long BJ, Freund R, Liu N, Rodriguez-Valenzuela C, Malkas LH. 1998. A unique form of proliferating cell nuclear antigen is present in malignant breast cells. Cancer Res 58:3264–3269.

Bell SP, Dutta A. 2002. DNA replication in eukaryotic cells. Annu Rev Biochem 71:333–374.

Biamonti G, Perini G, Weighardt F, Riva S, Giacca M, Norio P, Zentilin L, Diviacco S, Dimitrova D, Falaschi A. 1992. A human DNA replication origin: Localization and transcriptional characterization. Chromosoma 102:S24–S31.

Blow JJ, Gillespie PJ. 2008. Replication licensing and cancer—A fatal entanglement? Nat Rev Cancer 8:799–806.

Callejo M, Alvarez D, Price GB, Zannis-Hadjopoulos M. 2002. The 14-3-3 protein homologues from Saccharomyces cerevisiae, Bmh1p and Bmh2p, have cruciform DNA-binding activity and associate in vivo with ARS307. J Biol Chem 277:38416–38423.

Callejo M, Sibani S, Di Paola D, Price GG, Zannis-Hadjopoulos M. 2006. Identification and functional analysis of a human homologue of the monkey replication origin ors8. J Cell Biochem 99:1606–1615.

Cohen SM, Chastain PD II, Cordeiro-Stone M, Kaufman DG. 2009. DNA replication and the GINS complex: Localization on extended chromatin fibers. Epigenetics Chromatin 2:6.

Collins J, Glock M, Chu A. 1982. Nuclease S1 sensitive sites in potential deoxyribonucleic acid of cold-and temperature-sensitive mammalian cells. Biochemistry 21:3414–3419.

Conti C, Sacca B, Herrick J, Lalou C, Pommier Y, Bensimon A. 2007. Replication fork velocities at adjacent replication origins are coordinately modified during DNA replication in human cells. Mol Biol Cell 18:3059–3067.

de Campos Vidal B, Russo J, Mello ML. 1998. DNA content and chromatin texture of benzo[a]pyrene-transformed human breast epithelial cells as assessed by image analysis. Exp Cell Res 244:77–82.

DePamphilis ML. 1997. The search for origins of DNA replication. Methods 13:211–219.

Di Paola D, Price GB, Zannis-Hadjopoulos M. 2006. Differentially active origins of DNA replication in tumor versus normal cells. Cancer Res 66:5094–5103.

Di Paola D, Rampakakis E, Chan MK, Arvanitis DN, Zannis-Hadjopoulos M. 2010. Increased origin activity in transformed versus normal cells: Identification of novel protein players involved in DNA replication and cellular transformation. Nucleic Acids Res 38:2314–2331.

Dimitrova DS, Berezney R. 2002. The spatio-temporal organization of DNA replication sites is identical in primary, immortalized and transformed mammalian cells. J Cell Sci 115:4037–4051.

Dorn ES, Chastain PD II, Hall JR, Cook JG. 2009. Analysis of re-replication from deregulated origin licensing by DNA fiber spreading. Nucleic Acids Res 37:60–69.

Dutta A. 2007. Chaotic license for genetic instability and cancer. Nat Genet 39:10–11.

Edenberg HJ, Huberman JA. 1975. Eukaryotic chromosome replication. Annu Rev Genet 9:245–284.

Evrin C, Clarke P, Zech J, Lurz R, Sun J, Uhle S, Li H, Stillman B, Speck C. 2009. A double-hexameric MCM 2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. Proc Natl Acad Sci USA 106:20240–20245.

Feng D, Tu Z, Wu W, Liang C. 2003. Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. Cancer Res 63:7356–7364.

Frum RA, Chastain PD II, Qu P, Cohen SM, Kaufman DG. 2008. DNA replication in early S phase pauses near newly activated origins. Cell Cycle 7:1440–1448.

Frum RA, Khondker ZS, Kaufman DG. 2009. Temporal differences in DNA replication during the S phase using single fiber analysis of normal human fibroblasts and glioblastoma T98G cells. Cell Cycle 8:3133–3148.

Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labib K. 2006. GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nat Cell Biol 8:358–366.

Gan N, Du Y, Zhang W, Zhou J. 2010. Increase of Mcm3 and Mcm4 expression in cervical squamous cell carcinomas. Eur J Gynaecol Oncol 31:291–294.

Gavin EJ, Song B, Wang Y, Xi Y, Ju J. 2008. Reduction of Orc6 expression sensitizes human colon cancer cells to 5-fluorouracil and cisplatin. PLoS ONE 3:e4054.

Ge XQ, Jackson DA, Blow JJ. 2007. Dormant origins licensed by excess Mcm 2-7 are required for human cells to survive replicative stress. Genes Dev 21:3331–3341.

Gonzalez MA, Tachibana KE, Laskey RA, Coleman N. 2005. Control of DNA replication and its potential clinical exploitation. Nat Rev Cancer 5: 135–141.

Gonzalez S, Klatt P, Delgado S, Conde E, Lopez-Rios F, Sanchez-Cespedes M, Mendez J, Antequera F, Serrano M. 2006. Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. Nature 440:702–706.

Gorisch SM, Sporbert A, Stear JH, Grunewald I, Nowak D, Warbrick E, Leonhardt H, Cardoso MC. 2008. Uncoupling the replication machinery: Replication fork progression in the absence of processive DNA synthesis. Cell Cycle 7:1983–1990.

Gray SJ, Gerhardt J, Doerfler W, Small LE, Fanning E. 2007. An origin of DNA replication in the promoter region of the human fragile X mental retardation (FMR1) gene. Mol Cell Biol 27:426–437.

Hammoud ZT, Badve S, Saxena R, Kesler KA, Rieger K, Malkas LH, Hickey RJ. 2007. A novel biomarker for the detection of esophageal adenocarcinoma. J Thorac Cardiovasc Surg 133:82–87.

Hand R. 1978. Eucaryotic DNA: Organization of the genome for replication. Cell 15:317–325.

Honeycutt KA, Chen Z, Koster MI, Miers M, Nuchtern J, Hicks J, Roop DR, Shohet JM. 2006. Deregulated minichromosomal maintenance protein MCM7 contributes to oncogene driven tumorigenesis. Oncogene 25:4027–4032.

Hu L, Xu X, Valenzuela MS. 2004a. Identification of novel initiation sites for human DNA replication around ARSH1, a previously characterized yeast replicator. Biochem Biophys Res Commun 313:1058–1064.

Hu L, Xu X, Valenzuela MS. 2004b. Initiation sites for human DNA replication at a putative ribulose-5-phosphate 3-epimerase gene. Biochem Biophys Res Commun 320:648–655.

Huang XP, Zhang X, Su XD, Ma GW, Zhao JM, Rong TH. 2007. Expression and significance of MCM4 in esophageal cancer. Ai Zheng 26:96–99.

Ibarra A, Schwob E, Mendez J. 2008. Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. Proc Natl Acad Sci USA 105:8956–8961.

Itoh-Lindstrom Y, Leffak M. 1994. Alteration of in vitro DNA synthesis in the alpha globin locus of embryo fibroblasts due to in vivo activity of Rous Sarcoma Virus. Nucleic Acids Res 22:498–508.

Karakaidos P, Taraviras S, Vassiliou LV, Zacharatos P, Kastrinakis NG, Kougiou D, Kouloukoussa M, Nishitani H, Papavassiliou AG, Lygerou Z, Gorgoulis VG. 2004. Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: Synergistic effect with mutant p53 on tumor growth and chromosomal instability—Evidence of E2F-1 transcriptional control over hCdt1. Am J Pathol 165:1351–1365.

Kennedy BK, Barbie DA, Classon M, Dyson N, Harlow E. 2000. Nuclear organization of DNA replication in primary mammalian cells. Genes Dev 14:2855–2868.

Kikuchi J, Kinoshita I, Shimizu Y, Kikuchi E, Takeda K, Aburatani H, Oizumi S, Konishi J, Kaga K, Matsuno Y, Birrer MJ, Nishimura M, Dosaka-Akita H. 2011. Minichromosome maintenance (MCM) protein 4 as a marker for proliferation and its clinical and clinicopathological significance in non-small cell lung cancer. Lung Cancer 72:229–237.

Kumar S, Giacca M, Norio G, Biamonti G, Riva S, Falaschi A. 1996. Utilization of the same DNA replication origin by human cells of different derivation. Nucleic Acids Res 24:3289–3294.

Lagadec C, Romon R, Tastet C, Meignan S, Com E, Page A, Bidaux G, Hondermark H, Le Bourhis X. 2010. Ku86 is important for TrkA overexpression-induced breast cancer cell invasion. Proteomics Clin Appl 4:580–590.

Lau E, Jiang W. 2006. Is there a pre-RC checkpoint that cancer cells lack? Cell Cycle 5:1602–1606.

Lau E, Tsuji T, Guo L, Lu SH, Jiang W. 2007. The role of pre-replicative complex (pre-RC) components in oncogenesis. FASEB J 21:3786–3794.

Lau E, Chiang GG, Abraham RT, Jiang W. 2009. Divergent S phase checkpoint activation arising from prereplicative complex deficiency controls cell survival. Mol Biol Cell 20:3953–3964.

Lebofsky R, Heilig R, Sonnleitner M, Weissenbach J, Bensimon A. 2006. DNA replication origin interference increases the spacing between initiation events in human cells. Mol Biol Cell 17:5337–5345.

Liang S, Xu Y, Shen G, Liu Q, Zhao X, Xu Z, Xie X, Gong F, Li R, Wei Y. 2009. Quantitative protein expression profiling of 14-3-3 isoforms in human renal carcinoma shows 14-3-3 epsilon is involved in limitedly increasing renal cell proliferation. Electrophoresis 30:4152–4162.

Liontos M, Koutsami M, Sideridou M, Evangelou K, Kletsas D, Levy B, Kotsinas A, Nahum O, Zoumpourlis V, Kouloukoussa M, Lygerou Z, Taraviras S, Kittas C, Bartkova J, Papavassiliou AG, Bartek J, Halazonetis TD, Gorgoulis VG. 2007. Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. Cancer Res 67:10899–10909.

Machida YJ, Hamlin JL, Dutta A. 2005. Right place, right time, and only once: Replication initiation in metazoans. Cell 123:13–24.

Malkas LH, Herbert BS, Abdel-Aziz W, Dobrolecki LE, Liu Y, Agarwal B, Hoelz D, Badve S, Schnaper L, Arnold RJ, Mechref Y, Novotny MV, Loehrer P, Goulet RJ, Hickey RJ. 2006. A cancer-associated PCNA expressed in breast cancer has implications as a potential biomarker. Proc Natl Acad Sci USA 103:19472–19477.

Martin RG, Oppenheim A. 1977. Initiation points for DNA replication in nontransformed and simian virus 40-transformed Chinese hamster lung cells. Cell 11:859–869.

Mazzarelli P, Parrella P, Seripa D, Signori E, Perrone G, Rabitti C, Borzomati D, Gabbrielli A, Matera MG, Gravina C, Caricato M, Poeta ML, Rinaldi M, Valeri S, Coppola R, Fazio VM. 2005. DNA end binding activity and Ku70/80 heterodimer expression in human colorectal tumor. World J Gastroenterol 11:6694–6700.

McNairn AJ, Gilbert DM. 2005. Overexpression of ORC subunits and increased ORC-chromatin association in transformed mammalian cells. J Cell Biochem 96:879–887.

Mello ML, Russo P, Russo J, Vidal BC. 2007. 17-beta-estradiol affects nuclear image properties in MCF-10F human breast epithelial cells with tumorigenesis. Oncol Rep 18:1475–1481.

Moldovan GL, Pfander B, Jentsch S. 2007. PCNA, the maestro of the replication fork. Cell 129:665–679.

Montagnoli A, Tenca P, Sola F, Carpani D, Brotherton D, Albanese C, Santocanale C. 2004. Cdc7 inhibition reveals a p53-dependent replication checkpoint that is defective in cancer cells. Cancer Res 64:7110–7116.

Novac O, Matheos D, Araujo FD, Price GB, Zannis-Hadjopoulos M. 2001. In vivo association of Ku with mammalian origins of DNA replication. Mol Biol Cell 12:3386–3401.

Novac O, Alvarez D, Pearson CE, Price GB, Zannis-Hadjopoulos M. 2002. The human cruciform binding protein, CBP, is involved in DNA replication and associates in vivo with mammalian replication origins. J Biol Chem 22:22.

Oppenheim A, Martin RG. 1978. Initiation points for DNA replication in nontransformed and simian virus 40-transformed BALB/c 3T3cells. J Virol 25:450–452.

Pacek M, Tutter AV, Kubota Y, Takisawa H, Walter JC. 2006. Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Mol Cell 21:581–587.

Padmanabhan V, Callas P, Philips G, Trainer TD, Beatty BG. 2004. DNA replication regulation protein Mcm7 as a marker of proliferation in prostate cancer. J Clin Pathol 57:1057–1062.

Panning MM, Gilbert DM. 2005. Spatio-temporal organization of DNA replication in murine embryonic stem, primary, and immortalized cells. J Cell Biochem 95:74–82.

Parrella P, Mazzarelli P, Signori E, Perrone G, Marangi GF, Rabitti C, Delfino M, Prencipe M, Gallo AP, Rinaldi M, Fabbrocini G, Delfino S, Persichetti P, Fazio VM. 2006. Expression and heterodimer-binding activity of Ku70 and Ku80 in human non-melanoma skin cancer. J Clin Pathol 59:1181–1185.

Pollok S, Bauerschmidt C, Sanger J, Nasheuer HP, Grosse F. 2007. Human Cdc45 is a proliferation-associated antigen. FEBS J 274:3669–3684.

Prasanth SG, Mendez J, Prasanth KV, Stillman B. 2004. Dynamics of pre-replication complex proteins during the cell division cycle. Philos Trans R Soc Lond B Biol Sci 359:7–16.

Radhakrishnan VM, Martinez JD. 2010. 14-3-3gamma induces oncogenic transformation by stimulating MAP kinase and PI3K signaling. PLoS ONE 5:e11433.

Rampakakis E, Di Paola D, Zannis-Hadjopoulos M. 2008. Ku is involved in cell growth, DNA replication and G1-S transition. J Cell Sci 121:590–600.

Remus D, Beuron F, Tolun G, Griffith JD, Morris EP, Diffley JF. 2009. Concerted loading of Mcm 2-7 double hexamers around DNA during DNA replication origin licensing. Cell 139:719–730.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning. Woodbury, NY: Cold Spring Harbor Laboratory Press.

Schepers A, Papior P. 2009. Why are we where we are? Understanding replication origins and initiation sites in eukaryotes using ChIP-approaches. Chromosome Res 18:63–77.

Sclafani RA, Holzen TM. 2007. Cell cycle regulation of DNA replication. Annu Rev Genet 41:237–280.

Shreeram S, Sparks A, Lane DP, Blow JJ. 2002. Cell type-specific responses of human cells to inhibition of replication licensing. Oncogene 21:6624–6632.

Sibani S, Price GB, Zannis-Hadjopoulos M. 2005a. Decreased origin usage and initiation of DNA replication in haploinsufficient HCT116 Ku80+/− cells. J Cell Sci 118:3247–3261.

Sibani S, Price GB, Zannis-Hadjopoulos M. 2005b. Ku80 binds to human replication origins prior to the assembly of the ORC complex. Biochemistry 44:7885–7896.

Sporbert A, Domaing P, Leonhardt H, Cardoso MC. 2005. PCNA acts as a stationary loading platform for transiently interacting Okazaki fragment maturation proteins. Nucleic Acids Res 33:3521–3528.

Takihara Y, Matsuda Y, Hara J. 2000. Role of the beta isoform of 14-3-3 proteins in cellular proliferation and oncogenic transformation. Carcinogenesis 21:2073–2077.

Tao L, Nielsen T, Friedlander P, Zannis-Hadjopoulos M, Price G. 1997. Differential DNA replication origin activities in human normal skin fibroblast and HeLa cell lines. J Mol Biol 273:509–518.

Tao L, Dong Z, Leffak M, Zannis-Hadjopoulos M, Price G. 2000. Major DNA replication initiation sites in the c-myc locus in human cells. J Cell Biochem 78:442–457.

Tao L, Dong Z, Zannis-Hadjopoulos M, Price GB. 2001. Immortalization of human WI38cells is associated with differential activation of the c-myc origins. J Cell Biochem 82:522–534.

Tatsumi Y, Tsurimoto T, Shirahige K, Yoshikawa H, Obuse C. 2000. Association of human origin recognition complex 1 with chromatin DNA and nuclease-resistant nuclear structures. J Biol Chem 275:5904–5910.

Valenzuela MS, Hu L, Lueders J, Walker R, Meltzer PS. 2011. Broader utilization of origins of DNA replication in cancer cell lines along a 78 KB region of human chromosome 2q34. J Cell Biochem DOI: 10.1002/jcb.23336.

Vaziri C, Saxena S, Jeon Y, Lee C, Murata K, Machida Y, Wagle N, Hwang DS, Dutta A. 2003. A p53-dependent checkpoint pathway prevents rereplication. Mol Cell 11:997–1008.

Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, Dutta A. 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 290:2309–2312.

Yahyaoui W, Zannis-Hadjopoulos M. 2009. 14-3-3 proteins function in the initiation and elongation steps of DNA replication in Saccharomyces cerevisiae. J Cell Sci 122:4419–4426.

Yahyaoui W, Callejo M, Price GB, Zannis-Hadjopoulos M. 2007. Deletion of the cruciform binding domain in CBP/14-3-3 displays reduced origin binding and initiation of DNA replication in budding yeast. BMC Mol Biol 8:27.

Zurita M, Lara PC, del Moral R, Torres B, Linares-Fernandez JL, Arrabal SR, Martinez-Galan J, Oliver FJ, Ruiz de Almodovar JM. 2010. Hypermethylated 14-3-3-sigma and ESR1 gene promoters in serum as candidate biomarkers for the diagnosis and treatment efficacy of breast cancer metastasis. BMC Cancer 10:217.