the inability of these cells to recover from the hydroxyurea treatment. We did not detect abnormal DNA structures at early replicating origins in rad53 cells grown under normal conditions (Fig. 3c and data not shown), and therefore we must assume that hydroxyurea treatment greatly amplifies the presence of these abnormal intermediates. Although our approach may not be sensitive enough to detect a small amount of these structures, it is also possible that replication forks in the 305-rf in rad53 cells grown under normal conditions will never collapse, but rather that this event is restricted to natural pause sites in the genome26,28 or sites where the forks encounter a damaged template. From this perspective, we propose that the checkpoint response directly modulates the stability of replicating chromosomes, thus contributing to the prevention of genomically rearrangements, which are the most prominent hallmarks of cancer susceptibility in multicellular organisms.

Methods

We used the following strains: W303-1A (MATa ade2-1, trp1-1, leu2-3, 112 lys3-11, 15 ura3, can1-100) and its isogenic derivatives CY2034 (rad53-K227A-KanMX4), CY387 (pri1-M4), CY2059 (pri1-2) and CY2081 (cdk1-1)26. Strains CY2572 (vector) and CY2573 (GALrad53) were constructed by integrating in the W303-1A strain, respectively, the YPlac128 (LEU2) vector plasmid or its pCH12 derivative26, containing the EcoRI fragment carrying the rad53-D3394 mutation under the control of the GAL1 promoter. Strains CY3278 (mec1-td) and CY3281 (mec1-td, rad53-K227A) are isogenic to W303 and were constructed by replacing the wild-type copy of MECl with the mecl+ `degenerate allele as already described26.

Yeast protein extracts prepared by the TCA extraction method26 were resolved by 10% SDS–PAGE and the phosphorylation state of the Rad53 polypeptide was analysed by western blotting using anti-Rad53 antibodies (provided by C. Santocanale and J. Difley). DNA samples to be analysed with the neutral–neutral two-dimensional electrophoresis were isolated from cells grown under normal conditions in the absence of DNA synthesis. We did not detect abnormal DNA structures at early replicating origins in rad53 cells grown under normal conditions, which are the most prominent hallmarks of cancer susceptibility in multicellular organisms.

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Supplementary information is available from Nature’s World Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of Nature.

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In eukaryotic cells the histone histone methylase SUV39H1 and the methyl-lysine binding protein HP1 functionally interact to repress transcription at heterochromatic sites1. Lysine 9 of histone H3 is methylated by SUV39H1 (ref. 2), creating a binding site for the chromo domain of HP1 (refs 3, 4). Here we show that SUV39H1 and HP1 are both involved in the repressive functions of the retinoblastoma (Rb) protein. Rb associates with SUV39H1 and HP1 in vivo by means of its pocket domain. SUV39H1 cooperates with Rb to repression the cyclin E promoter, and in fibrobasts that are disrupted for SUV39, the activity of the cyclin E and cyclin A2 genes are specifically elevated. Chromatin immunoprecipitations show that Rb is necessary to direct metabolic control of histone H3, and is necessary for binding of HP1 to the cyclin E promoter. These results indicate that the SUV39H1–HP1 complex is not only involved in heterochromatic silencing but also has a role in repression of eucromatich genes by Rb and perhaps other co-repressor proteins.

The Rb protein functions as a repressor, at least partly, through the recruitment of histone deacetylase activity5–7. We considered that SUV39H1 and HP1 might also be involved in Rb-mediated
Figure 1 Rb interacts with methylase activity specific for H3 Lys 9. a, GST fusion proteins (2 μg) were used to purify histone methylase activity from 500 μg of HeLa nuclear extract. c.p.m., counts per minute. b, Rb-associated activity methylates H3 but not H4 or the arginine-methylase substrate GAR. c, Endogenous Rb associates with H3-specific methylase activity. HeLa nuclear extract was immunoprecipitated using a Rb-specific antibody or a control antibody (HA, 2 μg each). Immunoprecipitates were tested for associated methylase activity. d, Mutations in the Rb pocket disrupt the Rb–methylase interaction as they do not associate with methylase activity. e, H3 labelled by Rb-associated methylase was sequenced. Fractions corresponding to each amino-acid cycle were collected and analysed by scintillation counting.

Figure 2 SUV39H1 and Rb interact and regulate transcription. a, Rb purifies SUV39H1 from cells. HEK-293 cells were transfected with a HA-SUV39H1 or an empty expression vector (Mock). Extracts were incubated with GST or GST–Rb (4 μg) and washed. Bound SUV39H1 was western blotted using an anti-HA antibody. b, SUV39H1 and Rb form a complex in vivo. U2OS cell nuclear extract was immunoprecipitated with antibodies (10 μg) against SUV39H1, Rb or Gal4–DBD. Immunoprecipitates were western blotted with a Rb antibody. c, U2OS cells transfected with a Gal4–driven CAT reporter (0.33 μg) under the control of the major late promoter (MLP) together with an expression vector for Gal4–Rb or Gal4 alone (0.66 μg) plus increasing amounts of a SUV39H1 expression vector (0.13–2 μg). d, HeLa cells were transfected with a reporter containing the cyclin E promoter driving luciferase (5 μg) together with combinations of expression vectors for E2F1 (2 μg), Rb (2 μg), SUV39H1 (0.1 μg) and SUV39H1ΔSET (0.1 μg). e, RNA from wild-type and SUV39H1 and SUV39H2 double-knockout (DKO) mice was isolated. Equal amounts of RNA (RNA input) were analysed by RT-PCR (25 cycles) for the expression of cyclin E, cyclin A2, Cdc25, GAPDH and HPRT. The RT control lanes represent RT-PCR reactions in the absence of reverse transcription. The RNA used here was from cells of female mice, but identical results were obtained using RNA from cells of male mice (data not shown).
repression, as the SUV39H1 methylase has repressive potential. To establish whether Rb can associate with histone-methylase activity, a glutathione S-transferase (GST)–Rb fusion was incubated with nuclear extract, and any bound methylase activity was assayed on bulk histones as a substrate. Figure 1a shows that GST–Rb (but not GST alone) can purify histone-methylase activity, whereas GST fusions to transcriptional activators such as P/CAP, E2F1, p53 and ATF2 do not. The Rb-associated methylase activity is specific for histone H3 and does not recognize the GAR substrate for arginine methylases (Fig. 1b).

An antibody directed against Rb can precipitate histone-methylase activity that is specific for histone H3 (Fig. 1c). This methylase binds the pocket domain of Rb because tumour-derived mutations in the pocket (F706C), or truncations of the pocket (∆D28 and ∆737), abolish binding to the methylase (Fig. 1d). The Rb-associated methylase has specificity for Lys 9 of histone H3, as shown by Edman degradation of radioactively methylated histone H3 (Fig. 1e).

The SUV39H1 protein possesses lysine methylase activity, which resides within its conserved SET domain. As this enzyme has specificity for Lys 9 of histone H3 we investigated whether SUV39H1 could be the methylase associated with Rb. Figure 2a shows that a GST–Rb fusion can bind to transfected, haemagglutinin (HA)-tagged SUV39H1. Endogenous Rb also associates with endogenous SUV39H1, as shown by a co-immunoprecipitation analysis (Fig. 2b). As DNA is present in these reactions as a low-level contaminant, it remains a formal possibility that the interaction is facilitated by DNA; however, as histones are not co-immunoprecipitated this possibility seems unlikely (data not shown).

We next investigated whether SUV39H1 could act as co-repressor with Rb. Figure 2c shows that SUV39H1 represses the activity of a promoter bearing GAL4 sites in a concentration-dependent manner in vivo, but only when Gal4–Rb is present at the promoter. The co-repressor functions of SUV39H1 can also be seen on the cyclin E promoter, a natural target for Rb-mediated repression. This promoter can be stimulated by E2F (Fig. 2d, columns 1 and 3) and is not affected by SUV39H1 alone (columns 1 and 2). Under limiting conditions, where Rb represses E2F activity slightly (column 5), the SUV39H1 enzyme can further repress E2F activity in cooperation with Rb (column 6). When the methylase domain of SUV39H1 is removed the resulting SUV39H1ΔSET is unable to mediate repression (column 7). These results suggest that SUV39H1 uses its methylase activity to repress the cyclin E promoter when it is targeted there by Rb.

The repressive functions of SUV39H1 were verified using RNA isolated from fibroblasts that lacked both SUV39H1 and the closely related methylase SUV39H2 (double-knockout cells). Reverse transcription followed by polymerase chain reaction (RT-PCR) was used to show that cyclin E messenger RNA levels are elevated in the double-knockout cells compared with wild-type cells (Fig. 2e). mRNA of cyclin A2, a gene repressed by the Rb-related pocket proteins p107 and p130 (ref. 9), is also upregulated in double-knockout cells. In contrast, the expression of Cdk2, a gene regulated by E2F but not repressed by Rb, p107, or p130 (ref. 9), is unchanged in double-knockout cells. The expression of two unrelated house-keeping genes, GAPDH and HPRT, is also unchanged. Collectively, these results support the conclusion that the SUV39H1 methylase is a specific repressor of genes regulated by the Rb-pocket family.

SUV39H1 is known to be in a complex with the HP1 protein. Recently, HP1 function has been placed downstream of SUV39H1 histone methylation, as HP1 recognizes specifically, and binds to, histone H3 methylated at Lys 9 (refs 1, 4, 5). This mechanistic link prompted us to investigate the role of HP1 in Rb/SUV39H1-mediated repression. Rb and HP1 can interact in a two-hybrid screen in yeast, and it has been shown that there is an LXCXE motif (X is any amino acid) in HP1 (ref. 12). We therefore asked if HP1 binds to Rb in mammalian cells. Figure 3a shows that a GST–HP1 fusion can bind Rb that is present in nuclear extracts; Rb and

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**Figure 3** HP1 interacts with Rb in an LXCXE-dependent fashion. a, GST fusion proteins (4 μg) were tested for binding to Rb from HeLa nuclear extract. b, Endogenous HP1 and Rb interact. U2OS nuclear extract was immunoprecipitated with anti-HP1 serum, anti-Rb antibody, or a pre-immune serum, and western blotted for Rb. c, GST–Rb and GST–HP1 (2 μg) were tested for binding to H3-specific methylase from HeLa nuclear extract in the presence of an LXCXE or control (HIS) peptide. d, HP1 recruits Rb to H3 methylated at Lys 9. A H3 peptide methylated at Lys 9 (2 μg) immobilized on beads was incubated with recombinant GST–Rb (0.5 μg) in the absence and presence of His-HP1 (1 μg). Rb binding was detected by western blotting. e, Recombinant GST and SUV39H1 bind specifically to H3 methylated at Lys 9 (K9Me H3). Differentially modified H3 peptides were tested for binding of endogenous HP1 and SUV39H1 and transfected Rb from cell extracts. Input lanes contain 2% of total extract.
Figure 4 Rb is required for HP1 promoter recruitment. **a**, **b**, A specific nucleosome within the cyclin E promoter associates with HP1. Chromatin immunoprecipitations (ChIPs) from wild-type MEFs were performed with HP1 antibody, or a pre-immune (PI) control, and the purification of cyclin E promoter fragments (centred at +1 for cyclin E pr in a; −550 for cyclin Eup in b) was analysed by quantitative PCR. **c**, Characterization of an anti-H3 methyl Lys 9 antibody (anti-MeK9). Total cellular extract was visualized by Coomassie blue staining or probed with anti-MeK9. H3 peptide competition showed that the antibody was only effectively competed against by a histone H3 peptide when methylated at Lys 9. **d**, Chromatin immunoprecipitations show Rb dependence for HP1 recruitment to the cyclin E promoter. Crosslinked chromatin from Rb+/− and Rb−/− cells was immunoprecipitated with HP1 antisera, anti-MeK9 antibody or pre-immune control. Equal abundance of cyclin E promoter sequence in Rb+/− and Rb−/− nucleosomal preparations was determined by PCR from the input chromatin. **f**, The Cdc25c gene does not associate with HP1 or histone H3 methylated at Lys 9. Chromatin immunoprecipitations were performed as in e.

HP1 can associate in vivo, as determined by co-immunoprecipitation analysis (Fig. 3b). An LXCXE motif peptide can compete for the binding of histone H3 methylase activity to Rb, but does not affect the binding of H3 methylase activity to HP1 (Fig. 3c), which is consistent with the finding that the methylase activity is associated with the Rb pocket.

Figure 5 Role of the SUV39H1–HP1 complex in the transcriptional co-repressor function of Rb. Deacetylation of histone H3 at Lys 9 by Rb-associated deacetylase activity (HDAC) might be required as a preceding step to SUV39H1-mediated methylation.

We next assessed whether HP1 can recognize methylated lysines while associated with Rb. To address this, a histone H3 peptide methylated at Lys 9 was used as an affinity resin. Recombinant Rb does not bind to this methylated peptide (Fig. 3d, lane 2), but it can do so efficiently in the presence of recombinant HP1 (Fig. 3d, lane 3). This result confirms that HP1 can bind directly to Rb and that it can recognize Rb and methylated lysine simultaneously. A similar experiment was attempted using nuclear extracts as the source of protein. Figure 3e shows that the H3 peptide methylated at Lys 9 (but not unmethylated or Lys-4-methylated peptide) binds to HP1, SUV39H1 and Rb, as detected by western blotting.

The above results suggest that an Rb-regulated promoter such as cyclin E should be associated with HP1. To test this we performed chromatin immunoprecipitation analysis of the cyclin E promoter. Figure 4a shows that a nucleosome encompassing the cyclin E initiation site (cyclin Epr) that is known to be deacetylated (A.M. and R.H., personal communication) is associated with HP1 in fibroblast cells of mouse embryos. In contrast an upstream nucleosome (cyclin Eup) does not associate with HP1 (Fig. 4b). As the cyclin Epr nucleosome binds HP1, we next addressed whether this nucleosome contains histone H3 that is methylated at Lys 9. To test this we raised an antibody that recognizes histone H3 when methylated at Lys 9 (Fig. 4c, d). Figure 4e shows that in Rb+/− cells the cyclin Epr nucleosome contains methylated histone H3 and is associated with HP1. However, in Rb−/− cells histone H3 methylation and HP1 binding is significantly reduced. A nucleosome encompassing the Cdc25c initiation site (Fig. 4e, f) is found associated with HP1 (Fig. 4f). Thus, in the presence of Rb, methylase activity and HP1 are targeted to the cyclin E promoter.

Collectively, the results presented here implicate each of the components of the SUV39H1–HP1 complex in the repression functions of the Rb protein. In this model (Fig. 5) Rb brings to the promoter the SUV39H1 enzyme (and possibly other members of this family) to methylate Lys 9 of histone H3 and provide a binding site for HP1. Methylated by SUV39H1 cannot take place on an already acetylated lysine. Thus the deacetylase activity associated with Rb−/− may be a necessary preceding step to SUV39H1-mediated methylation. The precise function of HP1 in repression is unclear. HP1 may protect the methyl group on Lys 9 from attack from potential demethylases, it may bring in other repressive functions, or it may enhance the stability of the Rb-associated repressor complex.

HP1 is found associated with a number of transcriptional repressors, suggesting that it may have a role in repressing many other promoters. Thus, the results presented here extend the role of SUV39H1 and HP1 beyond heterochromatic gene silencing to a more general, genome-wide function in repressing gene transcription.

Methods
Cell culture, transfections and transcription assays
Cells (U2OS and HEK-293) were transfected using the calcium phosphate technique or FuGene (Roche) according to the manufacturer’s instructions. Twenty-four hours after
transfection, cells were collected and processed for CAT or luciferase activity using standard techniques15.

GST pull downs and protein sequencing

GST–Rb (wild type and mutants)16 and other GST fusion proteins were expressed and purified from *Escherichia coli* C490 (ref. 16). GST fusion proteins that were immobilized on glutathione-sepharose (Pharmacia), or H3-derived peptides2 bound to SulfoLink beads (Pierce), were incubated with extract in IPH buffer2. Complexes were washed four times in IPH buffer before processing for methylation assays or western blotting. Antibodies against HA (12CA5, Roche), Galα–DBD (DNA-binding domain; sc-510, Santa Cruz), SUV39H1 (M. Cleary), Rb (G3-245; ZXS5, PharMingen) or HP1 (ref. 3) were used. For immunoprecipitations antibodies were incubated with HeLa nuclear extract (Cell Culture Center) or U2OS nuclear extract in IPH buffer at 4°C (ref. 17). After 2 h a 50:50 mixture of protein A- and protein G-sepharose beads (Pharmacia) was added. To avoid the possibility that DNA mediates the interaction between SUV39H1 and Rb, the immunoprecipitations were probed for the presence of histones with negative results.

Histone methylase assays and protein sequencing

Precipitations from pull downs or immunoprecipitations were incubated with 20 µg histones (Sigma) and 1 µL [3H-Me]-S-adenosyl methionine (NEN, 80 Ci mmol−1) in PBS at 30°C for 1 h. Assays were analysed by SDS–PAGE followed by western blotting and autoradiography or were spotted onto P-81 cationic exchange paper (Whatman), washed in carbonate buffer and quantified by scintillation counting3. For amino-terminal Edman degradation (Protein Sequencing Facility, University of Cambridge). We counted fractions for the presence of tritium.

RNA purification and RT-PCR analysis

Total RNA (0.5 µg) was isolated from W12 (wild type) and D3 (SUV39H1 and SUV39H2 double knockout; D.O. and T.J., unpublished observations) female mouse cells, and was used for quantitative RT-PCR, following the Qiagen One Step protocol, for 20, 25 and 30 PCR cycles.

Antibody generation

Rabbits were immunized with a H3 N-terminal lysine-methylated peptide corresponding to amino acids 1–16. Immunoreactive serum was applied to a H3 Lys-9-methylated peptide column to affinity purify specific antibodies, as the antiserum crossreacted with H3 methylated at Lys 4.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed using HeLa cells and MEF cells essentially as described17. Immunoprecipitations were analysed for the presence of cyclin E or Cdc25C promoter fragments by PCR using primers specific for single nucleosomes. PCR reactions were repeated exhaustively using varying cycle numbers and different amounts of templates to ensure that results were within the linear range of the PCR. We have identified several items requiring correction or clarification in our paper on the sequencing of the human genome.

Six additional authors should have been included: Pieter de Jong, Joseph J. Catanese, and Kazutoshi Osoegawa (Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York 14263, USA; present address: Children’s Hospital Oakland Research Institute, 747 52nd street Oakland, California 94609, USA) and Hiroaki Shizuya, Sangdun Choi and Yu-Jun Chen (Division of Biology, California Institute of Technology, Pasadena, California 91125, USA). These investigators and their laboratories constructed the high-quality BAC libraries that were crucial in sequencing the genome, as described in Table 1. These libraries were not previously published. We apologize to our colleagues for this omission.

The Supplementary Information on *Nature*’s website has been revised. Changes to the original Supplementary Information are available in the Supplementary Information to this Correction. We have added 7 additional investigators to the full list of authors. We have also added 79 additional references, citing previously published sequences that were included in the draft genome sequence.

Table 27 reported 18 instances of apparently novel paralogues of genes encoding drug targets. We have carefully reviewed these 18 cases and found that two are incorrect: a parologue of an insulin-like growth factor-1 receptor gene and a parologue of the calcitonin-related polypeptide alpha gene. In both cases, we had incorrectly recorded the chromosomal location sequence of the known gene, thereby erroneously giving rise to an apparent parologue (the first instance was identified by J. Englebrecht and C. Kristensen (personal communication)). Of the 16 remaining apparent paralogues, two (calcium channel paralogue IGI_M1_c017137_10 and heparan N-deacetylase/N-sulphotransferase paralogue IGI_M1_c013263_18) have so far been confirmed as bona fide genes12.

Several correspondents have written to point out that a handful of clones listed as human sequence in the HTG division of GenBank (established to house ‘unfinished’ sequence data) are actually mouse sequence (about two dozen out of 30,000 clones). They asked...