H4K20 methylation regulates quiescence and chromatin compaction

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ABSTRACT The transition between proliferation and quiescence is frequently associated with changes in gene expression, extent of chromatin compaction, and histone modifications, but whether changes in chromatin state actually regulate cell cycle exit with quiescence is unclear. We find that primary human fibroblasts induced into quiescence exhibit tighter chromatin compaction. Mass spectrometry analysis of histone modifications reveals that H4K20me2 and H4K20me3 increase in quiescence and other histone modifications are present at similar levels in proliferating and quiescent cells. Analysis of cells in S, G\textsubscript{2}/M, and G\textsubscript{1} phases shows that H4K20me1 increases after S phase and is converted to H4K20me2 and H4K20me3 in quiescence. Knockdown of the enzyme that creates H4K20me3 results in an increased fraction of cells in S phase, a defect in exiting the cell cycle, and decreased chromatin compaction. Overexpression of Suv4-20h1, the enzyme that creates H4K20me2 from H4K20me1, results in G\textsubscript{2} arrest, consistent with a role for H4K20me1 in mitosis. The results suggest that the same lysine on H4K20 may, in its different methylation states, facilitate mitotic functions in M phase and promote chromatin compaction and cell cycle exit in quiescent cells.

INTRODUCTION

Proper formation of tissues and organisms requires that cells have the capacity to transition between a proliferative, cycling state and a resting state outside the proliferative cell cycle termed quiescence. Cells integrate cues from growth factors, other cells, and extracellular matrix proteins and interpret these signals as they decide whether to commit to proliferation or quiescence. The ability of cells to properly exit the cell cycle, retain viability during quiescence, and return to the cell cycle when needed is necessary for complex multicellular processes such as growth and healing. Cells that fail to quiesce properly can contribute to the formation of tumors.

The transition between an out-of-cycle, quiescent state and a proliferative state is associated with changes in gene expression patterns (Schneider et al., 1988; Coppock et al., 1993; Venezia et al., 2004; Coller et al., 2006) and, in some systems, changes in overall transcription rates (Jaehning et al., 1975). These changes in gene expression with quiescence may be accompanied and regulated by alterations in the packing of DNA as chromatin (Tokuyasu et al., 1968; Dardick et al., 1983; Setterfield et al., 1983). In particular, modifications of lysines on the tails of histones H3 and H4 play an important role in local control of transcriptional activation and silencing, and the information encoded in these tails may constitute a code interpreted by proteins that bind to specific modifications (Jenuwein and Allis, 2001).
When proliferating cells enter S phase, their histones are incorporated into chromatin unmodified. Most lysines are quickly modified, however, likely using the existing chromatin as a template to transfer information. One lysine in particular does not become modified immediately after deposition—lysine 20 on H4 (Pesavento et al., 2008; Zee et al., 2012). H4K20 becomes monomethylated only during M phase as a result of mechanisms that limit the expression and activity of PR-Set7, the methyltransferase (Fang et al., 2002; Nishioka et al., 2002). PR-Set7 is actively targeted for proteasome-mediated degradation in S phase (Julien and Herr, 2004; Abbas et al., 2010; Wu et al., 2010; Jorgensen et al., 2011). The M-phase specific phosphorylation of PR-Set7 at serine 29 occurs at the onset of mitosis and contributes to stabilization of the enzyme (Wu et al., 2010). In addition, PHF8, an H4K20me1 demethylase, is removed from chromatin in prophase, allowing for an accumulation of H4K20me1 (Liu et al., 2010). Dimethylated and trimethylated H4K20 do not fluctuate as widely as the monomethyl form during the cell cycle. H4K20me2 is the most abundant form in Drosophila (Yang et al., 2008) and human cells (Young et al., 2009), existing on ~80% of histones. Trimethylated H4K20 is the least abundant of the three forms and is associated with repeated sequences (Kourmouli et al., 2004; Martens et al., 2005; Phalke et al., 2009). Knockouts of the enzymes that modify H4K20 display a variety of phenotypes. PR-Set7–knockout mice lose all H4K20 modifications and are embryonic lethal. A conditional knockout in embryonic stem cells leads to chromatin condensation defects (Oda et al., 2009). Knockout of both Suv4-20h1 (the enzyme that converts the monomethylated form to the dimethylated form) and Suv4-20h2 (the enzyme that converts the dimethylated form to the trimethylated form) leads to loss of the H4K20me2 and H4K20me3 histone forms and is perinatal lethal (Schotta et al., 2008). Loss of these modifications in mouse cells leads to telomere elongation (Benetti et al., 2007; Marion et al., 2011).

Studies examining the distribution of bulk levels of histone modifications in quiescent and proliferating lymphocytes have revealed differences in the levels of specific histone modifications between the two states. In response to antigen, lymphocytes can be stimulated from their quiescent state to divide. This reactivation is associated with an unpacking of the tightly wound heterochromatin and physical relocation of specific histone modifications within the nucleus (Tokuyasu et al., 1968; Dardick et al., 1983; Setterfield et al., 1983; Grigoryev et al., 2004). Activation of B cells is associated with increased levels of multiple histone modifications (Baxter et al., 2004). Lymphocyte activation is also associated with changes in the intranuclear localization of specific histone modifications. H4K12Ac, for instance, was excluded from centromeric heterochromatin in quiescent lymphocytes and was redistributed more uniformly upon activation (Grigoryev et al., 2004).

Primary dermal fibroblasts represent another cell type that can transition between a quiescent and a proliferative state. Fibroblasts are often quiescent in vivo. Their physiological role is to secrete extracellular matrix proteins that give tissue its strength and resilience. Fibroblasts can also proliferate, for instance, to replenish dead cells, and upon activation in the context of a wound. Early studies indicated a likely transition in chromatin structure upon stimulation of quiescent fibroblasts based on circular dichroism (Chiu and Baserga, 1975). We therefore set out to address whether fibroblasts undergo changes in chromatin compaction and histone modifications upon quiescence. We used fluorescence in situ hybridization (FISH) for two loci on opposite arms of a single chromosome to quantitatively assess chromatin compaction. We also used a mass spectrometry–based method (Plazas-Mayorca et al., 2009) to measure the levels of ~50 histone modifications. With these approaches, we characterized changes in chromatin compaction and histone modifications as fibroblasts transition from proliferation to contact inhibition–induced quiescence. We further defined changes in histone modifications with time during quiescence, over the course of the cell cycle, and in cells that are terminally arrested and have entered senescence. Our findings highlight the importance of methylation on H4K20. The singly methylated form of H4K20 is enriched in mitotic cells, whereas in quiescent cells, the levels of H4K20me2 and H4K20me3 increase. This specific lysine, in its different forms, plays a role in mitosis and during quiescence, facilitating compaction and the transition between proliferative and quiescent cell cycle states.

RESULTS
Contact inhibition in fibroblasts is associated with chromatin compaction
We established a model system of quiescence in which primary human dermal fibroblasts are sampled either proliferating or after being induced into quiescence by contact inhibition (Lemons et al., 2010). We sought to determine whether the quiescent state achieved when primary human fibroblasts are contact inhibited is associated with a change in chromatin compaction. We collected proliferating (P), 14 d contact–inhibited (14dCI), and restimulated fibroblasts (Figure 1, A–C) and used dual-color FISH with probes for two loci on chromosome 16 to monitor the extent of chromatin compaction. This technique has been used as a global measure for chromatin compaction (Bystricky et al., 2004; Chambeyron and Bickmore, 2004; Centore et al., 2010). The distance between the two probes was measured in ~50 cells in each state, and the average distance was calculated. In quiescent, contact-inhibited fibroblasts, the average distance between these loci was shorter than in proliferating fibroblasts (Figure 1, D and E). Fibroblasts that were restimulated to enter the cell cycle contained the largest average interlocus distance. Thus, entry into quiescence in response to contact inhibition results in more tightly packed chromatin that is reversibly unpacked upon stimulation.

H4K20 is differentially methylated in quiescent fibroblasts
Given our observation that contact-inhibited fibroblasts pack chromatin more tightly than proliferating or restimulated fibroblasts, we sought to uncover histone modification changes that are associated with quiescence in this system. To quantitatively measure the global changes in steady-state levels of histone modifications between P and 14dCI fibroblasts, we analyzed histones by liquid chromatography–mass spectrometry (LC-MS/MS). Mass spectrometry allows for a highly quantitative analysis of ~50 histone modifications in a single experiment, eliminating the need to select specific modifications for analysis before performing the experiment. Histones were extracted from primary human fibroblasts using acid extraction (Shechter et al., 2007) and modified with propionic anhydride to block unmodified and monomethylated lysines. Histones were digested with trypsin (cleaving only at arginine residues) and analyzed by LC-MS/MS. The intensity values for all modification states of a given peptide were used to calculate the fraction of the peptide in each individual modification state. Differential labeling of peptide N-termini with hydrogen-containing or deuterium-containing propionic anhydride was used to analyze two samples simultaneously and calculate fold changes between conditions, similar to microarray analysis.

Surprisingly, after 14 d of contact inhibition, despite the fact that the proliferating but not quiescent cells must replace their histone modifications with each cell division, the majority of histone
Some lysines did show differential levels of histone modifications between proliferating and quiescent fibroblasts (Figure 2A). Of the 48 modification states monitored, there were 44 with less than a twofold change. This suggests that when fibroblasts transition between proliferation and quiescence, they mostly maintain similar steady-state histone modification levels. The results do not rule out a difference in the rates at which these modifications are added and removed, and we explore this question elsewhere (Evertts et al., 2013). It also does not rule out changes in the locations of the modifications, which can be addressed using chromatin immunoprecipitation (ChIP) sequencing.

Some lysines did show differential levels of histone modifications between P and 14dCI (Figure 2, C and D). On histone H3, K9 and K27 were more likely to be methylated in quiescent fibroblasts than proliferating fibroblasts. Lysine 9 methylation was increased in quiescent cells, as indicated by a loss of unmodified H3K9. For H3K27, the most prominent change was that quiescent fibroblasts contained higher levels of H3K27me2 and H3K27me3, especially in combination with modified K36. The largest changes between P and 14dCI histones occurred on H4K20 (Figure 2, C and D, and Supplemental Figure S1). H4K20 can exist in four distinct forms with no methylation or one, two, or three methyl groups, each of which has been reported to play a different cellular role. In quiescent fibroblasts, the fraction of H4K20 that is unmethylated or contains a single methyl group decreased >2-fold, whereas the fraction of H4K20 that is modified with two or three methyl groups increased 2- to 10-fold, respectively. We used an antibody specific for the trimethyl form to confirm and validate the increase in H4K20me3 during quiescence and also found that the modification level is reversed after 48 h of restimulation (Figure 2B and Supplemental Figure S2). Immunofluorescence with the same antibody indicated that H4K20me3 was higher in abundance in the nucleus of quiescent versus proliferating fibroblasts, although a recognizable difference in the distribution pattern was not observed between the two states (Supplemental Figure S3). Thus, although most histone modifications are found at similar steady-state levels in proliferating and contact-inhibited fibroblasts, there were some histone lysines with reproducibly different levels of methylation.

**G1-enriched fibroblasts show differential H4K20 methylation patterns compared with quiescent fibroblasts**

The asynchronous proliferating cells that we monitored were a mixture of cells in all phases of the cell cycle, whereas quiescent cells were mostly in the G0/G1 cell cycle phase (Figure 1). To determine whether there are histone modifications associated specifically with quiescence or G0, we compared histone modifications in quiescent cells with histone modifications in a purified population of G1 cells, based on the expectation that quiescent cells exit the cell cycle from G1 (Pardee, 1974). In this way, we eliminated the effects of changes in histone modifications over the cell cycle. Fibroblasts were synchronized with serum starvation and hydroxyurea treatment. Cells were released from a G1/S block via hydroxyurea and collected 12 h later in early G1. The G1-enriched fibroblasts were compared with 14dCI fibroblasts using LC-MS/MS (Figure 2A). Most lysines had similar levels of modifications in G1-enriched fibroblasts compared with 14dCI fibroblasts, which is in accord with the finding that P and 14dCI cells had similar levels of most histone modifications. The increase in methylation on K27 observed in P versus 14dCI cells was reduced with G1-enriched fibroblasts, suggesting that K27 methylation fluctuates moderately over the cell cycle. We address this further later. For H4K20, however, 14dCI fibroblasts contained higher levels of dimethylated and trimethylated forms when compared not only to proliferating cells but also to G1-enriched cells. Thus these changes are associated with quiescence and do not reflect a stalling in G1.

We identified six modifications that changed significantly in 14dCI versus G1-enriched fibroblasts, including H4K20me2 and H4K20me3 (Figure 2, C and D). All six modifications also change significantly between 14dCI and P cells. Unmodified H3K9/K14 and unmodified H3K27/K36 were statistically significant but very modestly repressed in quiescent compared with G1 cells. H3K36me1 and H3K27me3K36me1 were statistically significantly but very modestly increased in quiescent compared with G1 cells. In contrast, large changes were observed for H4K20. 14dCI fibroblasts contained a twofold increase in the fraction of lysines in the dimethylated form. Because many histones in the cell are in the H4K20me2 form, this twofold change translates to ∼34% increase in the amount of histones present in the dimethylated form in 14dCI cells (Figure 2D). H4K20me3 is a rare modification representing only 0.2% of H4K20 in proliferating fibroblasts. Its levels were increased approximately eightfold in 14dCI fibroblasts, in which it represented ∼1.4% of all H4K20.

**FIGURE 1:** Contact-inhibited fibroblasts exhibit increased chromatin compaction. (A–C) Proliferating, contact-inhibited (14 d), and restimulated fibroblasts were collected and analyzed by propidium iodide staining and flow cytometry. FlowJo analysis was performed to estimate the fraction of cells in G1, S, and G2/M phases. (D) Cells were fixed, and dual-colored FISH probes were used to visualize 16q22 and 16p13. Approximately 30–100 cells were measured, and the average distance between the foci was determined. Mean and SE are plotted. Contact-inhibited fibroblasts exhibited a smaller interprobe distance than proliferating (p = 7.5 × 10⁻⁴) and restimulated (p = 6.4 × 10⁻⁸) cells. (E) Representative images of FISH on both copies of chromosome 16 are marked with a different color to visualize the distance between the arms. Scale bar, 2 μm.
To address this question, we monitored histone modifications over the cell cycle in primary fibroblasts using LC-MS/MS (Schulze et al., 2009). Cells were synchronized with serum starvation and hydroxyurea treatment and collected in S, G2/M, and G1 phases (Figure 3, B–E). Histones were extracted and analyzed together with 14dCI histones to generate a fold-change difference between 14dCI and each cell cycle phase. The fold change was normalized across each modification so that decreases represent lower amounts of the modification relative to the average of all phases, and increases represent higher amounts of the modification relative to the average of all phases. The majority of modifications do not change as the cells pass through S phase and into other phases (Figure 3A). This suggests that most lysines are rapidly modified at the replication fork or soon after and that the cell maintains comparable levels of most modifications throughout the cell cycle. For instance, the different modified forms of two lysines that have been studied extensively in the literature for their roles in transcription, H3K9 and H3K27, are present at relatively stable levels across cell cycle phases, with some slight variations that are most pronounced in the dimethylated forms (Pesavento et al., 2008; Pesavento et al., 2008; Schulze et al., 2009).

**Histone modification patterns are maintained during contact inhibition**

We then tested whether the changes in histone modifications observed at 14 d of contact inhibition would be maintained if the cells remained contact inhibited for a longer period of time. We compared the pattern of histone modifications in 14dCI fibroblasts with the pattern in 21dCI fibroblasts (Figure 2A). An additional 7 d of contact inhibition did not further increase or decrease histone modification levels, including on H4K20, suggesting that the changes in histone modification levels achieved by 14 d of contact inhibition were preserved when cells were maintained in a contact-inhibited state for a longer period of time.

**H4K20me1 levels increase over the course of the cell cycle and are converted to H4K20me2 and H4K20me3 in quiescence**

Our discovery that there were differences in histone modifications between a G1-enriched cell population and an asynchronous proliferating population suggested that there are changes in histone modifications during cell cycle progression (Pesavento et al., 2008; Pesavento et al., 2008; Schulze et al., 2009). To address this question, we monitored histone modifications over the cell cycle in primary fibroblasts using LC-MS/MS (Figure 3A). Cells were synchronized with serum starvation and hydroxyurea treatment and collected in S, G2/M, and G1 phases (Figure 3, B–E). Histones were extracted and analyzed together with 14dCI histones to generate a fold-change difference between 14dCI and each cell cycle phase. The fold change was normalized across each modification so that decreases represent lower amounts of the modification relative to the average of all phases, and increases represent higher amounts of the modification relative to the average of all phases. The majority of modifications do not change as the cells pass through S phase and into other phases (Figure 3A). This suggests that most lysines are rapidly modified at the replication fork or soon after and that the cell maintains comparable levels of most modifications throughout the cell cycle. For instance, the different modified forms of two lysines that have been studied extensively in the literature for their roles in transcription, H3K9 and H3K27, are present at relatively stable levels across cell cycle phases, with some slight variations that are most pronounced in the dimethylated forms (Figure 4, A and B).
FIGURE 3: Cell cycle–dependent changes in histone modification levels. The relative abundances of histone modifications were determined for S phase–enriched, G2/M-enriched, and G1-enriched fibroblasts using LC-MS/MS. (A) The log2 fold change for each phase relative to the average of all phases for each modification in heat map format. Data represent means from three independent experiments for S and G1 and two independent experiments for G2/M. (B–E) Propidium iodide staining and flow cytometry were used to generate cell cycle profiles for (B) asynchronously proliferating fibroblasts and cells enriched in (C) S phase, (D) G2/M phase, and (E) G1 phase. Data represent the mean of three independent experiments. Error bars indicate SE.

FIGURE 4: Modification states of H4K20 exhibit the greatest fluctuation across the cell cycle among histone lysines. The relative percentage of modified histones was calculated using mass spectrometry data for histone modifications in S, G2/M, G1, and 14dCl. The relative distribution of methylated forms is plotted for (A) H3K9, (B) H3K27, (C) H4K20, and (D) acetylated H4 on lysines 5–16. Error bars indicate SE.
One lysine, H4K20—the same lysine that exhibited the largest changes between proliferating and quiescent fibroblasts—did display large changes in modification levels across the cell cycle (Figure 4C). This is consistent with reports that H4K20 methylation status is cell cycle dependent (Jorgensen et al., 2007; Pesavento et al., 2008; Oda et al., 2009; Abbas et al., 2010; Centore et al., 2010; Wu et al., 2010). The unmodified form of histone H4K20 was present at its highest level among the cell cycle states during S phase. It decreased as the cells moved into G2/M and G1 and remained low in quiescent cells. Levels of H4K20me1 were low in S phase and progressively increased in G2/M. H4K20me1 levels were lower in 14dCI than in G1 cells, although the result is not statistically significant. Levels of H4K20me2 were relatively stable throughout the cell cycle but increased in quiescence. Levels of H4K20me3 were also relatively constant and very low over the course of the cell cycle but rose in quiescent fibroblasts. Other lysines on H4, such as H4K5-K16, did not exhibit the same pattern of increased modification over the course of the cell cycle as H4K20 (Figure 4D). The results indicate that H4K20, in its unmodified and monomethylated states, is the lysine that exhibits the greatest changes over the course of the cell cycle and exhibits the largest changes with quiescence.

Modification levels in senescence are similar to those in quiescence
Quiescent fibroblasts have the ability to reenter the cell cycle and proliferate after a period of cell cycle arrest. Other cells, such as senescent or terminally differentiated cells, remain in a state of cell cycle arrest and do not routinely reexpress proliferation-associated genes. We sought to compare histone modifications in reversibly arrested cells with those of permanently arrested cells by extending our analysis to senescent fibroblasts. A retroviral vector expressing an oncogenic form of the Ras protein (G12V) was introduced into fibroblasts. RasG12V-overexpressing fibroblasts grew rapidly and then ceased division and became senescent. Immunoblotting confirmed higher Ras levels in engineered fibroblasts compared with cells transduced with a control vector (Figure 5A). β-Galactosidase staining confirmed that the RasG12V-overexpressing cells had entered senescence (Figure 5B). LC-MS/MS analysis of histones in senescent fibroblasts indicated that the levels of H4K20 modifications are similar between quiescence and senescence (Figure 5C). This suggests that changes in the pattern of H4K20 methylation occur during other types of cell cycle arrest and not just during quiescence.

Overexpression of Suv4-20h1 causes an increase in the fraction of cells in G2
Most H4K20 lysines are in a dimethylated form in fibroblasts that were contact inhibited for 14 d. To assess whether a shift from the monomethylated to dimethylated form of H4K20 contributes to cell cycle exit, we overexpressed the enzyme that catalyzes the transition from H4K20me1 to H4K20me2 (Suv4-20h1) in fibroblasts (Figure 6). Fibroblasts were transduced with a retrovirus containing Suv4-20h1 driven by a cytomegalovirus promoter and allowed to recover for 24 h after selection with puromycin. Fibroblasts overexpressing Suv4-20h1 were larger and more flattened than control cells (Figure 6, B and D). The cell cycle profile of control and Suv4-20h1-overexpressing fibroblasts was determined by staining cells with propidium iodide, followed by flow cytometry analysis. Overexpression of Suv4-20h1 caused a decrease in the fraction of cells in S phase and an increase in the fraction of cells in G2/M (p = 0.02, paired t test; Figure 6, A, C, and E), whereas overexpression of Su4-20h2 did not alter the cell cycle profile significantly (Figure 6F).

Knockdown of Suv4-20h1 and Suv4-20h2 results in loss of compaction
To further explore the possible importance of the trimethylated form of H4K20, the form that displays the largest increase in quiescent
cells, we also generated fibroblasts with retroviral vectors containing knockdown of both Suv4-20h1 and Suv4-20h2. Mass spectrometry was used to monitor the reduction of both modifications. H4K20me2 was reduced by ∼30%, and H4K20me3 was reduced by ∼70% (Figure 7A). We analyzed intrachromosome distances using dual-color FISH, as described earlier, in shControl and shSuv4-20h1/h2 cells and discovered that this distance increased 1.2-fold in the knockdown cells, \( p = 1.2 \times 10^{-6} \) (analysis of variance [ANOVA]; Figure 7B). Representative images show the decreased compaction observed in shSuv4-20h1/h2 cells (Figure 7C). A more extreme example of the decreased compaction is shown in Figure 7C, right, and was observed only in shSuv4-20h1/h2 cells. We also monitored the amount of compaction in cells with individual knockdowns of Suv4-20h1 and Suv4-20h2 using small interfering RNAs (siRNAs). We found that a reduction of either methyltransferase caused an ∼15% increase in the distance between FISH probes, which is similar to the effect observed with the shSuv4-20h1/h2 double knockdown. The Suv4-20h1 siRNA increased the distance between FISH probes but did not achieve statistical significance, whereas treatment with the Suv4-20h2 siRNA did produce a statistically significant change in compaction (\( p = 0.01 \); paired t test). These results are consistent with previous reports that the trimethyl form of H4K20 promotes compaction (Lu et al., 2008), as intrachromosomal locus distances increased in cells with lower levels of this modification.

Knockdown of Suv4-20h1 and Suv4-20h2 results in increased S-phase cells and defects in quiescence entry

To determine the effect of the loss of H4K20me2 and H4K20me3 on proliferation and quiescence, we transfected cells with a pool of four siRNAs targeting Suv4-20h1 and Suv4-20h2 (four sequences for each transcript). Cells were transfected twice, which resulted in a reduction in the levels of the targeted transcripts (Supplemental Figure S7). At 24 h after the second transfection, cells were treated with the modified nucleotide 5-ethyl-2'-deoxyuridine (EdU) for 2 h. The incorporated EdU was detected using a chemically bound fluorophore and analyzed by flow cytometry (Click-iT EdU). The percentage of cells that were in S phase or entered S phase in the 2-h labeling was significantly higher for fibroblasts transfected with siSuv4-20h1/h2 than for a control set of siRNAs (Figure 8A). We next assessed whether knockdown of Suv4-20h1/h2 affects the ability of fibroblasts to exit the proliferative cell cycle in response to quiescence cues. We serum starved fibroblasts and monitored the fraction in S phase 24 h later with Click-iT EdU. We discovered that cell populations in which Suv4-20h1/h2 was depleted contained a greater fraction of cells in S phase than controls (Figure 8A). By performing the experiment with pools of siRNAs specific for either Suv4-20h1 or Suv4-20h2, we discovered that Suv4-20h2 was the enzyme responsible for the S-phase phenotype (Figure 8A), as a reduction in Suv4-20h1 showed similar levels of S-phase cells, and knockdown of Suv4-20h2 resulted in more S-phase cells. We then tested whether Suv4-20h2 knockdown alone could lead to resistance to contact inhibition and found that after 48 h of contact inhibition, the population of cells transfected with the siSuv4-20h2 contained more cells in S phase (Figure 8A). To eliminate the possibility of off-target effects from the siRNA pools, we performed knockdown experiments with individual siRNAs for both the control and Suv4-20h2 (Figure 8B). Transfection with three of the four Suv4-20h2 sequences resulted in more S-phase cells compared with the control sequences, suggesting that the results are not a consequence of off-target effects of the siRNAs. These findings indicate that not only do the levels of the higher methylated forms of H4K20 increase with quiescence, but the methylation state of H4K20 plays a functional role in the control of cell cycle progression, with the trimethyl H4K20 maintaining cells in a noncycling state (Figure 9).

**DISCUSSION**

Previous studies in lymphocytes (Baxter et al., 2004; Grigoryev et al., 2004) and mesodermal precursors (Schubeler et al., 2000; Zhang et al., 2002; Caretti et al., 2004; Mal, 2006) revealed that quiescence in these cell types is associated with large changes in the levels of many histone modifications. Our data, in contrast, demonstrate that proliferating and contact-inhibited fibroblasts contain similar steady-state, global levels of most histone modifications. Our data are in accord with a recent study in which quiescence in T-lymphocytes was not associated with changes in the global levels of histone modifications but instead with chromatin condensation by the condensin II complex (Rawlings et al., 2011). Our results indicating similar levels of histone modifications in proliferating and quiescent fibroblasts for most modifications are also consistent with our previous data.
indicating that contact-inhibited fibroblasts both repress and activate genes upon entering quiescence (Coller et al., 2006; Pollina et al., 2008; Lemons et al., 2010) and maintain high metabolic rates (Lemons et al., 2010). Although global levels do not change for most modifications, however, the positions of the modifications within chromatin could shift dramatically between proliferating and quiescent fibroblasts. ChIP-sequencing experiments using antibodies that bind these histone modifications would address whether quiescence is associated with a redistribution of activating or repressive marks among promoters to accommodate a new transcriptional program.

Histone modifications have been shown to vary across the cell cycle. One report shows that H3K79me2 levels peak in Saccharomyces cerevisiae at G2/M and the enzyme responsible for creating the mark (Dot1) is dependent on SBF, a cell cycle-regulated protein complex (Schulze et al., 2009). Knockdown of Dot1L in small-cell lung cancer cells resulted in a proliferation block and display of senescence characteristics, further linking this modification to the cell cycle (Kim et al., 2012). We did not measure a large change in either H4K79me2 or H4K79me3 with our methods in human fibroblasts.

In contrast, the methylation status of H4K20, a lysine that is not conserved in S. cerevisiae, varied dramatically over the course of the cell cycle in the human fibroblasts we studied. Previous reports based on mass spectrometry analysis in HeLa cells showed that new H4K20 is unmodified in S phase and becomes monomethylated only in M phase (Pesavento et al., 2008; Zee et al., 2012) via specific regulation of PR-Set7. We also show an increase of H4K20me1 during G2/M and early G1 relative to S phase. H4K20 then shifts from being highly enriched with monomethyl to predominantly dimethylated and trimethylated forms.

The H4K20 methylation profile is distinct in quiescent fibroblasts from its profile at any point in the cell cycle. In quiescent fibroblasts, the dimethylated and especially trimethylated forms accumulate (Sarg et al., 2002; Kourmouli et al., 2004). This methylation pattern...
is likely associated with both reversible and irreversible cell cycle exit. Other nondividing states, such as differentiation of murine myogenic and neural lineages, also show increased levels of H4K20me3 (Biron et al., 2004; Tsang et al., 2010), and mass spectrometry analysis of differentiating embryonic stem cells showed a correlation between H4K20 methylation and loss of pluripotency (Phanstiel et al., 2008). In our experiments, contact-inhibited and senescent fibroblasts had a similar H4K20 profile despite the fact that senescent and quiescent cells have very different chromatin structures (Rai and Adams, 2012). Recent analysis of senescence-associated heterochromatin foci, however, demonstrate that these structures are not dependent on changes in the levels of repressive chromatin marks such as H3K9me3 (Chandra et al., 2012; Chandra and Narita, 2013), making increases in H4K20 unlikely to drive chromatin changes associated with senescence. The aging process often involves cellular senescence within an organism, and H4K20me3 was found to increase in 450-d-old rat livers (Sarg et al., 2002). H4K20me1 was shown to decrease in 12-mo-old mouse brains (Wang et al., 2009), which could be indicative of a shift to H4K20me2 and H4K20me3. These studies further highlight the link between nondivision and increases in H4K20me2 and H4K20me3.

We discovered that overexpression of Suv4-20h1, the methyltransferase that creates the dimethylated form of H4K20 at the expense of the monomethylated form, caused cell cycle arrest in G2. This phenotype could reflect an overabundance of dimethylated H4K20 or a relative depletion of monomethylated H4K20 (Supplemental Figure S4). Previous reports indicated that a lack of H4K20me1 caused by inactivation of PR-Set7 results in activation of a G2/M checkpoint (Abbas et al., 2010) and abnormal chromosomes during mitosis (Rice et al., 2002; Oda et al., 2009). The phenotypes associated with a lack of H4K20me1 could reflect the importance of H4K20me1 in chromatin condensation, as subunits of the condensin II complex and other proteins have been found to bind H4K20me1 and can induce chromatin condensation in vitro (Trojer et al., 2007; Liu et al., 2010). Further, H4K20me1 can recruit L3MBTL1, which preferentially binds monomethylated and dimethylated lysines and induces chromatin compaction to negatively regulate gene expression (Trojer et al., 2007; Kalakonda et al., 2008).

A reduction in Suv4-20h2 resulted in defects in both S-phase cell number and cell cycle exit. Knockdown cells cultured in the presence of full serum contained an increased fraction of cells in S phase compared with controls. Further, in response to serum starvation or contact inhibition, there were more S-phase cells in knockdown than control cell populations. Increased numbers of cells in S phase could result from cells progressing through S phase more slowly or cells being blocked in S phase. Recent reports suggest a role for H4K20 in origin licensing and specifically demonstrate binding of H4K20me3 to ORC components (Vermeulen et al., 2011; Beck et al., 2012). Therefore it is possible that a loss of H4K20me3 may interfere with normal origin firing and lead to defects in S-phase progression. Alternatively, the propensity to proliferate in cells in which Suv4-20h2 is knocked down may be due to a loss of H4K20me3 at critical regions of the genome, such as cell cycle regulatory genes. H4K20me3 deposition has been shown to be dependent on Rb (Gonzalo et al., 2005), and loss of the modification may affect expression of E2F genes. We observed additional S-phase cells at the expense of G1/G0 cells, suggesting that cells are likely to be exiting G1/G0 and progressing into S phase faster.
Modifications of H4K20 may be functionally important for quiescence. Dimethylated H4K20 serves as a binding site for the DNA-damage recognition protein 53BP1 and thereby helps to mediate repair of double-strand breaks (Sanders et al., 2004; Botuyan et al., 2006). Mouse embryonic fibroblasts containing null alleles for the two Suv4-20h histone methyltransferases exhibit increased sensitivity to damaging stress as a result of inadequate double-strand break repair (Schotta et al., 2008). Quiescent cells cannot engage in the high-fidelity DNA repair mechanism of homologous recombination because there are no sister chromosomes from which to perform homologous recombination (Blanpain et al., 2011). Instead, they must rely on the error-prone pathway of non-homologous end joining. Higher H4K20me2 levels in quiescent cells could help them to initiate DNA repair events, which might help to protect them from DNA damage.

H4K20me3, the mark most significantly enriched in quiescent chromatin, is colocalized with H3K9me3 at centromeres (Martens et al., 2005), telomeres (Benetti et al., 2007), and pericentric heterochromatin (Schotta et al., 2004). The methyltransferase for H4K20me3 may be recruited to sites of H3K9me3 via interaction with the H3K9me3-binding protein Hp1 (Schotta et al., 2004). Depletion of H4K20me3 by knockdown of Suv4-20h1 and Suv4-20h2 results in depletion of dimethyl and trimethylated H4K20, and chromosomes are less compactly organized, consistent with such a role for this modification. We did not detect significant changes in the levels of H3K9me3 between proliferating and quiescent cells, but with H4K20me3 constituting <2% of H4, we might not have detected such a small change in H3K9me3.

Loss of trimethylation at H4K20 is a common hallmark of human cancer (Fraga et al., 2005; Pogribny et al., 2006; Van Den Broeck et al., 2008; Schneider et al., 2011). In a large panel of cancer cells and matched tumors and normal tissue, histone H4 consistently exhibited decreased trimethylation in cells derived from tumors (Fraga et al., 2005). In one study of bladder cancer, H4K20me3 levels decreased with increasing tumor grade (Schneider et al., 2011). In lung tumors, an association was observed between low levels of H4K20me3 and decreased levels of Suv4-20h2 (Van Den Broeck et al., 2008). Decreased expression of H4K20me3 could reflect the proliferative state of the tumor cells. Alternatively, lower levels of H4K20me3 could promote tumorigenesis by preventing the repression of genes that control cell cycle progression and thereby inhibiting formation of a proper out-of-cycle state. A better understanding of the changes in chromatin dynamics as cells enter, maintain, and exit a quiescent state is likely to provide important insights into the control of cellular proliferation and how this process is altered in developmental abnormalities, aging, and cancer.

MATERIALS AND METHODS

Cell culture

Primary human foreskin fibroblasts (HFFs) were isolated from donor foreskins as previously described (Legesse-Miller et al., 2009). All experiments were performed in cells with a passage number of <13. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum unless otherwise indicated. Proliferating cells were plated at 50% confluence and harvested after 24 h to avoid contact inhibition. Contact-inhibited cells were plated at 50% confluence and incubated for the indicated number of days with media changes every 3 d. Cells used for histone analysis were scraped in phosphate-buffered saline (PBS) from tissue culture plates and flash frozen in liquid nitrogen.

Cell synchronization

HFFs were plated at 30–40% confluence and maintained in a 37°C incubator for 16 h. Cells were initially washed 2X with PBS and serum-starved for 24 h in DMEM with 0.1% fetal bovine serum (FBS) to synchronize them in G1. Cells were then washed 2X with PBS and incubated with DMEM + 10% FBS + 2 mM hydroxyurea to release them from serum starvation–induced arrest and block them at the G1/S transition. After incubation for 18 h, cells were washed 2X with PBS, and fresh DMEM with 10% FBS was added to allow a synchronized exit into other cell cycle phases. Cells were harvested at 3 h (S phase), 6.5 h (G2/M phases), and 12 h (G1 phase).

Senescence model

A total of 5 × 10⁶ Phoenix cells was transfected with 5 μg of Amphi helper plasmid (Imgenex, San Diego, CA) and 5 μg of either pBABE or pBABE-RasGTP using Arrest-In (Open Biosystems, Huntsville, AL). Viral supernatant was collected 48 h posttransfection and filtered with a 0.45-μm filter. Cycling human fibroblasts were infected for 24 h with viral supernatant plus Polybrene at 2.6 μg/ml. Cells were selected for 48 h in DMEM + 10% FBS + 2 μg/ml puromycin. After selection, cells were passaged approximately three times until cell division ceased. Cells were fixed between 1 and 14 d after cell cycle arrest and stained with X-gal for 8 h to visualize β-galactosidase–positive cells.

Flow cytometry

HFFs were removed from plates with PBS + 0.05% trypsin-EDTA. For propidium iodide staining, cells were fixed and permeabilized by adding one volume of PBS to two volumes of 100% ethanol and stored at 4°C for >24 h. Ethanol was removed, and cells were incubated with propidium iodide (PI; 40 μg/ml; EMD Chemicals, Gibbstown, NJ) and RNase A (200 μg/ml; Roche, Basel, Switzerland) in PBS for 1 h in the dark. For Click-iT analysis, cells were incubated for 2 h in 10 mM EdU. Cells were pelleted, fixed with 4% paraformaldehyde, and treated with Alexa 488 azide (Invitrogen). DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). All cells were analyzed with a FACScaliber flow cytometer (BD Biosciences, San Jose, CA). At least 20,000 cells were analyzed per sample. The software FlowJo (version 8.8.2, Watson algorithm) was used to estimate the fraction of cells in G1, S, and G2/M for PI-stained cells. Paired t tests were used to determine whether the fraction of cells in different phases of the cell cycle were significantly different in knockdown cells and controls.

Histone isolation and preparation for MS

Histones were purified using acid extraction as previously described (Shechter et al., 2007). Briefly, cell pellets were thawed and resuspended in 10 volumes (for every volume of cell pellet) of nuclear isolation buffer (15 mM Tris-HCl at pH 7.5; 60 mM KC1; 15 mM NaCl; 5 mM MgCl2; 1 mM CaCl2; 250 mM sucrose; 1 mM DTT; 5 μM microcystin; 500 μM 4-(2-aminoethyl) benzenesulfonic acid hydrochloride; 10 mM sodium butyrate) with 10% NP-40 and incubated for 5 min. Nuclei were washed 2X with nuclear isolation buffer lacking NP-40 and then mixed with five volumes (for every volume of nuclei) of 0.4 N H₂SO₄ and incubated for 3–4 h at 4°C. Histones were precipitated with trichloroacetic acid and washed once with acetone containing 0.1% HCl, followed by two washes with 100% acetone. Histones were air dried overnight and resuspended in H₂O. Histones were processed using methods previously described (Plazas-Mayorca et al., 2009). A solution containing 100 μg of purified histones was reduced to 40 μl using a vacuum concentrator and mixed with 20 μl of 3:1 anhydrous isopropanol to propionic anhydride.
Histones were separated by reversed-phase high-performance liquid chromatography (HPLC) on an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA) using a 75-μm-inner diameter fused silica column packed with 10–15 cm of 5-μm C18 (Michrom, Auburn, CA). A gradient of 0.7–30% buffer B in buffer A (buffer A, 0.1 M acetic acid; buffer B, 95% acetonitrile in 0.1 M acetic acid) for 35 min followed by 30–98% buffer B for 30 min was used to elute peptides, which were ionized via electrospray ionization. Peptides were analyzed in a LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA). Full scans of m/z = 290–1000 with a resolution of 70,000 were measured in the Orbitrap. Collision-induced dissociation was used to fragment ions corresponding to isotopic acetylated peptides (H3K9ac or K14ac [528.296, z = +2] and H3K18ac or K23ac [570.841, z = +2]) in segments where those peptides elute; in other segments data-dependent fragmentation was performed on the seven most intense ions. Extracted ion chromatograms from Orbitrap data were integrated to yield intensity values for all histone peptides of interest. The values for all modified forms of a particular peptide were used to find the relative abundance of individual modified forms for that peptide.

Dual-color fluorescence in situ hybridization
Cells were harvested and incubated at 37°C for 30 min in 0.59% KCl. Cells were fixed in ice-cold methanol:acetic acid at a 3:1 ratio and spread on glass slides. Slides were prepared for FISH using fluorescently labeled probes specific for the arms of chromosome 16 (16q22, red; 16p13, green) according to the manufacturer’s instructions (LPH 022; Cytocell, Cambridge, United Kingdom). Coverslips were mounted, and DNA was detected with 0.2 μg/ml DAPI/anti-sense detection (Cytocell). Fluorescence images were captured with an Orca AG cooled charge-coupled device camera (Hamamatsu, Hamamatsu, Japan) mounted on a Nikon TI (Melville, NY)/Yokagawa (Tokyo, Japan) CSU-10 spinning disk confocal microscope with a 100×, 1.4 numerical aperture objective. A series of 0.25-μm optical sections was collected in the z-axis for each channel (DAPI, fluorescence, and Texas red). Intrachromosome distances under each condition were measured with SlideBook analysis software (SlideBook, Denver, CO). Approximately 30–100 cells were used to measure intrachromosome distances for each condition, and three biological replicates were scored.

Immunoblotting and immunofluorescence
We separated 10 μg of acid-extracted histones on a 15% polyacrylamide gel. Primary rabbit anti-H4K20me3 (Novus Biologicals, Littleton, CO) was incubated for 16 h at 4°C. A secondary antibody was incubated for 1 h at room temperature at 1:10,000 dilution, and the membrane was exposed using an enhanced chemiluminescence detection kit. For antibody testing, modified peptides (AnaSpec, Fremont, CA) were spotted onto a polyvinylidene fluoride membrane. Primary and secondary conditions were identical to those described earlier except that streptavidin–horseradish peroxidase (PerkinElmer, Waltham, MA) was used instead of a secondary antibody. For immunofluorescence, cells were grown on glass slides (EZ slide; EMD Millipore, Billerica, MA) and fixed using 4% formaldehyde for 15 min. After permeabilization for 20 min with methanol, cells were treated with blocking buffer and antibodies as described for Western blotting. Anti–rabbit-488 was used for detection of antibodies, and cells were stained with DAPI at 1 μg/ml to visualize the nucleus.

Overexpression/knockdown
The CDS of Suv4-20h1 (variant 1) was PCR amplified from human cDNA (forward, 5’-CCGGTttaattaaATGAAGTGGTTGGGAGAA-TC5AAGA-3’; reverse, 5’-CCGGGgattcTTAGCATTAGGCT-TAAAGACTGA-3’) and cloned into retroviral vector pQCXIP using PacI and BamHI restriction sites. Vectors that express short hairpin RNAs (shRNAs) against Suv4-20h1 (GTTTGTGTCACCTTGCAGATACAGCAT) and Suv4-20h2 (CGACCGTAGTGCAGCGGTGAAGAAGCTGTG) were purchased from OriGene (Rockville, MD). Virus was generated and collected as described (senescence model). For overexpression, fibroblasts were transduced with pQCXIP– or pQCXIP-Suv4-20h1–derived virus and selected for 48 h with 2 μg/ml puromycin. For the Suv4-20h1/h2 knockdowns, an shRNA against green fluorescent protein (puromycin resistance gene) and a scrambled shRNA (blasticidin resistance gene) were used as controls. Both control viruses or viruses derived from shRNAs to Suv4-20h1/h2 were used to coinfect fibroblasts. Infected cells were selected with 2 μg/ml puromycin and 30 μg/ml blasticidin for 5 d. Cells expressing shRNAs were passaged three or more times to sufficiently reduce histone modification levels before analysis. Cells expressing Suv4-20h1 were maintained for 24 h without selection media and analyzed. siRNAs were purchased from Thermo Scientific (Laayette, CO) as siGENOME SMARTpools targeting Suv4-20h1 and Suv4-20h2. We transfected 100 nM of each siRNA pool or 200 nM of control siRNAs into HFFs using Oligofectamine (Invitrogen, Carlsbad, CA). Cells were expanded for 48 h, transfected again, and either analyzed 24 h later as proliferating cells or serum starved for 24 h and then analyzed.

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