OPINION

Replication licensing and cancer — a fatal entanglement?

J. Julian Blow and Peter J. Gillespie

Abstract | Correct regulation of the replication licensing system ensures that chromosomal DNA is precisely duplicated in each cell division cycle. Licensing proteins are inappropriately expressed at an early stage of tumorigenesis in a wide variety of cancers. Here we discuss evidence that misregulation of replication licensing is a consequence of oncogene-induced cell proliferation. This misregulation can cause either under- or over-replication of chromosomal DNA, and could explain the genetic instability commonly seen in cancer cells.

To prevent the occurrence of potentially cancer-causing alterations to the genome, it is crucial that chromosomal DNA is precisely duplicated during S phase of the cell division cycle. Because of the large size of animal chromosomes, it is necessary for them to be replicated by thousands of replication forks initiated at replication origins scattered throughout the genome. The activity of these replication origins must be carefully regulated to ensure precise chromosome duplication. If too few replication origins are active, there is a danger that the chromosomal DNA will not be completely replicated during S phase, which can potentially lead to DNA strand breaks and gross chromosomal rearrangements in surviving daughter cells. It is equally important to ensure that no replication origin initiates more than once in each cell cycle, as this would lead to re-duplication (amplification) of the DNA in the vicinity of the over-firing origin and other consequent chromosomal rearrangements.

Correct regulation of the replication licensing system is responsible for ensuring the proper regulation of replication origins during cell cycle progression[1–3]. Origin licensing, which occurs before S phase in late mitosis and early G1 (FIG. 1), involves the stable loading of the minichromosome maintenance (MCM) complex comprising six replication proteins — MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7 (termed MCM2–7) — onto DNA at replication origins. MCM2–7 are essential replication fork proteins that probably provide the helicase activity to unwind the template DNA ahead of the fork[4,5]. Because of this behaviour, MCM2–7 complexes move away from each origin as it initiates, thereby leaving the origin in an unlicensed state. In the absence of DNA-bound MCM2–7, replication origins cannot initiate (FIG. 1). Therefore, to prevent replicated origins from being re-licensed (and hence re-replicated) after they have initiated, it is necessary for the replication licensing system to be shut down before entry into S phase.

Replication licensing requires at least three proteins in addition to MCM2–7: the origin recognition complex (ORC), cell division cycle 6 (CDC6) and CDT1 (REFS 1–3,6). ORC first binds to DNA at replication origins and recruits CDC6 and CDT1. These proteins then act together to load MCM2–7 onto DNA, plausibly by opening up the ring-shaped MCM2–7 complex and clamping it around the DNA[7]. Consistent with this model, once MCM2–7 have been loaded onto the DNA, ORC, CDC6 and CDT1 are no longer required for MCM2–7 to remain bound and for the origin to remain functionally licensed[2]. The complex of ORC, CDC6, CDT1 and MCM2–7 at replication origins is termed the pre-replicative complex or pre-RC.

ORC, CDC6 and CDT1 are each subject to cell-cycle regulation that could individually contribute to the inactivation of the licensing system on entry into S phase. In most animal cells, however, it appears that downregulation of CDT1 at the G1/S transition is the key event that prevents re-licensing of replicated origins. CDT1 is regulated in at least two different ways. First it is subject to cell cycle-dependent proteolysis during S phase and G2 (REFS 8–11). Second, CDT1 activity is inhibited by the tight binding of a small regulatory protein called geminin[12–14]. The levels and activity of geminin are cell cycle-regulated, so that geminin only binds to CDT1 during S, G2 and early mitosis. In most cell types, it is sufficient to prevent CDT1 degradation or geminin inhibition for significant re-licensing and re-replication of DNA to occur[15–21].

In addition to this cell-cycle control, the licensing system is also downregulated when cells exit from the cell division cycle[22–26]. Many of the cells in an adult human are not actively engaged in the cell division cycle but have withdrawn from it, either temporarily into G0 or permanently as a consequence of terminal differentiation or senescence. When this happens, replication origins are converted into the unlicensed state. In addition, MCM2–7 and other pre-RC proteins are degraded (FIG. 1). Because MCM2–7 proteins are abundant throughout the cell division cycle, they are a unique marker of cells with proliferative capacity, providing great potential for histopathology[24–27]. FIGURE 2a shows a cross-section through normal cervical epithelium stained for MCM5. The basal proliferating layers of the cervical squamous epithelium contain high levels of MCM5, which are lost as cells differentiate and migrate towards the outer surface.

Licensing protein expression in cancer

Many studies have now shown that there is inappropriate expression of MCM2–7 and other pre-RC proteins in a wide variety of premalignant dysplasias and cancers[24–26,28,29]. This is typically associated with an increased number of cells expressing MCM2–7 or cells expressing MCM2–7 where they should not normally do so. For example, in low- and high-grade squamous intraepithelial

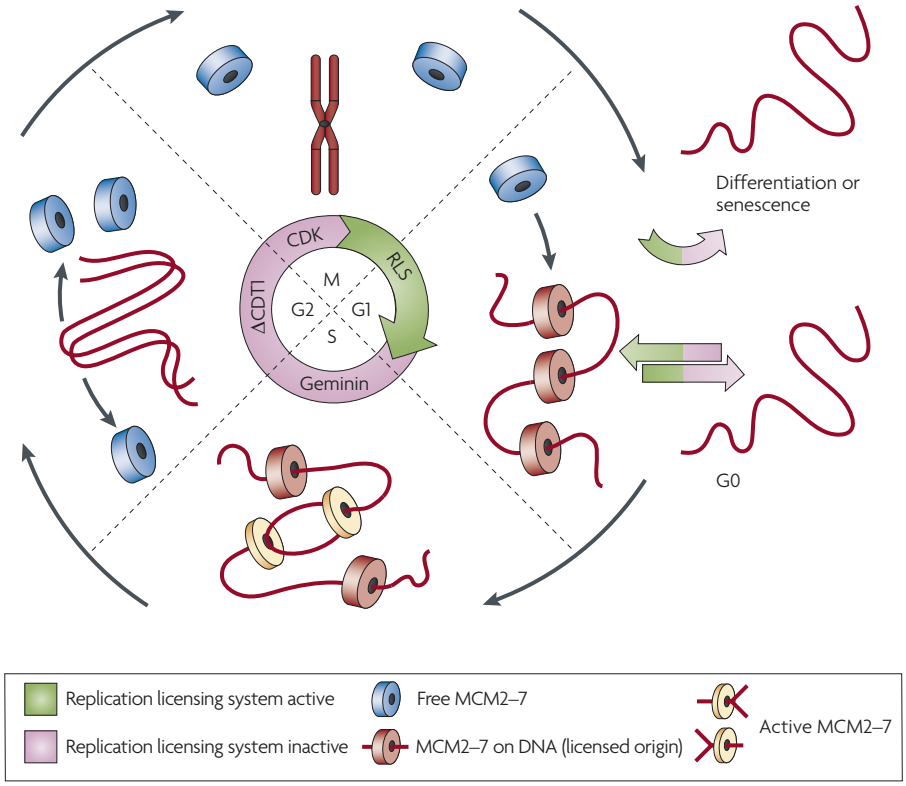

Figure 1 | **Overview of origin licensing during the cell division cycle.** A small segment of chromosomal DNA, encompassing three replication origins, is shown during the cell cycle. Minichromosome maintenance 2–7 (MCM2–7) complexes (here shown as coloured rings) are loaded onto origins during G1 when the licensing system is active (green). During S phase, MCM2–7 complexes at licensed origins are activated to form part of the replication fork. When replication forks terminate, MCM2–7 is displaced from the DNA. Before entry into S phase, the licensing system is shut down (pink) so MCM2–7 cannot be loaded onto replicated DNA. This occurs as a consequence of geminin activation and CDT1 degradation during S and G2 and high cyclin-dependent kinase (CDK) activity during mitosis. On exit from the cell cycle into G0, terminal differentiation or senescence, the licensing system is inactivated and MCM2–7 are degraded. RLS, replication licensing system.

lesions of the cervix, cells with proliferative potential are seen at increasing distances from the basal layer, and this is mirrored by immunostaining for MCM5 (FIG. 2b,c). Similar overexpression of MCM2–7 and other licensing proteins have been seen in a wide variety of different cancers, including oral, laryngeal, oesophageal, pulmonary, mammary, ovarian, renal, prostatic, urothelial, colorectal and haematological cancers[24,26]. This protein overexpression also appears to be reflected in overexpression of mRNA levels. Expression of MCM2–7 has prognostic value and can help predict survival in patients with a range of different cancers[24–26].

It is currently unclear why dysplastic and malignant cells express licensing proteins inappropriately. One possibility is that it may reflect the failure of cells to exit the cell cycle properly. During malignant transformation the cell cycle is disrupted, typically with increased activity of cyclin-dependent kinases (CDKs) that drive cell cycle progression. This may result in fewer cells following their normal differentiation programme and exiting the cell cycle, with the consequence that an increased proportion of cells remain in-cycle and express licensing proteins. In this view, the increased expression of MCM2–7 and other licensing proteins in cancers is a consequence of oncogene-induced stimulation of cell division that decreases the proportion of cells that are in a quiescent (out-of-cycle) state. As such, MCM2–7 would not be directly involved in oncogenic progression, but would provide a powerful marker for in-cycle cells.

An alternative and intriguing possibility, supported by recent data, is that misregulation of the licensing system may have a causal role in the development of cancer. Not only do licensing proteins themselves show oncogenic activity but genes that are commonly mutated in cancer such as Ras, cyclin D1 and cyclin E can cause misregulation of the licensing system. Oncogenes can either induce the re-licensing of replicated DNA or can allow cells to enter S phase with an insufficient number of licensed replication origins. In either case, the integrity of chromosomal DNA is compromised. The possible ways in which oncogenes might misregulate the licensing system are explored in the remainder of this Perspective.

Re-licensing in S and G2

If the licensing machinery is not completely shut down before entry into S phase, replication origins can be re-licensed after they have initiated a pair of forks, allowing the origin to fire a second time in S phase. This is distinct from endoreduplication, where a failure of mitosis leads to a second S phase in the absence of cell division[23]. The main route by which the licensing system is downregulated in S phase in animal cells is proteolysis of CDT1 and activation of the CDT1 inhibitor geminin. Overexpression of CDT1 or the loss of geminin is sufficient to misregulate the licensing system and to induce re-replication[15–21]. It is therefore essential that both CDT1 and geminin activities are well-controlled, especially during S and G2 phase, such that a balance between the two activities is maintained[1–3].

Cell cycle-regulated proteolysis of CDT1 is mediated by its ubiquitylation, which is controlled by at least two distinct mechanisms. In the first mechanism, CDT1 is a substrate for the CUL4–DDB1–CDT2 (also known as DTL) ubiquitin ligase that requires CUL4–DDB1–CDT2 to be recruited to the DNA polymerase processivity factor PCNA[11,19,30–37]. This means CDT1 ubiquitylation is coupled to ongoing DNA synthesis, thus limiting the likelihood of inappropriate origin re-licensing during DNA replication or repair. In the second mechanism, the SCF^{SKP2} (S-phase kinase associated protein 1 (SKP1)/Cullin/F-Box protein; S-phase kinase associated protein 2 (SKP2)) complex promotes CDT1 proteolysis during S and G2 phases in a manner dependent on the phosphorylation of CDT1 by CDK2–cyclin A[9,38–40]. As CDK2–cyclin A is active during S phase and G2, and is involved in promoting the initiation of DNA replication, this provides a second mechanism by which CDT1 destruction is functionally coupled to S phase progression. Loss of DDB1, CDT2 or CUL4, but not SKP2, is sufficient to induce re-replication[10,32,33,36] suggesting that CDT1 degradation mediated

by CUL4–DDB1–CDT2 is a major control mechanism for preventing re-replication. CDT1 is also a substrate of the anaphase-promoting complex (APC/C) ubiquitin ligase, which is active from anaphase through to late G1 (REFS 20,41). APC/C activity does not lead to a complete loss of CDT1 in G1, but may serve to prevent excessive accumulation of active CDT1 at this time, before S phase entry and geminin accumulation.

Geminin is a small protein that binds to CDT1 and blocks its ability to load MCM2–7 onto DNA[12–14] and is active during S, G2 and M phases of the cell cycle[3,12,42,43]. At the metaphase-to-anaphase transition geminin is ubiquitylated by the APC/C, which leads either to its proteolysis or inactivation. This establishes a period of licensing competence during late M phase and G1. Loss of geminin promotes re-licensing and re-replication in many, but not all, cell types[15,17,20,21,44]. Misregulation of APC/C activity can therefore potentially lead to re-replication as a consequence of a failure to correctly regulate geminin. Loss of the APC/C regulator EMI1 (also known as FBXO5) promotes re-replication in this way[45–48]. EMI1 serves to inhibit APC/C activity in late G1 so that sufficient CDK activity can accumulate to permit S phase entry. Loss of EMI1 destabilizes geminin during S phase as APC/C activity is maintained, and thus provides a licensing-competent environment. Although cyclin A and cyclin B activity is reduced in the absence of EMI1, cyclin E activity is increased to a level at which replication initiation can be supported[45,49].

Both ORC and CDC6 activity are regulated in animal cell cycles, although in most cell types this does not appear to have a major role in preventing the re-licensing of replicated DNA[2,3,50]. A proportion of CDC6 is exported from the nucleus during S and G2, but the extent of this appears to vary between cell types[51–53]. There is some evidence for limited re-replication of DNA occurring as a consequence of deregulation of CDC6 in some, but not all, cell types[16,54–56]. Overexpression of CDC6 does, however, significantly increase the degree of re-replication that occurs on de-regulation of CDT1, suggesting that low levels of CDC6 in the nucleus during S and G2 have a supporting role in preventing re-replication[16,54,57]. A potential role for ORC regulation in preventing re-replication has not been extensively studied.

Stabilization of CDT1 and CDC6, or loss of either geminin, EMI1, CDT2, DDB1 or CUL4, can promote re-replication. Re-replication induced by misregulation of these factors can cause accumulation of double-strand DNA breaks and activation of the DNA damage response (DDR)[15–17,20,21,32,33,46,49,58,59]. The DDR activates cell cycle checkpoints, which delays progression through S phase and the onset of M phase or promotes apoptosis (FIG. 3).

The DNA structures generated by re-replication may depend on the frequency of origin reinitiation[3]. Infrequent origin reactivation would generate local regions of DNA that had been re-replicated just once (FIG. 4a). Forks from these origins will eventually stall or collapse (FIG. 4d). Alternatively, frequent reinitiation would drive multiple rounds of re-replication occurring from a single origin (FIG. 4b,c). Experiments in *Xenopus laevis* suggest that consecutive forks travelling in the same direction from the same parental origin have a high probability of colliding[58]. Such 'head-to-tail' fork collisions result in the accumulation of extruded linear DNA strands (FIG. 4c).

It is currently unclear what ultimately happens to these aberrant DNA structures. It is likely that they will be recognized and processed by the DDR machinery, possibly becoming a substrate for homologous recombination. Likely outcomes are that either an extrachromosomal DNA fragment is generated (FIG. 4e) or that the re-replicated fragment is recombined to generate a localized intrachromosomal duplication (FIG. 4f). As the re-replicated DNA contains an origin of replication, this instability may be heritable. Heritable instability would be amplified if specific replication origins were more likely to support re-replication. Partial misregulation of the licensing system in *Saccharomyces cerevisiae* results in detectable re-replication occurring preferentially at specific chromosomal loci[60]. Propagation of this amplification over several generations could result in repeated duplication of this particular locus, causing significant genetic instability.

Consistent with these ideas, both CDT1 and CDC6 are oncogenes[55,61–63]. Overexpression of CDT1 in cells injected into nude mice results in tumour formation[61] and mice specifically overexpressing CDT1 in T-cells develop thymic lymphoblastic lymphoma in p53-null mice[63]. It is important to note that, although replication licensing is essential for cell division, there is no evidence to suggest that it is a rate-limiting step for progression through G1. Therefore, increasing the rate of loading MCM2–7 onto DNA in G1 is unlikely to increase the rate of cell division by itself. Instead it is more likely that the oncogenic activity of CDT1 is a consequence of the genetic instability it induces. Consistent with this idea, tumour cells derived from cells overexpressing CDT1 displayed severe chromosomal aberrations and genetic instability.

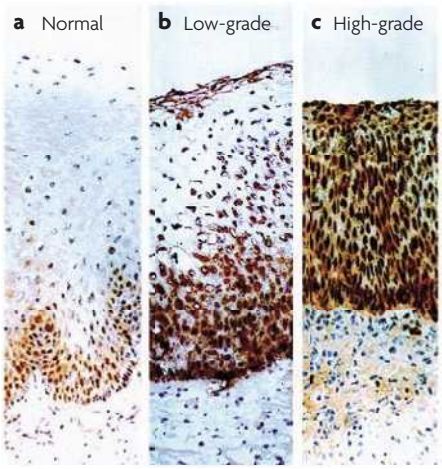

Figure 2 | **Minichromosome maintenance 5 (MCM5) in normal and dysplastic cervical epithelium.** Frozen sections of normal cervix (**a**), low-grade (**b**) and high-grade (**c**) squamous intraepithelial lesions stained with antibodies against MCM5. Reproduced, with permission, from REF. 27 © National Academy of Sciences (1998).

The role of CDC6 in tumorigenesis is potentially more complex. In addition to facilitating re-replication, CDC6 overexpression causes heterochromatinization and repression of the *INK4/ARF* tumour suppressor locus (*CDKN2A*)[62]. CDC6 has also been shown to have a role in activating checkpoint kinases in response to replication inhibition[64–67]. Furthermore, CDC6 may stimulate inappropriate recovery from a cell cycle arrest mediated by p21 (also known as CIP1, encoded by *CDKN1A*) in response to DNA damage by releasing p21 from CDK[68]. CDC6 overexpression may therefore exert a plethora of effects, each of which could lead to genetic instability[69]. Nevertheless it is possible that the oncogenic activity of CDC6 is in part mediated by its ability to promote re-replication.

Re-replication caused by mutation of components of the licensing system may therefore contribute to the genetic instability seen in cancer. However, there is no evidence that such mutations are commonly seen in cancer cells (for example, see Supplementary information S1 (table)). A lack of mutations in the licensing system might be a consequence of animal cells primarily

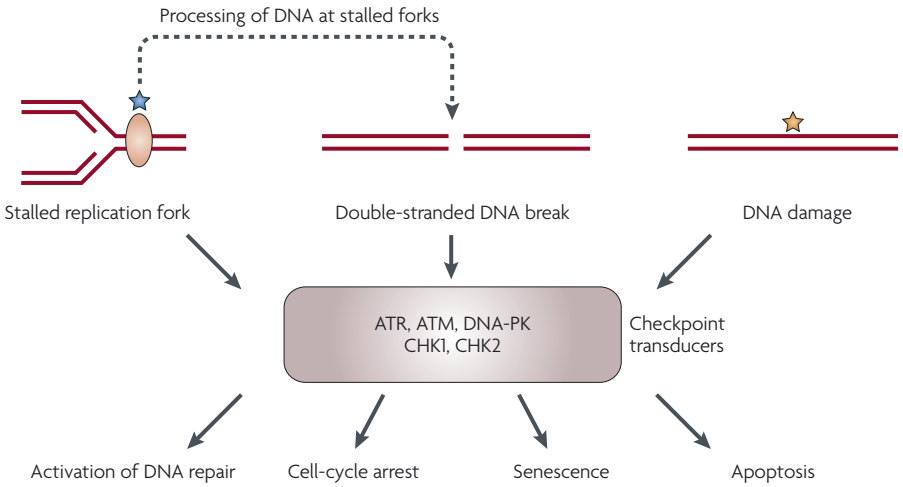

Figure 3 | **The DNA damage response.** Cartoon summarizing major components of the DNA damage response system that are active during S phase. Defects occurring at the DNA are sensed and, through the activation of the signal transducers, lead to cellular responses. ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; DNA-PK, DNA-dependent protein kinase.

using downregulation of CDT1 in S and G2 phases to prevent re-licensing of replicated DNA. Focusing all of the regulation on CDT1 might make it harder for mutations to arise that lead to genetic instability, as loss of geminin or stabilization of CDT1 typically cause lethal levels of re-replication[70].

However, recent evidence suggests that activation of oncogenes that are more commonly involved in tumorigenesis can interfere with the mechanisms that normally shut down replication licensing in S phase and G2. This causes low levels of re-replication that are sufficient to cause genetic instability but are compatible with cell survival. One such case is cyclin D1, a potent oncogene that is frequently mutated in human tumours. Overexpression of a mutant form of cyclin D1 has recently been shown to cause origin re-licensing and re-replication in a single cell cycle[71]. This cyclin D1-induced re-replication appears to be caused by loss of CUL4 expression and consequent stabilization of CDT1 in S phase.

A second case involves the *HRAS* oncogene. Ectopic expression of HRAS in primary cells induces them to undergo oncogene-induced senescence[72]. Senescent cells arrest with partially replicated DNA and strong induction of the DDR. Fluorescence *in situ* hybridization showed that the senescent cells had more than the expected two copies of certain chromosomal loci, which suggests that DNA re-replication had occurred. In addition, there was evidence for a high rate of replication fork stalling and the activation of dormant replication origins. Similar DDR activation, fork stalling and induction of oncogene-induced senescence were seen when the *MOS* oncogene or *CDC6* were ectopically expressed in primary cells[73]. Suppression of the DDR after these oncogenes had induced senescence led to reactivation of proliferation and tumour formation, providing an appealing model for the early events of tumorigenesis[72–74].

It is currently unclear how frequently re-replication is induced by oncogene activation in primary cells. One possibility is that oncogenes activating growth-regulatory pathways upstream of RB behave like cyclin D1 and HRAS and prevent full inactivation of the licensing system in S phase and G2, thus leading to re-replication, DNA damage and DDR activation. This could well be the first part of the recent two-step model of tumorigenesis[72–74]. Oncogene-induced re-replication could drive cells into oncogene-induced senescence, from which they would only emerge as a consequence of additional mutations in the DDR system, thereby providing an explanation for the observed phenotypes of cells early in tumorigenesis. It would be of great interest to test this idea and to determine which pathways controlling licensing activity are misregulated by oncogenes.

Insufficient origin licensing

An alternative consequence of misregulation of the licensing system might be to reduce the loading of MCM2–7 onto chromatin before entry into S phase. MCM2–7 are essential replication proteins so DNA replication cannot occur if their loading onto DNA is completely prevented [75–80]. However, reducing (rather than abolishing) the quantity of MCM2–7 loaded onto DNA can have complicated consequences by reducing the number of replication origins that the cell can use.

Eukaryotic cells use a significantly larger number of replication origins than seems strictly necessary to complete replication in the time available for S phase. For example, typical mammalian somatic cells use replication origins spaced on average 30–150 kb apart, even though the forks initiated from a single origin could potentially replicate ~1.5 Mb of DNA over the entire period of S phase. There are probably several reasons for the excessive number of replication origins used, but one important reason is probably addition of a degree of redundancy to the system to deal with problems that may occur during S phase. Forks encountering DNA damage or tightly associated DNA–protein complexes can irreversibly stall[81], and if two converging forks stall it is difficult for the intervening DNA to be replicated (FIG. 5a). If MCM2–7 loading is reduced, fewer replication origins are used and this results in DNA strand breaks, checkpoint activation, genetic instability and cell death[82–90], consistent with the idea that abundant origins are required to compensate for replication fork failures.

Not only is there an excess of replication origins over the minimum number required to complete S phase in a timely manner, but there are 10–20 times more MCM2–7 molecules loaded onto DNA in G1 than there are active replication origins[83,91–93]. Cells continue to synthesize DNA at normal rates when the level of MCM2–7 is reduced[94–96] and in *X. laevis* egg extracts normal replication rates are maintained when MCM2–7 levels are reduced to only ~2 per origin[66,92,93].

Recent work has provided evidence that these excess MCM2–7 are required for cells to properly cope with replicative stresses that might induce replication fork stalling[96–98]. When two converging replication forks irreversibly stall (FIG. 5a) it is not possible to license a new origin between the two stalled forks because the licensing system needs to be inactivated before entry into S phase (FIG. 1). If further licensing were allowed at this stage there would be a high risk of DNA being re-replicated, as there is no known mechanism that could direct the MCM2–7 to the unreplicated portion of DNA rather than the replicated DNA. It is possible that homologous recombination could be

used to restart the stalled forks, but this creates the risk of DNA strand breakage or chromosome rearrangement. However, inhibition of replication forks promotes the activation of dormant origins that do not fire in unperturbed S phases[96–102]. The activity of these dormant origins is dependent on the full complement of MCM2–7 being loaded onto DNA[96,97]. In the absence of replicative stress, these dormant origins are passively replicated by forks from neighbouring origins, so do not normally fire (FIG. 5b). However, dormant origins become essential for complete replication and cell survival under conditions of replicative stress[96–98] (FIG. 5c).

Consistent with these ideas, mice heterozygous for an *Mcm4* hypomorphic mutation that apparently destabilizes MCM4 protein (*Mcm4*^{Chaos3}) showed greatly increased rates of chromosome breakage in response to a replication inhibitor[103]. *Chaos3* mutant mice also showed increased levels of micronuclei, another sign of increased chromosome instability. Significantly, 80% of *Chaos3* females died of mammary adenocarcinomas. In a separate study, cells from a mouse strain that produces lowered levels of transgenic MCM2 (MCM2$^{IRES-CreERT2}$) also showed increased micronuclei and increased γH2AX foci (a sign of double-strand DNA breaks)[104]. MCM2$^{IRES-CreERT2}$ cells proliferated at normal rates, though they appeared to have a severe stem cell deficiency in various tissues. MCM2$^{IRES-CreERT2}$ mice die of cancer at an early age but, unlike the *Chaos3* mice, this was predominantly owing to T- and B-cell lymphomas.

These studies are consistent with the idea that insufficient origin licensing can promote the development of cancer. The virtually normal proliferative capacity of cells with mutant MCM2–7 coupled with their increased sensitivity to replication inhibitors and increased levels of spontaneous DNA damage is consistent with the idea that they are unable to license a sufficient number of dormant origins and so cannot deal properly with sporadic replication defects. This would create genetic instability and thereby promote the development of cancer. As discussed above, misregulation of the licensing system appears to be an early event in the development of many cancers. To date this has been noted as an increased proportion of cells expressing MCM2–7 and other licensing proteins. However, it would be important to know whether individual cells are expressing adequate levels of the licensing proteins and are being driven through S phase with a sufficient number of licensed origins.

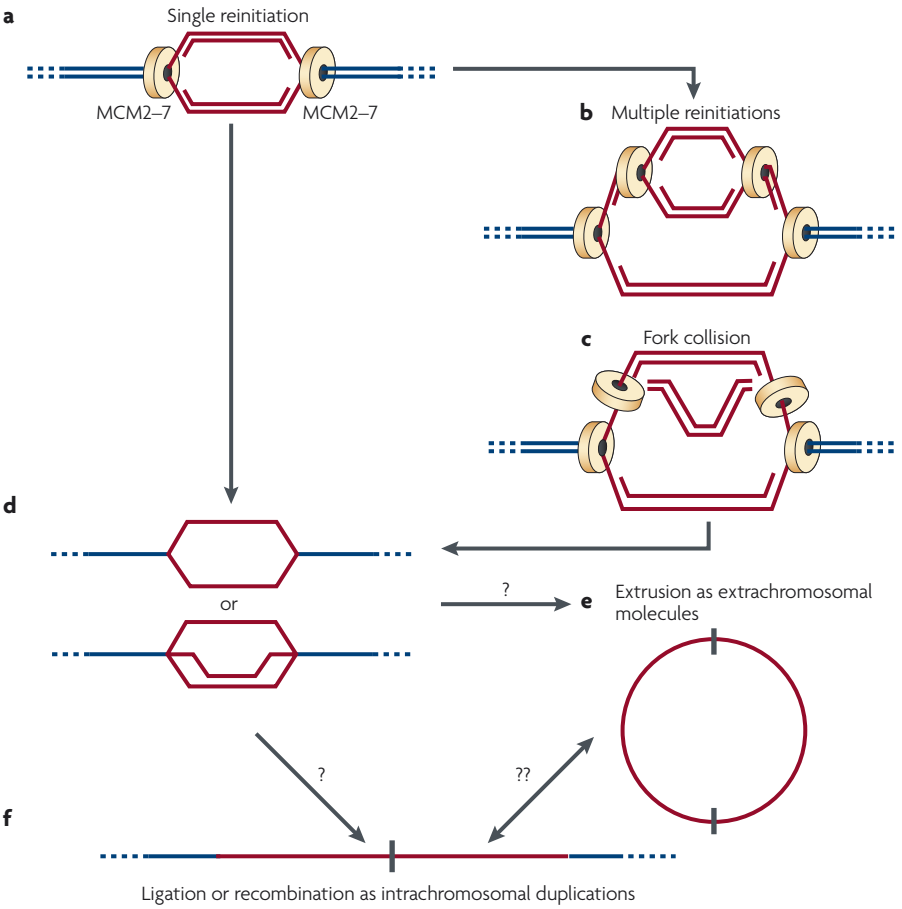

Figure 4 | **Consequences of re-replication.** A small segment of chromosomal DNA containing a replication origin is shown. A single reinitiation event generates a bubble of re-replicated DNA (**a**). If the rate of reinitiation is high, forks will be initiated sufficiently close together that they undergo head-to-tail collision (**b**,**c**)[58]. The resultant bubble structures (**d**) may undergo recombination to form either extrachromosomal circles (**e**) or tandem chromosomal duplications (**f**). Lines in **a**–**c** indicate single DNA strands and lines in **d**–**f** indicate double DNA strands.

When cyclin E is overexpressed in cells, they show a reduction in the amount of MCM2–7 loaded onto DNA during late mitosis and G1 (REF. 78). Consistent with this causing a reduction in the number of origins licensed, cyclin E-overexpressing cells also show decreased rates of S phase progression, genetic instability and accelerated tumorigenesis[78,105,106]. Like HRAS overexpression[72], cyclin E overexpression in primary cells caused DDR activation and oncogene-induced senescence[73]. It would be interesting to investigate how common it is for oncogene activation to cause insufficient MCM2–7 loading in G1, and the mechanism by which cyclin E overexpression causes this.

The results discussed here and in the section above suggest that oncogenes can induce DNA damage and DDR activation both by re-licensing origins in S and G2 and by insufficient origin licensing in G1 (REFS 71–73,78). Both ways of misregulating the licensing system lead to stalled replication forks that generate structures recognized by the DDR machinery that can potentially drive cells into oncogene-induced senescence[72–74]. Cells escaping from this by acquiring a secondary mutation in the DDR then have a lethal combination of high proliferative capacity due to oncogene activation and genetic instability due to oncogene-induced licensing defects and loss of adequate DDR responses.

The licensing checkpoint

As it is crucial that cells load sufficient MCM2–7 onto DNA before they embark on S phase, it is plausible that they have a feedback system that could delay progression into S phase until a sufficient number of origins are licensed. This idea has been addressed using a number of

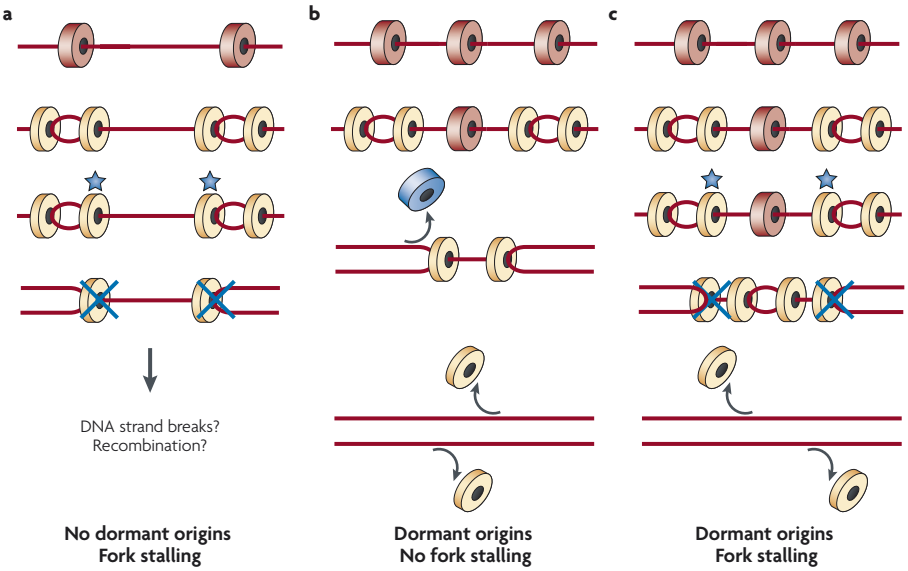

Figure 5 | **Dormant origins and replication fork stalling.** A small section of chromosomal DNA replicated by 2–3 origins is shown. Minichromosome maintenance 2–7 (MCM2–7) double hexamers are shown as ovals. **a** | If two converging forks stall (indicated with star) with no dormant origin between them, the result is likely to be DNA breakage or rescue by recombination. **b** | In the absence of fork stalling, the dormant origin between the two active origins does not initiate, and its MCM2–7 proteins are passively displaced as they are replicated. **c** | If the converging forks stall, the dormant origin between them can initiate and allow complete replication of the chromosomal segment.

different approaches to reduce the quantity of functional MCM2–7 loaded onto DNA[86,88,89,107–109]. The response to inhibition of licensing, either by forced expression of geminin or by RNA interference against licensing proteins, depended on the cell type[86,88]. Primary cell lines responded to licensing inhibition by delaying entry into S phase, thereby keeping cells at a cell cycle stage where further licensing could potentially occur. This suggests that primary cells have a licensing checkpoint that delays entry into S phase if insufficient MCM2–7 has been loaded onto DNA. By contrast, when licensing was inhibited in a range of different cancer cell lines they did not delay progression into S phase but instead entered an S phase that they were unable to complete, and which was therefore lethal. Some less-transformed cancer cells with active checkpoint mechanisms underwent a relatively rapid apoptosis, whereas more transformed cells survived longer but ultimately died at a later cell cycle stage with partially replicated chromosomes[86,89]. A similar difference between normal and cancer cells was seen when cells were treated with small interfering RNA targeting the CDC7 protein kinase[109]. As the essential function of CDC7 is to phosphorylate and activate MCM2–7, this treatment may have many similar effects to reducing the total amount of MCM2–7. The observation that the licensing checkpoint appears defective in many cancer cell lines suggests that they frequently experience inefficient origin licensing.

The way that primary cells detect and respond to decreased MCM2–7 levels is currently unclear. In *X. laevis* egg extracts, a feedback loop has been described that promotes entry into S phase only when MCM2–7 have been loaded onto DNA[110]. MCM2–7 on chromatin stimulates the loading of an essential nuclear pore protein ELYS/MEL-28; as nuclear pore function is required both for progression into S phase and for the activation of geminin as a licensing inhibitor[42,43] this creates a feedback loop. It is unlikely that this sort of mechanism will operate in somatic cells with a lengthy G1 phase. Instead, the licensing checkpoint appears to depend on downregulating CDK activity in late G1 of somatic cells. Normal cells treated with the licensing inhibitor geminin or with small interfering RNA targeting one of the ORC subunits caused cell-cycle arrest in a G1-like state with low cyclin E–CDK2 activity and induction of the CDK inhibitor p21 (REFS 86,89,108). Activation of this 'licensing checkpoint' may involve a novel pathway that blocks activation of S phase CDKs without involving the classical DDR pathway (J. Cook, personal communication).

Licensing as an anticancer target

A large number of chemotherapeutic drugs target, either directly or indirectly, the process of DNA replication (Supplementary information S2 (table)). The antimetabolite class of drugs, for example, directly affect the supply of dNTPs to the replicative DNA polymerases, whereas many DNA-damaging agents, such as alkylating agents, are primarily recognized by the DDR while the DNA is being replicated[111]. The replication fork, which is the target of these chemotherapeutic drugs, appears to be essentially normal in cancer cells but, as discussed above, the replication licensing system may be frequently misregulated. An intriguing possibility is that the effectiveness of these chemotherapeutic drugs is due to misregulation of the licensing system in cancer cells. For example, cancer cells that license a reduced number of origins in G1 would be expected to be hypersensitive to a range of replication inhibitors[96,97].

Further, if there is a licensing checkpoint that is defective in cancer cells, then small-molecule inhibitors of the replication licensing system will specifically kill these cancer cells but only delay the proliferation of normal cells. Normal cells finding their origins unlicensed would respond by activating the licensing checkpoint and arresting temporarily in a G1-like state. When the drug was removed or metabolized, re-licensing of origins and entry into S phase could occur. Cancer cells lacking the licensing checkpoint would suffer a different fate, as they would pass into S phase with an insufficient number of licensed replication origins to complete replication. It is impossible for these cells to regain viability, because even if the inhibitor were subsequently removed or metabolized, no further origin licensing could take place once the cells had progressed into S phase. As replication licensing is essential for cell proliferation, cancer cells could not become resistant to licensing inhibitors by using an alternative pathway. Licensing inhibitors might also be expected to synergize well with existing chemotherapeutic drugs. These considerations make replication licensing an attractive anticancer target.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

DATABASES
Entrez Gene:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
CDC6 | CDC7 | CDK2 | *CDKN1A* | *CDKN2A* | CDT1 | cyclin D1 | DDB1 | DTL | FBXO5 | geminin | *HRAS* | MCM2 | MCM3 | MCM4 | MCM5 | MCM6 | MCM7 | MOS | p53 | PCNA
National Cancer Institute: http://www.cancer.gov/
colorectal cancer | mammary cancer | oesophageal cancer | oral cancer | ovarian cancer | prostatic cancer | renal cancer

FURTHER INFORMATION
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http://www.lifesci.dundee.ac.uk/people/julian_blow/

SUPPLEMENTARY INFORMATION
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OPINION

Bone morphogenetic protein signalling in colorectal cancer

James C. Hardwick, Liudmila L. Kodach, G. Johan Offerhaus and Gijs R. van den Brink

Abstract | Much of the current understanding of colorectal cancer stems from the study of rare, inherited colorectal cancer syndromes. Mutations in the bone morphogenetic protein (BMP) pathway have been found in juvenile polyposis, an inherited polyposis syndrome that predisposes to colorectal cancer. The hamartomas that develop in these patients and in BMP pathway mutant mice have a remarkable mesenchymal component. Further evidence in mice suggests a primary role for mesenchymal loss of BMP signalling in hamartoma development. Here, we examine this evidence and question its relevance to sporadic colorectal carcinogenesis.

The initiation and progression of colorectal cancer development is characterized by the gradual accumulation of genetic and epigenetic changes. The list of these changes is long and continues to grow, but for most it is not known at what stage the modification is required, or whether they are the cause or result of the progression to cancer. By contrast, there are a small number of genes thought to be causative in the initiation of colorectal carcinogenesis[1]. This makes the study of these genes and the signalling pathways to which they belong particularly interesting. Known initiators have mostly been identified in families with inherited polyposis syndromes. Tumours that develop in these patients are rare examples of neoplasms with a known initiating genetic alteration. The classic example is familial adenomatous polyposis (FAP) resulting