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Dormant origins as a built-in safeguard in eukaryotic DNA replication against genome instability and disease development

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Abstract

DNA replication is a prerequisite for cell proliferation, yet it can be increasingly challenging for a eukaryotic cell to faithfully duplicate its genome as its size and complexity expands. Dormant origins now emerge as a key component for cells to successfully accomplish such a demanding but essential task. In this perspective, we will first provide an overview of the fundamental processes eukaryotic cells have developed to regulate origin licensing and firing. With a special focus on mammalian systems, we will then highlight the role of dormant origins in preventing replication-associated genome instability and their functional interplay with proteins involved in the DNA damage repair response for tumor suppression. Lastly, deficiencies in the origin licensing machinery will be discussed in relation to their influence on stem cell maintenance and human diseases.

Keywords

MCM2-7; Dormant replication origins; Replication stress; Replication-associated genome instability; Stem cells; Cancer; Rare human genetic diseases

1. Introduction

Given the large size of genomes and their organization into multiple chromosomes, eukaryotic cells must initiate DNA synthesis from hundreds or thousands of sites known as replication origins to generate duplicates of their genomes within a given time frame of the cell cycle. Successful completion of DNA replication allows faithful transmission of the genetic information to progeny through cell division, supporting the survival of unicellular species and proper development of multiple cellular organisms. Having numerous replication origins brings not only benefits but also challenges, since their distribution and activity must be under fine control. Eukaryotic cells have adopted a multi-layered system to meet this necessity for precise DNA replication. This includes dividing the genome into many regions (or domains) to be replicated at different times during S phase and

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implementing the mechanism that executes origin licensing separately from firing at distinct stages of the cell cycle [1–6].

Origin licensing is a prerequisite for DNA replication in S phase in all eukaryotes, which takes place exclusively from the late M to G1 phases [4–8]. During this period, a pair of heterohexameric complexes of MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7 (thereafter called MCM2-7) are loaded onto DNA to license replication origins [9–12]. While all licensed origins have the potential to initiate DNA synthesis, only a subset of them actually fire in the following S phase when two origin-bound MCM2-7 hexamers individually assemble into active helicases along with CDC45 and GINS (i.e., CMG helicase) to establish bidirectional replication forks [12–16]. Thereafter, CMG helicases travel as a component of replisomes at replication forks, placing fired origins into an unlicensed state. Moreover, re-licensing of fired origins is prevented, as loading of MCM2-7 hexamers onto DNA is prohibited upon S phase entry [17–19]. This temporal separation of origin licensing from firing ensures that origins fire only once per cell cycle, thereby preventing over-replication of the genome. Concurrently, under-replication of the genome must also be avoided. Origin licensing allows eukaryotic cells to generate many more origins than they use in S phase through the preparation of countless dormant origins. Dormant origins constitute the vast majority of licensed origins, which mostly remain unused but occasionally fire as backups to resolve problems that prevent or impede replication fork progression [20–22]. Therefore, the abundance of dormant origins acts as a safeguard against under-replication. In particular, it is well known that dormant origins are more frequently used due to high demand under conditions of replication stress [23–25]. In contrast, the backup role of dormant origins is relatively under-appreciated in normal S phase despite the fact that a reduction in their number has significant consequences at the cellular and whole animal levels [26–28].

In this perspective, we will shed light on dormant origins in unperturbed conditions with respect to their contribution to genome stability and their functional interplay with DNA damage repair responses mainly in mammalian cells. Moreover, we will discuss how these impact on normal development and tumor suppression in mice. Lastly, we will review current knowledge to better understand rare human genetic disorders associated with defects in origin licensing and firing.

2. Origin licensing and firing

There are excellent recent reviews on the regulation of DNA replication in eukaryotes [4–8]. Concurrently, dormant origins have also been the topic of previous reviews including the prequel to this perspective [20–22,29]. Therefore, we will briefly summarize the fundamental processes that regulate DNA replication with relevance to the function of dormant origins.

From the late M to the G1 phase of the cell cycle, origin licensing occurs by step-wise actions involving the origin recognition complex (ORC), CDC6, and CDT1 for loading MCM2-7 complexes onto DNA [4–8]. ORC comprises six subunits (ORC1-ORC6) and first binds origin DNA before recruiting CDC6. In budding yeast, ORC binds DNA in a

sequence-specific manner [30], whereas ORC binding sites in humans are enriched at open chromatin without any consensus motif [31]. CDT1 helps loading of two copies of MCM2-7 hexamers onto ORC-bound DNA in a head-to-head orientation to assemble the pre-replicative complex (pre-RC) [9–12]. Upon loading of MCM2-7 double hexamers, ORC, CDC6 and CDT1 are no longer required for initiation of DNA replication [15,32–34]. Since the MCM2-7 double hexamers loaded onto DNA outnumber the ORCs, the mechanism(s) for this to occur may influence the distribution of licensed origins [35–39]. Moreover, recent studies suggest a mobile nature of MCM2-7 double hexamers on DNA, which could be redistributed by active transcription without losing their functionality as seen in budding yeast and fly [40,41]. Therefore, any site bound with the MCM2-7 double hexamer has the potential to act as a replication origin regardless of DNA sequences. By the end of G1 phase, the amount of DNA-bound MCM2-7 double hexamers reaches the maximum level, far exceeding (3–20 fold) the number of active origins that cells use in a given S phase [39,40,42–46]. A prevailing idea to explain this phenomenon is that excess MCM2-7 double hexamers license dormant origins for backup use in case problems arise during replication fork progression [24,25]. During S phase, a small fraction of MCM2-7 double hexamers assemble into two active CMG helicases to fire origins promoted by CDC7 kinase and cyclin-dependent kinase (CDK) [12–16]. The rest of unused MCM2-7 double hexamers (residing at dormant origins) are likely removed by active replication forks, but it is unclear how this occurs [47,48]. At replication termination, CMG helicases are unloaded at converging forks after the final ligation step, which is regulated by polyubiquitilation of MCM7 contained within the CMG helicase, as revealed by recent studies on budding yeast and *Xenopus* egg extracts [49–51].

The mammalian genome is divided into large regions that contain domains (several hundred Kb) with multiple replicons, which are replicated at different time periods during S phase [1–3,52,53]. Distribution of replication origins (that actually fire) does not appear random throughout the genome, as early replicating domains display a high density of efficient origins and late-replicating domains are observed with low origin activity [54]. Consistently, early-replication domains are far more enriched with ORC binding sites than late-replicating domains [31]. Replicons within a domain are coordinately activated at their expected replication timing, and it has been proposed that regulation of dormant origin firing occurs domain by domain [3,55]. Each replicon may contain multiple licensed origins (likely within early-replicating domains) but fire only one among them in normal S phase [3]. Under replication stress, additional origins (i.e. dormant origins) are allowed to fire within actively replicating domains, but origin firing is entirely suppressed in those that have not started replication [55]. This systematic process most likely adjusts the number of replication forks in response to the limited resources available to complete replication of active domains, while minimizing stalled forks to preserve genome stability [1,55]. It is not well understood how a reduction of dormant origins impacts replication of each domain or the entire genome. However, it is logical to postulate that large replicons with intrinsically fewer licensed origins are more susceptible to under-replication in the absence of dormant origins. Such regions may not be fully replicated if replication forks stall or progress at a much slower rate unless an alternative mechanism intervenes. This idea is analogous to the basis for fragility observed for specific chromosome loci known as common fragile sites. Origin paucity along

with late-replicating timing make these loci prone to under-replication as well as chromosome aberrations upon replication stress [56–58]. In contrast, a genome-wide study reported that ~65% reduction in MCM2 level in mouse embryonic fibroblasts (MEFs) caused fewer initiation events more prominently at early-replicating domains [59]. These identified loci are also well correlated with chromosome regions that are frequently deleted in mouse tumors [59]. Therefore, it appears that dormant origins influence genome stability regardless of replication timing.

3. Dormant origin deficiency and genome instability

If mammalian cells happen to license very few origins, it is known that they activate a "licensing checkpoint" that prevents S phase entry [60]. Initiating DNA replication under such conditions is a high risk and may cause cell death in the worst case scenario [60,61]. However, it is currently unclear what the threshold is for licensed origins to trigger the activation of this checkpoint. When one subunit of the MCM2-7 complex is depleted by RNA interference (RNAi) or mutations, it typically reduces the amount of other MCM proteins. A modest reduction of MCM2-7 proteins (50–60%) does not prevent S phase entry of human and mouse cells. This level of reduction rarely results in detectable changes in the density of active origins in unperturbed S phase [24,27,28,62], suggesting that a decrease in overall licensed origins results in a loss of dormant origins. Under the conditions of replication stress, a reduction of dormant origins has a more profound effect on origin usage [24,25]. It has been reproducibly observed that cells treated with a low dose of hydroxyurea (HU) or aphidicolin (APH) fire a significantly increased number of replication origins, displaying shorter inter-origin distances compared to untreated cells when assayed by DNA fiber techniques [24,27,28,62] (Fig. 1). The increase in origin usage under replication stress is attributed to the activation of dormant origins, since partial depletion of MCM2-7 proteins diminishes the ability of cells to increase the density of active origins [24,27,28]. Most importantly, cells with partial depletion of MCM2-7 exhibit poor survival or proliferation in the presence of HU or APH [24,25]. This suggests that dormant origin firing not only rescues stalled forks but also compensates for slower fork progression by increasing the number of replication forks, thereby raising the chance for cells to complete DNA replication under conditions of replication stress [24,25].

Even in unperturbed conditions, replication forks stall when they encounter endogenous DNA lesions [63]. Dormant origins play a major role in the recovery of stalled forks in normal S phase [28] (Fig. 1). Therefore, a reduction of dormant origins still causes a detectable level of genome instability in unperturbed conditions despite no detectable changes in active origin density [25,28,64]. MEFs homozygous for *Mcm4*^{chaos3} (*Mcm4*^{c3}, see below) display a ~50% loss of chromatin-bound MCM2-7 complexes relative to isogenic wild-type cells, exhibiting a modest but significant increase in replication-associated genome instability [28]. Under-replication of chromosome loci causes an elevated incidence of spontaneous micronuclei (MN) in *Mcm4*$^{c3/c3}$ cells [28] (see Fig. 2). MN are a well-established marker of chromosome damage, arising from acentric fragments or lagging chromosomes that are not incorporated into one of the daughter nuclei following anaphase [65]. Not only MN-containing acentric fragment(s) increased but also MN with whole chromosome(s) increased in *Mcm4*$^{c3/c3}$ cells compared to wild-type MEFs [28]. This

suggests that chromosomes containing under-replicated loci often manifest themselves as lagging chromosomes during anaphase, potentially causing aneuploidy. Under-replicated loci may also be converted to chromatin lesions during mitosis, which would then be transmitted to daughter cells to form 53BP1-NBs in the ensuing G1 phase [66,67]. *Mcm4*$^{c3/c3}$ cells consistently display a slightly higher level of basal 53BP1-nuclear bodies (53BP1-NBs) relative to wild-type [68]. Likewise, partial depletion of MCM5 or MCM2 in human cancer cell lines also increases the number of 53BP1-NBs [69,70]. The formation of 53BP1-NBs tends to occur at large replicons that are highly susceptible to under-replication [69]. Within 53BP1-NBs, under-replicated loci are supposedly protected from degradation [66,67], as depletion of 53BP1 decreases survival of cells with dormant origin deficiency [69]. Most interestingly, *Mcm4*$^{c3/c3}$ cells display an elevated incidence of isolated DNA synthesis in early M phase [68], which is similar to mitotic DNA repair synthesis that is strongly induced upon replication stress [71,72]. Consistent with this observation, partial depletion of MCM5 in human cells also results in a significant increase in mitotic DNA synthesis [69]. It will be of great interest to investigate a potential link between mitotic DNA synthesis and small deletions found in mouse tumors with *Mcm* mutations (see below). Taken together, these findings indicate a critical role of dormant origins in suppressing replication-associated genome instability even in normal S phase. This role of dormant origins may partially or indirectly explain the strong tumor predisposition observed in mouse mutant strains that exhibit dormant origin deficiency [28,73,74].

4. Dormant origins and tumor suppression in mice

Since the *Mcm2-7* genes are essential for DNA replication, homozygosity for a null allele of the respective *Mcm* gene causes embryonic lethality [62,73,75]. Only hypomorphic alleles such as *Mcm4*^{c3} and *Mcm2*$^{IRES–CreERT2}$ can result in viable homozygous mice surviving into adulthood [73,74]. However, the majority of these mice still die prematurely due to the development of spontaneous tumors [28,73,74]. The *Mcm4*^{c3} allele was identified from a phenotype-based screen for an elevated frequency of spontaneous micronuclei in erythrocytes [73,76]. *Mcm4*^{c3} encodes a Phe345Ile change, which lowers the efficiency of MCM2-7 complex assembly but does not confer detectable helicase defect *in vitro* [28]. MEFs homozygous for *Mcm4*^{c3} exhibit ~50% reduction in chromatin-bound MCM2-7 proteins relative to isogenic wild-type cells, which causes a lesser ability to activate dormant origins after treatment with a low dose of APH [28,75]. A recent study reported that SV40-immortalized *Mcm4*$^{c3/c3}$ MEFs display less stable association of MCM2-7 at replication forks relative to wild-type cells [77]. It is uncertain if this truly reflects helicase activity in primary, un-immortalized *Mcm4*$^{c3/c3}$ MEFs, since they display replication fork speeds comparable to wild-type cells [28]. The *Mcm2*$^{IRES–CreERT2}$ allele (*Mcm2*^{Cre}) was engineered to express a tamoxifen-inducible form of Cre recombinase (CreERT2) that is inserted into the 3′-untranslated region (3′-UTR) of the endogenous *Mcm2* locus [74]. This modification is apparently responsible for ~65% reduction of MCM2 in *Mcm2*$^{Cre/Cre}$ MEFs compared to wild-type [74]. This reduction level of MCM2 nearly abolishes the activation of dormant origins even in the presence of HU [27]. Overall, these two mouse models with dormant origin deficiency are phenotypically similar to one another. However, they display a striking difference with respect to tumor latency. All *Mcm2*$^{Cre/Cre}$ mice in a 129 Sv

background succumb to T-cell lymphoblastic lymphomas within 12 weeks of age. In contrast, *Mcm4*$^{c3/c3}$ mice show a longer tumor latency (~one year) in all genetic backgrounds tested [28,68,73,78]. This difference is likely attributed to the severity of dormant origin deficiency as well as the respective mutant gene they harbor. A reduction of *Mcm2* gene dosage (by heterozygosity for a null allele termed *Mcm2*^{Gt}) decreases mRNA expression of its own and of other *Mcm* genes [75]. Reportedly, ~75% of *Mcm2*$^{Gt/+}$ mice succumb to spontaneous tumors after a long latency (up to 18 months of age) [75]. Therefore, *Mcm2* mutations that lower its expression in general may have a profound effect on MCM2-7 protein levels, leading to severe dormant origin deficiency as seen for *Mcm2*$^{Cre/Cre}$ mice. By mouse crossing, the *Mcm2*^{Gt} allele was introduced into *Mcm4*$^{c3/c3}$ mice to generate *Mcm2*$^{Gt/+}$;*Mcm4*$^{c3/c3}$ offspring. This reduction of *Mcm2* gene dosage declined the viability of offspring to ~30% of *Mcm4*$^{c3/c3}$ mice [75]. Those rare individuals that survived into adulthood were severely growth-retarded and died before 6 months of age with early onset of tumors including T-cell lymphomas [75]. Surprisingly, a reduction of Mcm3 gene dosage (also by heterozygosity for a null allele; *Mcm3*^{Gt}) apparently rescues dormant origin deficiency to some extent, substantially delaying or suppressing tumor formation in *Mcm4*$^{c3/c3}$ mice [75]. This unexpected observation has been explained by better nuclear retention of MCM2-7 proteins caused by *Mcm3* heterozygosity, increasing the ability of *Mcm4*$^{c3/c3}$ cells to license dormant origins [75]. Together, these findings suggest that an increasing severity of dormant origin deficiency contributes to shorter tumor latency. Moreover, *Mcm2* and *Mcm3* appear to have additional roles beyond origin licensing in regulating overall expression or nuclear retention of MCM2-7 proteins.

Genetic backgrounds also influence tumor spectra in these mice [27,28,68,73,74,78]. Initially, mammary tumors were predominantly observed in *Mcm4*$^{c3/c3}$ females in an inbred C3HeB/FeJ (C3H) background [73]. *Mcm4*$^{c3/c3}$ mice also develop a variety of spontaneous tumors depending on genetic backgrounds including histiocytic sarcomas in a C57BL/6J (B6) background [28,78]. Similarly, a fraction of *Mcm2*$^{Cre/Cre}$ mice survived beyond 15 weeks of age when bred into a 129 Sv:BALB/c mixed background, developing lung and liver tumors in addition to lymphomas [27]. Although it is not known for *Mcm2*$^{Cre/Cre}$ mice, *Mcm4*$^{c3/c3}$ mice show a clear difference in tumor predisposition between males and females. In general, *Mcm4*$^{c3/c3}$ females develop a variety of tumors faster than males [73,78]. It will be interesting to investigate this difference in relation to the roles of female hormones in regulating MCM2-7 expression [79,80].

Different from other mouse *Mcm* mutant alleles which are either recessive or potentially haplo-insufficient, a *Mcm4* allele known as spontaneous dominant leukemia (*Sdl*) is very unique given its dominant nature in causing cancer. *Mcm4*^{Sdl} is a spontaneous mutation that was discovered in a mouse breeding colony due to its ability to cause T cell lymphoblastic leukemia/lymphoma in the majority of *heterozygous* carriers before 6 months of age [81]. Notably, all tumors examined retain the wild-type allele of *Mcm4*, suggesting that *Mcm4*^{Sdl} alone is not compatible with cell viability. Consistently, *Mcm4*^{Sdl} homozygotes die during early embryonic development (before 8.5 dpc) and its corresponding allele in budding yeast fails to rescue *mcm4* deficiency [81]. Unlike other *Mcm* mouse models, *Mcm4*^{Sdl} heterozygous MEFs show no detectable changes in MCM2-7 protein levels. Rather, the encoded change by *Mcm4*^{Sdl} (Asp573His) is proposed to render the replicative helicase

inactive due to its location within the Walker B motif of MCM4 [81]. Presumably, the inactive helicase could have a profoundly strong effect on overall DNA replication processes. Therefore, the more severe phenotypes of *Mcm4*^{Sdl} mice most likely reflect a complex problem than dormant origin deficiency alone. This idea remains to be tested, since it is currently unknown how *Mcm4*^{Sdl} heterozygosity influences origin usage and replication fork movement. Finally, *Mcm4*^{Sdl} and *Mcm4*^{c3} mice provide a good example that different alleles can cause divergent phenotypes even when the same gene is mutated.

The *Mcm* mouse models are also useful for the identification and functional characterization of cancer-related genes. Array-based comparative genome hybridization (aCGH) performed on tumors that arose in these mice have revealed recurrent copy number alterations (CNAs) involving known or putative cancer genes in humans [81–84]. The majority of CNAs found in T-cell lymphoblastic lymphomas developed in *Mcm2*$^{Cre/Cre}$ mice are mainly deletions averaging < 500 kb in size, far smaller than those found in other mouse models [82,85]. Given the small size of deletions along with their highly recurrent nature, it has been proposed that the discovery of genes and pathways responsible for lymphomagenesis can be achieved at high resolution using a small number of animals [82]. In fact, all analyzed tumors bear bi-allelic deletions involving the *Pten* locus [82], which is also inactivated in T-cell acute lymphoblastic lymphomas in humans [85–87]. Overall, small recurrent deletions found in *Mcm2*^{Cre} tumors are correlated with sites that exhibit a substantially reduced ability to initiate DNA replication in *Mcm2*$^{Cre/Cre}$ cells [59]. While amplifications are relatively rare in *Mcm2*^{Cre} tumors [82], T-cell lymphoblastic lymphomas formed in *Mcm4*^{Sdl} mice contain many small deletions as well as amplifications (averaging 110 kb in size) [81]. In particular, *Mcm4*^{Sdl} tumors display intra-genic deletions at the *Notch1* locus, which are most likely responsible for the activation of *Notch1* pathway in these tumors [81,88]. Interestingly, these similar deletions located within the *Notch1* locus were also found in *Mcm2*^{Cre} tumors [82], and more relevantly *NOTCH1*-activating mutations were found in > 50% of T-cell acute lymphoblastic lymphomas [89]. These findings suggest that these *Mcm* mouse models well recapitulate tumorigenic pathways in human lymphomas.

Genomic analyses of mammary tumors found in *Mcm4*^{c3} mice also unveiled highly recurrent CNAs, which cause deletions of tumor suppressors that may also be involved in the development of sporadic breast cancer in humans [83,84]. One such CNA leads to homozygous or heterozygous loss of the tumor suppressor *Nf1* (Neurofibromin 1) in nearly all *Mcm4*^{c3} tumors [83]. Consistent with NF1 being a negative regulator of the RAS signaling pathway, *Mcm4*^{c3} tumors exhibit hyper-activated RAS [83]. Importantly, re-analysis of the Cancer Genome Atlas (TCGA) data also revealed *NF1* deletions or mutations in ~30% of human breast cancers [83]. Moreover, it was recently reported that ~70% of *Mcm4*^{c3} tumors have mono-allelic deletion of *Arid1a*, resulting in its lower expression [84]. This gene encodes a subunit of the mammalian SWI/SNF chromatin-remodeling complex, which has been implicated as a haplo-insufficient tumor suppressor in breast cancer [90–92]. Supporting this idea, *Mcm4*^{c3} tumor cells with restored *Arid1a* expression display a significantly reduced ability to form tumors by transplantation assays [84]. This function of *Arid1a* depends on *Trp53*, the master tumor suppressor that in turn also relies on a wild-type level of *Arid1a* expression to properly activate its downstream pathways [84]. This study reveals a possible co-dependency between *Arid1a* and *Trp53* in tumor suppression. In

addition to mechanistic investigation of tumorigenic pathways, these *Mcm* mouse models will also be useful for the development of therapeutics for lymphomas and breast cancers [83,84].

5. Functional interplay between dormant origins and DNA damage repair responses

Cells with dormant origin deficiency moderately accumulate at the G2/M phases of the cell cycle, constitutively activating DNA damage repair response (DDR) pathways at a low level [27,73,93]. In particular, $Mcm2^{Cre/Cre}$ and $Mcm4^{c3/c3}$ cells slightly upregulate the basal expression of p21 [27,93], a major downstream target of *Trp53* [94]. To understand the role of *Trp53*-mediated DDR in these cells, *Trp53* was in-activated in $Mcm2^{Cre/Cre}$ mice by mouse crossing. Resulting $Mcm2^{Cre/Cre}$ offspring lacking *Trp53* was recovered at a substantially reduced number (~20% of the expected by the Mendelian ratios), and rare surviving mice developed tumors with a significantly shorter tumor latency than those with *Trp53* deficiency alone [27]. Similarly, introduction of *Trp53* nullizygosity into $Mcm4^{c3/c3}$ mice also caused semi-synthetic lethality and synergistically accelerates tumorigenesis [93]. Together, these findings suggest that functional *Trp53* is required not only for tumor suppression but also the development of mice that suffer dormant origin deficiency. This function of *Trp53* may be partially mediated through p21, since its deficiency in $Mcm4^{c3/c3}$ mice slightly exacerbates tumor formation [78]. ATM is another central DDR protein, which is activated in response to the induction of double strand breaks as well as replication stress [95–98]. The lack of ATM causes ~60% of $Mcm4^{c3/c3}$ mice to die at late embryonic stages [78]. Surviving $Atm^{-/-}$;$Mcm4^{c3/c3}$ mice succumb to lymphomas at 2–4 months of age much like $Atm^{-/-}$ mice do [78,99,100], suggesting that dormant origin deficiency negatively impacts the development of $Atm^{-/-}$ embryos far more than tumorigenesis in adult $Atm^{-/-}$ mice. CHK2 is a downstream effector of ATM [101,102], but its deficiency does not increase the formation of spontaneous tumors in mice unlike *Atm* deficiency [103]. However, *Chk2* deficiency in $Mcm4^{c3/c3}$ female mice causes a slight but significant decrease in tumor latency with a stronger predisposition to mammary tumors than $Mcm4^{c3/c3}$ females that mainly develop histiocytic sarcomas in a mixed background involving B6 and C3H [78]. This observation is intriguing with respect to the fact that *CHEK2* is a human breast cancer susceptibility gene with moderate penetrance [104].

Fanconi anemia is a rare genetic disorder characterized with developmental defects, bone marrow failure, chromosome instability and highlighted cancer susceptibility [105–107]. To date, 21 genes have been identified to cause FA and the products of these genes are required for efficient repair of DNA inter-strand crosslinks (ICLs)[105–108]. The FA pathway is intrinsically activated in $Mcm4^{c3/c3}$ MEFs [28,68]. This finding is in line with an expanding number of studies supporting the role of FA proteins in maintaining genome stability under conditions of replication stress [105–108]. Even in normal S phase, basal activation of FA pathway is observed when the FA core complex (involving FANCA, −B, −C, −E, −F, −G, −L, and −M) mono-ubiquitinates FANCD2, promoting the latter's chromatin loading and focus formation [109,110]. A low dose of APH or HU causes a drastic increase in FANCD2 foci, which are preferentially found at common fragile sites during the G2/M phases [111–

114]. While the exact mechanism remains to be fully elucidated, the FA proteins are required for efficient suppression of gaps and breaks at common fragile sites in the presence of APH [111]. One major factor that accounts for the fragility of these loci is a paucity of replication origins, making them vulnerable to under-replication [56–58]. A reduction of dormant origins may increase chromosome loci with fewer origins, explaining a higher number of spontaneous FANCD2 foci in *Mcm4*$^{c3/c3}$ MEFs relative to wild-type [28,68]. This activation of the FA pathway is functionally significant, as its disruption exacerbates genome instability in *Mcm4*$^{c3/c3}$ cells [68]. In particular, a lack of the FA pathway leads to post-natal lethality of *Mcm4*$^{c3/c3}$ mice in the B6 background where a reduction of dormant origins unusually results in a significant decrease of active origin density in unperturbed S phase [68,93]. When bred in this sensitized background, *Mcm4*$^{c3/c3}$ and FA-deficient mice share common phenotypes including partial lethality and microphthalmia [93,115,116]. Therefore, the observed synthetic lethality suggests functional dependency between the FA pathway and dormant origins during mouse development.

A subset of FA proteins such as FANCD2 and its interacting partner FANCI display physical interactions with the MCM2-7 proteins and exert their regulatory roles in origin firing and fork progression independently of the FA pathway [117–119]. Upon replication stress, FANCD2 is required to impede replication fork speed possibly through its transient interaction with the MCM2-7 helicase [117,119]. Notably, this function of FANCD2 is independent of its mono-ubiquitination, whereas FANCI does not seem to have this role [117,119]. It has also been described that the absence of FANCD2 influences origin usage [118–121]. However, it is not clear how FANCD2 is involved in this process. A recent study unveiled FANCI as a regulator of dormant origin firing depending on the severity of replication stress [119]. Upon ICL or HU treatment, FANCI is phosphorylated by ATR followed by monoubiquitination via the FA core complex [119,122–124]. However, it is unmodified FANCI that supports dormant origin firing under conditions of mild replication stress that are less likely to trigger the activation of ATR [119]. In turn, as replication stress gets severe, ATR-mediated phosphorylation of FANCI negatively regulates its ability to fire dormant origins [119]. Further studies are required to better understand the newly discovered roles of FANCD2 and FANCI.

6. Dormant origins and stem/progenitor cells

In vivo analysis of *Mcm2*$^{Cre/Cre}$ mice has first revealed a role of dormant origins in maintaining stem/progenitor cell populations. In *Mcm2*$^{Cre/Cre}$ mice, stem cell numbers are greatly decreased in the sub-ventricular zone of the brain, small intestinal crypt and skeletal muscle with a modest increase of DNA damage relative to wild-type mice [74]. Similarly, neural stem/progenitor cells isolated from *Mcm4*$^{c3/c3}$ embryos show an upsurge of γH2AX and 53BP1 foci with accumulation at the G2/M phases, leading to a reduced ability to form neurospheres when cultured *in vitro* [125]. Consistently, *Mcm4*$^{c3/c3}$ mice display a defect in embryonic neurogenesis. In the developing brains of *Mcm4*$^{c3/c3}$ embryos, the renewal of stem cells appears normal, but the number of intermediate progenitor cells is significantly reduced due to an increase of apoptotic cells in the sub-ventricular and intermediate zones [125]. This ultimately stunts ventral forebrain growth and substantially reduces the viability of *Mcm4*$^{c3/c3}$ embryos [125]. These studies indicate that a full expression of MCM2-7

proteins is needed to support the proper functions of stem/progenitor cells by minimizing replication-associated genome instability. Intriguingly, aging hematopoietic stem cells even in wild-type mice suffer from replication stress, explaining their declined functionality [126]. This is because these old stem cells have reduced expression of MCM2-7 proteins relative to their younger counterparts, resulting in chromosome instability and cell cycle defects [126]. Supporting this finding, mice homozygous for a hypomorphic allele of *Mcm3* (*Mcm3*^{Lox}) were recently found to be late embryonic lethal due to fetal anemia [62]. The *Mcm3*^{Lox} allele was generated by modifying the endogenous *Mcm3* locus to contain loxP sites flanking exons 14–17 followed by the insertion of a luciferase reporter at its 3′-UTR. Similar to the *Mcm2*^{Cre} allele, this modification by itself results in lower expression of MCM3 in heterozygous and homozygous mice (~70% and ~30% of wild-type, respectively) [62]. Accordingly, *Mcm3*$^{Lox/Lox}$ MEFs are less able to increase active origin density upon treatment with a low dose of APH, exhibiting dormant origin deficiency. Interestingly, this reduction of MCM3 has no effect on other MCM protein levels, which may be related to the observation seen for *Mcm3*^{Gt} heterozygosity [75]. *Mcm3*$^{Lox/Lox}$ embryos suffer from impaired maturation of red blood cells in fetal liver which shows a wide-spread presence of DNA damage [62]. Declined functionality of hematopoietic stem cells in these embryos was also revealed by transplantation assays [62]. Embryonic lethality of *Mcm3*$^{Lox/Lox}$ mice can be partially rescued by CHK1 overexpression, supporting that intrinsic replication stress is the underlying cause of lethal anemia [62].

7. Human genetic diseases associated with origin licensing and firing

A rare human *MCM4* germline mutation results in growth retardation, adrenal insufficiency and classical natural killer (NK) cell deficiency in an autosomal recessive manner [127–129]. This mutant allele produces truncated forms of MCM4 with disruption in its N-terminal serine/threonine-rich domain [127,128]. Patient-derived cells show an altered cell cycle profile with increased ploidy even in untreated conditions, and they are sensitized to a low dose of APH [127]. It is not clear if dormant origin deficiency is involved in these cellular phenotypes, because patient-derived cell lines display no difference in the formation of MCM2-7 complex and its chromatin binding relative to control cell lines [127]. Of note, the N-terminal serine/threonine-rich domain of MCM4 is conserved in vertebrates [127], and several serine and threonine residues in this domain are phosphorylated by CDK, influencing biochemical properties of MCM2-7 helicase [130–132]. More recently, it was reported that over-expression of mutant MCM4 lacking this domain allows human cells to bypass CDC7 requirement for origin firing [119]. These findings suggest a regulatory role of this domain, possibly providing a clue to understand the symptoms of these patients [127].

Meier-Gorlin syndrome (MGS) is a rare disease characterized by the triad of short stature, small ears and absent/small patellae with variable expressivity [133–139]. MGS occurs in an autosomal recessive manner due to germline mutations in one of the five genes encoding replication licensing factors ORC1, ORC4, ORC6, CDT1, and CDC6 [133–135,137,140]. Moreover, MGS can also be caused in an auto-somal dominant manner, as three patients harbor *de novo* mutations in *GMNN* which stabilizes its product geminin, the inhibitor of the licensing factor CDT1 [141]. Expression of geminin is regulated to occur in a restricted time from the beginning of S-phase to late mitosis to avoid re-licensing of origins [142,143].

Its untimely presence in the G1 phase is expected to inhibit proper origin licensing. Surprisingly, MGS-causing genes are not restricted to those involved in regulating origin licensing but include *CDC45*, which encodes a component of the CMG helicase [144]. MGS with *CDC45* mutations is uniquely distinguished by the frequent presence of craniosynostosis [144]. Based on its function, partial loss-of-function mutations in *CDC45* are expected to impair origin firing even though patient cells are able to license a normal number of origins. Together with insights from functional studies on identified *ORC* mutations, it seems likely that impaired DNA replication by reduced origin usage is unable to support rapid cellular proliferation during development, primarily leading to primordial dwarfism of MGS patients [133,134,145–149]. Additional defects such as cilia formation and centrosome reduplication may also contribute to symptoms seen in the disease [150,151].

It was recently reported that mutations in *MCM5* are associated with Meier-Gorlin syndrome (MGS) [152]. The etiology of NK cell deficiency was also linked with mutations in *GINS1*, which encodes a subunit of CMG helicase [153]. More interestingly, a missense variant in *MCM2* has been described to be associated with a dominant disorder characterized by progressive hearing loss [154]. Future discoveries will help us fully understand the basis for the respective pathogenesis of these different diseases.

8. Future perspectives

Dormant origins are now recognized as a major safeguard against under-replication of the genome. Firing of dormant origins plays a central role in the rescue of stalled forks, contributing to faithful DNA replication. Despite their significance, the functional interplay between dormant origins and other mechanisms (such as translesion synthesis and homology-mediated fork restart) is largely unknown. Moreover, pathway choice between dormant origins and these mechanisms in response to different types of fork-stalling lesions also remains to be investigated. Most intriguingly, a reduction of dormant origins by itself induces mitotic DNA synthesis in human and mouse cells. Elucidation of the underlying mechanism(s) will provide key information to better understand the functions of dormant origins in DNA replication, stem cell maintenance, and suppression of diseases.

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Abbreviations

HU	hydroxyurea
APH	aphidicolin
MN	micronuclei
53BP1-NBs	53BP1-nuclear bodies
MEFs	mouse embryonic fibroblasts
B6	C57BL/6
C3H	C3HeB/
CNAs	copy number alterations
ICLs	inter-strand crosslinks
DDR	DNA damage repair/response
FA	Fanconi anemia
MGS	Meier-Gorlin syndrome

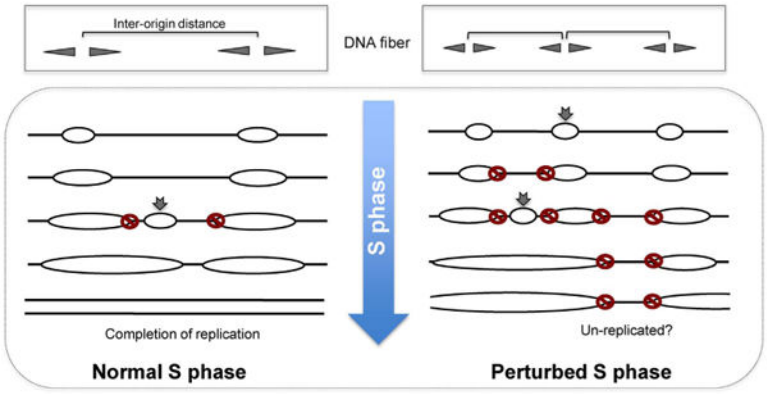

Fig. 1.

Dormant origin firing contributes to the completion of DNA replication. The top section displays expected DNA fiber images in both normal and perturbed S phase in which a pair of triangles represents DNA synthesis by bi-directional replication forks from fired origins at the beginning of S phase in the bottom section. Note that slower fork progression activates a greater number of dormant origins in perturbed S phase, which would result in shorter inter-origin distances in DNA fiber experiments relative to normal S phase. In the bottom section, each bubble on the line represents origin firing that generates replication forks moving bi-directionally as S phase progresses. Dormant origin firing (indicated by grey arrows) rescues stalled forks both in normal (left) and perturbed S phase (right). However, cells may still experience under-replication due to an increased incidence of stalled forks in perturbed S phase with replication stress.

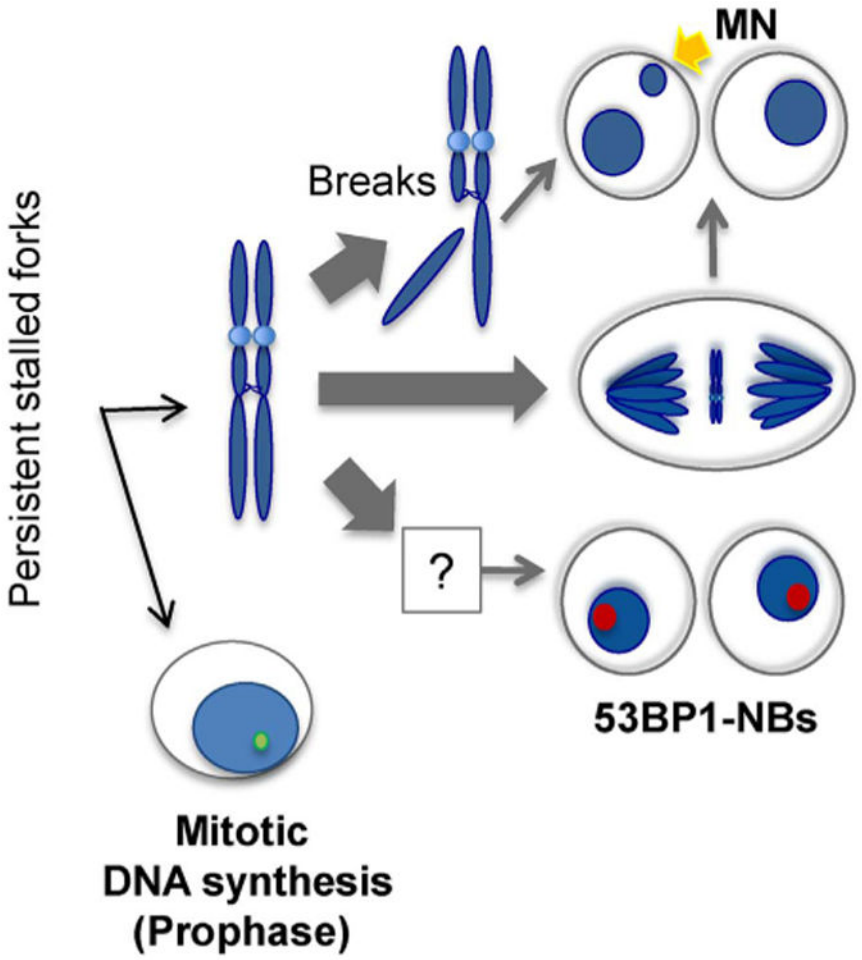

Fig. 2.

Multiple consequences of unresolved stalled replication forks. Dormant origin deficiency increases the quantity of stalled forks persistent into M phase, as illustrated in sister chromatids containing under-replicated DNA. As a cell progresses through M phase, the sister chromatids may form acentric fragments or lagging chromosomes during anaphase. If these fail to be incorporated into main nuclei, the formation of micronuclei (MN, shown by yellow arrow) occurs in daughter cells. Alternatively, unresolved stalled forks may also generate 53BP1-NBs in G1 daughter nuclei (shown in red) via yet unknown mechanisms. When formed in both daughter nuclei, 53BP1-NBs often display a symmetrical appearance. Persistent stalled forks may be resolved by mitotic repair synthesis that occurs in prophase (shown as a small green dot). Note that mitotic repair synthesis in prophase rarely results in the formation of MN or 53BP1-NBs [71].