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Regulation of the initiation of DNA replication in human cells.

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Abstract

The origin of species would not have been possible without high fidelity DNA replication and complex genomes evolved with mechanisms that control the initiation of DNA replication at multiple origins on multiple chromosomes such that the genome is duplicated once and only once. The mechanisms that control the assembly and activation of the replicative helicase and the initiation of DNA replication in yeast and *Xenopus* egg extract systems have been identified and reviewed [1,2]. The goal of this review is to organize currently available data on the mechanisms that control the initiation of DNA replication in human cells.

Preface for a general audience.

DNA replication is targeted by chemotherapeutic drugs as cancer cells generally proliferate faster than most normal cells, and most of them have acquired mutations that inactivate the mechanisms that ensure genome stability. A precise understanding of the mechanisms that initiate DNA replication will allow the rational design of clinical trials of new agents and combinations that target DNA replication. At this time our understanding of the mechanisms that initiate DNA replication is largely derived from experiments performed in highly tractable yeast and *Xenopus* egg extract systems. The objective of this review is to summarize what is currently known about the initiation of DNA replication in human cells.

Complexity and timing of the initiation of DNA replication.

The human genome evolved with mechanisms that assemble and activate the replicative helicase to initiate DNA unwinding and replication at $\sim 10^4$ origins (indirect estimations showed $\sim 22,000$ in HeLa [3], $\sim 50,000$ in HeLa Kyoto [4]). The MCM2-7 hexamer is loaded onto DNA to license origins in G1 phase and Cdc7 (working in complex with Dbf4 and also

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known and DDK for Dbf4-dependent kinase) and Cdk2 kinase activities in S phase initiate the assembly of CDC45, MCM2-7 and GINS and activation of the replicative helicase CMG [5]. The spatiotemporal pattern of replication foci identified by replisome components and pulses of labelled nucleotides is consistent from one cell cycle to the next and identifies regions of the genome that replicate in early, middle and late S phase [3, 6].

Origins of replication in human cells have been mapped only partially [7-9], and it appears that sets of active origins may be different for different human cell lines [8]. The ~50,000 origins that replicate the human genome are selected from a large excess of “licensed” origins (origins that have loaded MCM helicases). Quantitative studies identified ~ 10 fold excess of chromatin-loaded MCM complexes over active origins in Xenopus egg extract [10] and ~20-50 fold in budding yeast [11]. However, there are few estimations for human cells. Bukhari et al. [12] in their classic widely cited study demonstrated that about 10-20% of 10^6 MCM complexes in HeLa cells were tightly bound to DNA, making it 2-4 fold excess over active origins. It is worth noting that this study was performed on asynchronous cells by quantifying western blots after cell fractionation, and therefore may not be very accurate. Another study by Ibarra et al. [13] showed that knocking down MCM subunits to 5-10% of their normal level was enough for cell survival, which indirectly implies a 10-20 fold excess. Further investigation is required to determine the exact excess of loaded MCM over the number of active origins in human cells. This excess is different in different human cell types and is expected to define their sensitivity to replication stress.

Activation of additional replicative helicases at origins that would otherwise be passively replicated is observed after stress [14]. This plasticity in origin use is a simple mechanism to recover DNA replication between stalled and collapsed replication forks. Origin use is also affected by oncogene expression [15].

The mechanisms that determine the spatiotemporal sequence of origin firing and identify which origins fire in any given region of the genome are not clear. Several models attempt to describe the observed replication dynamics.

A stochastic model of origin firing is supported by computational modeling [16-18]. The most recent model assumes that origins in euchromatin and facultative heterochromatin regions fire stochastically with a domino-like progression that places later firing origins in close proximity to recently fired origins. This model requires an unidentified mechanism that inhibits origin firing at a distance of 7 to 120 kbp around the replication fork, a distance that corresponds to the chromatin loops [16]. This model was able to accurately predict the observed spatiotemporal pattern of replication foci, leaving the underlying mechanism(s) to be elucidated.

An MCM loading efficiency-dependent model of origin firing is supported by observations made in yeast [19], where a higher density of MCM loading on chromatin correlates with earlier origin firing [20]. It is proposed that the number of MCM complexes loaded per origin defines its probability of firing [21]. This model doesn't contradict the stochastic model, but rather defines the starting point for the domino-like progression, suggested by

computational modelling. To date, there is no evidence that a higher density of MCM loading on chromatin correlates with earlier origin firing in human systems.

Chromatin structure has been shown to be a major factor regulating the initiation of DNA replication. Rif1 (Rap 1-interacting-factor-1) has been identified as a critical component of replication timing regulation in human cells [22]. Rif1 has been shown to regulate chromatin loop structures and to be co-localized with mid-S-phase replication foci [22]. Rif1 depletion causes increased Cdc7 activity and increased DNA synthesis in early S-phase as well as major changes in replication timing domains [22]. Since Rif1 defines chromatin loop structures, it is likely a component of the mechanism that inhibits origin firing across a loop, that is predicted by the stochastic computational model, possibly through the regulation of Cdc7 activity.

Topologically associating domains (TADs) are conserved 3D domains within the genome that were identified by studying 3D chromatin organization using Hi-C approach [23]. Recently TADs have been shown to correlate with replication domains [24]. Depending on the cell line, a TAD may be an early or late replicating unit, but the replication timing tends to be the same for all the parts of the domain: it depends on domain's association with lamina and transcriptional repression/chromatin state. TADs and their association with replication timing were demonstrated in yeast as well [25].

Epigenetic regulation is an important factor determining the initiation of DNA replication. While some histone modification marks are common for all active origins, others are specific for a certain subset of replication initiation sites. For example, it has been shown that the activation of origins near transcription start sites require H3K14 acetylation by BRPF3-HBO1 [26]. Recently, H4K20 tri-methylation in heterochromatin has been shown to be critical for the replication timing program [27]. Another histone modification that controls the replication timing program is acetylation. One of the critical origin firing factors – treslin/TICRR – has been shown to directly interact with the acetyl-histone binding proteins BRD2 and BRD4. Disruption of treslin recruitment to acetylated chromatin results in abnormal or mixed replication patterns [28].

Some proteins that regulate origin firing by unknown mechanisms have been identified. RepID was shown to bind a subset of replication origins in human cells and be crucial for their activation [29]. The authors suggest that RepID may be a member of a family of proteins that regulate different subsets of origins in mammalian cells. SIRT1 phosphorylated at T530 was shown to prevent excessive origin firing [30]. SIRT1 is a deacetylase that may prevent the initiation of DNA replication by removing the acetylation marks from the surrounding chromatin. ATR kinase activity is also implicated in the regulation of DNA replication in the absence of stress by recent observations by our group and others that ATR kinase inhibition induces origin firing [31-33].

Replication complex assembly

The assembly of the replisome in human cells and all eukaryotic systems begins with origin licensing in the G1 phase of the cell cycle (Figure 1a).

MCM loading on chromatin in G1 phase human cells, just like yeast, requires ORC (Origin Recognition Complex)[34, 35], Cdt1 [36], and Cdc6 [37] and these proteins need to be tightly regulated to prevent re-loading of the replicative helicase and re-replication in S phase [38]. However, the mechanisms preventing re-replication are quite different between species. While yeast ORC complex remains bound to replication origins throughout the cell cycle, in mammalian cells ORC1 subunit is selectively degraded starting at the onset of S-phase[39], and Cdk1/Cyclin A-dependent phosphorylation prevents its binding to chromatin until mitosis is complete[40]. While yeast Cdc6 is rapidly degraded in S-phase, its human counterpart is very stable and exported from the nucleus in a Cdk2 phosphorylation-dependent manner [41-43]. Cdt1 is quickly degraded at the onset of S-phase in human cells [44], partial conservation of this mechanism is reviewed in [45]. In human cells [46] and *Xenopus* egg extracts [47] Cdt1 can also be inhibited by binding to an inhibitor protein - geminin. Geminin is absent in yeast.

MCM loading on chromatin requires additional proteins in human cells:

- HBO1 (human acetylase binding to ORC1; also known as KAT7 and MYST2) has been shown to interact with ORC, Cdt1 and MCM and possess specific H4-acetyltransferase activity that is critical for MCM chromatin loading [48].
- SNF2H (sucrose nonfermenting 2 homolog) is a non-essential chromatin remodeler that functions in MCM loading downstream of Cdt1 [49].
- GRWD1 (glutamate-rich WD40 repeat containing 1) protein was identified as a Cdt1 interacting partner and was subsequently shown to function in MCM loading, presumably through histone binding and chromatin remodeling [50].
- WDR18 (WD repeat domain 18) or hIPI3 (human homolog of yeast Ipi3p) was shown to interact with ORC and MCM subunits and to function in MCM loading [51].

Three of these additional proteins are chromatin remodelers, suggesting that histone modification is important for MCM loading in humans.

While ORC marks replication origins and directly participates in MCM loading on DNA, it appears that MCM and ORC do not co-localize [52]. MCM helicases seem to not localize to replication foci in the live cell imaging experiments either [53]. These observations together with the excessive loading of MCM on chromatin, discussed above, constitute the MCM paradox reviewed in [54].

Origin licensing in G1 phase is followed by the assembly of the replicative helicase, CMG, through the recruitment of CDC45 and GINS to the loaded MCM (Figure 1b).

CDC45 is an essential component of the CMG helicase that facilitates RPA loading on ssDNA in human cells [55]. CDC45 appears to be rate-limiting for the initiation of DNA replication in human cells: overexpression of CDC45 causes increased origin firing and ssDNA accumulation [56]. However, there are complex mechanisms regulating CDC45 recruitment to MCM.

It has been clearly established that in yeast Cdc45 loading requires Sld3, Sld7 proteins and DDK kinase activity [57]. Treslin (main candidate for the role of Sld3 homolog in human cells) and TopBP1 were demonstrated to interact with Cdc45 [58, 59] and be required for its recruitment to chromatin in human cells [59, 60]. Treslin was also shown to be phosphorylated by cyclin A/Cdk2 and this promoted the treslin-TopBP1 interaction and CDC45 loading [58, 61]. It has been demonstrated that MTBP (MDM2 binding protein) forms a complex with treslin and TopBP1 and that MTBP functions in CMG assembly in human cells [62].

In contrast, bimolecular fluorescence complementation (BiFC) analyses revealed that TopBP1 is dispensable for CMG formation in human cells [63]. This observation contradicts both the data described above and the finding that TopBP1-deficient cells had lower levels of Cdk2 activity, impaired loading of replication components on chromatin, and failed to enter S-phase [64], so further investigation is required to resolve this contradiction.

More recently two more players in Cdc45 loading were identified in *Xenopus*: DUE-B (DNA unwinding element-binding protein) [65] and GEMC1 [66]. DUE-B is another candidate for the functional role of yeast Sld3 in human cells, and its C-terminal phosphorylation by Cdc7 (that can be reversed by PP2A) was shown to be important for Cdc45 chromatin association in human cells [67]. While a human homolog of *Xenopus* GEMC1 has been identified [68], there's no evidence that it plays a role in the initiation of DNA replication in human cells.

Thus, the proteins that are likely required for CDC45 loading in humans are Cdk2-phosphorylated treslin, Cdc7-phosphorylated DUE-B, MTBP and TopBP1.

It has been shown that CDC45 loading at origins near transcription start sites in human cells requires H3K14 acetylation by BRPF3-HBO1 [26]. A recent study [69] demonstrated that demethylation of histone H3K9 by Kdm4d was critical for CDC45 recruitment while it does not affect origin licensing step. This observation suggests that one of the players in the "CDC45 recruitment team" described above is regulated by this chromatin modification. Another study showed that treslin interacts with acetylated chromatin [28]. This may suggest that CMG assembly is regulated by histone acetylation through the recruitment of treslin to facilitate CDC45 incorporation.

The GINS complex is an essential component of CMG helicase [70], but little is known about the mechanism(s) underlying the recruitment of GINS to MCM/CDC45 in human cells. DNA polymerase epsilon (pol ϵ) is required to recruit GINS to MCM helicase in yeast [71], but there is no evidence that this is the case in human cells. It has also been shown that the binding of GINS and CDC45 to CMG may be facilitative, that is the binding of GINS promotes the binding of CDC45, and vice versa [63]. The recruitment of GINS to CMG is one of the least studied aspects of the initiation of DNA replication in human cells.

Additional factors demonstrated to be important for origin firing in human cells are MCM10, RECQ4 and And-1 (Ctf4 in yeast) (Figure 1d). MCM10, RecQ4, and And-1 form a complex in a Cdc7- and Cdk2-dependent manner, and the recruitment of this complex to CMG helicase is an essential step in origin firing in human cells [72]. MCM10 also interacts

with ssDNA [73], MCM2-7 [74] and CDC45 (in a DNA-dependent manner) [75], while And-1 has been shown to form a stable complex with DNA polymerase alpha [76] and interact with GINS in a manner dependent on Sld5 phosphorylation by Cdc7, but independent of DNA [33]. These data indicate that RECQ4-MCM10-And-1-polymerase alpha complex is recruited to the CMG helicase through the Cdc7-dependent interaction between And-1 and GINS, and that this is facilitated by MCM10 binding to the MCM hexamer. A DNA-dependent interaction between MCM10 and CDC45 stabilizes the complex once the helicase is activated and ssDNA is available.

The recruitment of the main replicative DNA polymerases - delta and epsilon - to origins is not well studied in human cells. While in yeast polymerase epsilon is recruited as a part of a well-characterized complex with GINS and other proteins [71], no such complex has been described in human cells. Interactions between the human GINS complex and three DNA polymerases – alpha, delta and epsilon – has been demonstrated using purified proteins [77] and in human cells [74], as in *Xenopus* [78], polymerase epsilon was shown to be loaded after Cdc45, but before polymerase alpha. The molecular mechanism underlying the recruitment is not known.

Timeless (TIM) is a homolog of the fruit fly gene responsible for the circadian clock system [79]. TIM and its interacting partner TIPIN have been shown to be essential for DNA replication in humans [80] and their yeast homologs Tof1 and Csm3 are required for maximum processivity in yeast [81]. While both yeast [82] and human [83] TIM-TIPIN complexes have been shown to travel with the replication fork, no molecular mechanism for their recruitment has been identified. A recent study [84] indicates that TIM directly interacts with the MCM helicase before it is loaded on chromatin, and that disruption of TIM leads to MCM loading on chromatin and CMG assembly outside of S-phase as well as delayed initiation of DNA replication. These data suggest that TIM-TIPIN are recruited to the replication complex together with the MCM complex at the time of origin licensing in G1 phase and while they are not essential for MCM loading, they are required for timely and efficient replication initiation.

The signaling required for the initiation of DNA replication

The kinases Cdc7 [85, 86] and Cdk2 [72] play critical roles in the assembly and activation of pre-replication complexes in human cells (figure 1c) and as such their regulation determines the initiation of DNA replication. CMG helicase activation in yeast and human cells requires MCM phosphorylation by Cdc7/DDK. Dbf4-dependent kinase (DDK) (Figure 2c, d) is a complex of Cdc7 and either Dbf4 or Dbf4b (Drf1) [87]. Dbf4 or Dbf4b is required for maximum Cdc7 kinase activity and substrate recognition. In *Xenopus*, Dbf4b is proposed to play a critical role in the initiation of DNA replication that is more important than that of the canonical Dbf4 [88]. Human Dbf4b, unlike its *Xenopus* homolog, doesn't bind chromatin, but appears to play an important role in the S and G2/M phases of the cell cycle [89, 90].

It has been demonstrated that Treslin stimulates MCM2 phosphorylation by Cdc7 and that this may contribute to MCM gate opening [91]. In addition to Treslin, at least two more proteins appear to be important for Cdc7 recruitment to CMG in human cells: both Claspin

[26] and Cdt1 [92] have been shown to directly interact with Cdc7 and recruit it to CMG helicase. Recent data [33] indicate that efficient MCM4 phosphorylation by Cdc7 requires the presence of And-1, however there's no evidence of a direct interaction between And-1 and either Cdc7 or Dbf4. And-1 has been demonstrated to interact with Claspin [93], so one possible explanation is that And-1 is required to recruit Claspin, which in turn brings Cdc7 to the replication origins. However, there's a controversy in Cdc7 activity being crucial for the recruitment of And-1 [72]. One possible explanation would be that both Claspin and Cdc7 interact with RecQ4-MCM10-And-1-polymerase alpha complex and Cdc7 phosphorylates its components before the recruitment resulting in stronger affinity for the CMG, however more research is required to investigate this process as it is inconsistent with Cdc7-dependent phosphorylation of DUE-B at the CDC45 recruitment step.

Cdk2 (Figure 2a, b) is required for both the assembly and activation of CMG and RecQ4-MCM10-And-1 recruitment during the initiation of DNA replication (discussed above). Cdk2 is a cyclin dependent kinase that is associated with either Cyclin E (in G1/S transition) or Cyclin A (during S-phase) [94] and the active kinase is phosphorylated on Thr160 and dephosphorylated on Tyr15 [95]. Cdk2 Tyr15 is phosphorylated by Wee1 kinase and dephosphorylated by Cdc25A phosphatase. This dephosphorylation appears to be the critical step in Cdk2 activation [96]. Cdk2 is an abundant protein in human cell (approximately 8.8×10^5 Cdk2 copies per HeLa cell compared to 2.1×10^4 copies of Cdc7 [97]) and with increased levels of cyclins during G1/S phase Cdk2 activity is unlikely to be rate-limiting for the initiation of DNA replication. Surprisingly, Cdk2 knockout mice are viable [98]. Cdk1 appears to be able to substitute for the absent Cdk2, in most of its functions, however, a study with analog sensitive Cdk2 mutant [99] showed an essential and non-redundant role for Cdk2 in G1/S transition, that includes outcompeting Cdk1 for binding to cyclins.

Recent observations that localized PP1 phosphatase activity is required for the spatiotemporal sequence of origin firing [100] suggests that the assembly and activation of CMG and the replisome is controlled by the gradients of kinase and phosphatase activities across the nucleus. While Cdc7 is localized to origins in a Claspin- or Cdt1-dependent manner, the opposing activities of PP1 family phosphatases, which removes Cdc7-dependent phosphorylations, is localized to origins though an interaction with Rif1 [100, 101]. The stochastic model of origin firing discussed above requires a unidentified mechanism that inhibits origin firing at a specific distance (7 to 120 kbp) around replication fork, a distance that corresponds to the chromatin loop size [16]. Since Rif1 is the main structural determinant of chromatin loops, the following model seems feasible: Rif1 recruits PP1 family phosphatases to the actively replicating loops to repress the local origin firing. While many questions remain, including the mechanism of PP1 recruitment to replicating but not replication loops that have been replicated or are yet to replicate, the Rif1-PP1 interaction may be the unidentified mechanism that inhibits origin firing at a specific distance predicted by the functional modeling of replication.

Regulation of replication initiation after DNA damage

Key elements of the DNA damage response include cell cycle arrest and inhibition of the initiation of DNA replication (Figure 3). The regulation of DNA replication after DNA

damage is a very complex process. While mild replication stress activates the initiation of replication from dormant origins, higher levels of damage completely block origin firing. Current models for this phenomenon suggests that low levels of ATR/Chk1 activity block the activation of replication in the new “replication factories” while allowing dormant origins to fire within active factories [102]. The main target of the replication checkpoint is Cdk2 kinase. Cdc25A phosphatase, that is critical for Cdk2 dephosphorylation and activation, is a known target of Chk1 and Chk2 kinases. Cdc25A is phosphorylated by Chk2 [103] and/or Chk1 [104] at serines 123, 178, 278, and 292 in response to DNA damage, and these phosphorylations lead to Cdc25A ubiquitylation and proteasomal degradation. In the absence of Cdc25A, Cdk2 remains in its Y15 phosphorylated inactive form and is unable to facilitate replication initiation.

Another pathway that contributes to Cdk2 inactivation after DNA damage is p53-dependent. The tumor suppressor protein p53 is stabilized and activated in response to DNA damage in ATM/ATR and Chk1/Chk2 dependent manner. Active p53 acts as a transcription factor, inducing the expression of multiple DNA damage response genes which impact cell cycle arrest, senescence and apoptosis. One of these genes is the Cdk inhibitor p21 which directly interacts with and inhibits Cdk2 kinase activity [105] which blocks its ability to initiate DNA replication. Cdk2 can also be inhibited by p27 [106], but there is no evidence to suggest that p27 is regulated in response to replication stress.

While the Chk1/2-Cdc25A-Cdk2 axis signaling is considered the main mechanism that blocks the initiation of DNA replication after DNA damage, additional pathways have emerged. It has been demonstrated that in response to the replication stress induced by benzo[a]pyrene dihydrodiol epoxide (BPDE) the initiation of DNA replication is blocked in the absence of any noticeable Cdc25A degradation. In this instance, the block of replication initiation was found to be due to Chk1-dependent inhibition of CDC45 recruitment to licensed origins [107]. One possible explanation of this phenomenon is that Chk1-dependent phosphorylation of Treslin in response to replication stress disrupts the interaction of Treslin with TopBP1 that is known to be critical for CDC45 recruitment to CMG from yeast to *Xenopus* and human [61, 108].

Many studies have investigated the regulation of Cdc7-Dbf4 activity in response to DNA damage, but the results are largely inconclusive. While in yeast Rad53-dependent phosphorylation of Dbf4 [109] has been demonstrated to contribute to the replication checkpoint, ATR-dependent regulation of DDK activity in human cells and *Xenopus* cell free extract remains highly controversial [110]. Some studies claim that the DDK complex is dissociated, and Cdc7 activity is decreased in response to replication checkpoint signaling induced by etoposide in *Xenopus* cell-free extracts [60], and even in human cells [111], while others detect no effect of ATR/Chk1 activation on Cdc7-Dbf4 interaction in human cells after UVC irradiation [112], and no reduction in Cdc7 activity after treatments with hydroxyurea or etoposide [113]. However, Dbf4 overexpression appeared to abrogate the UVC-induced (but not IR-induced) replication checkpoint [112]. These overexpression experiments are hard to interpret as Dbf4 appears to be one of the limiting factors in the regulation of the initiation of DNA replication that is stabilized at the G1/S transition [114], so its overexpression may have affected the cell cycle and DNA synthesis rate in general.

Dbf4 has been demonstrated to be a direct substrate of both ATM/ATR [110] and Chk1 [112] kinases in human cells, and mutant unphosphorylatable Dbf4 was able to partially suppress IR-induced replication arrest [110]. However the role of ATM/ATR-dependent phosphorylations on Dbf4 in inhibiting the initiation of DNA replication after DNA damage is not clear, as they do not affect DDK activity or stability, and appear to only prevent rereplication [110]. One study suggests that DDK activity during replication stress acts upstream of ATR/Chk1 signaling as it is shown that Dbf4 overexpression inhibits replication checkpoint in both human and *Xenopus* systems [115].

The variability in the type of damage as well as the DNA synthesis assays used to study the impact of DDK on the replication checkpoint and the frequent use of Dbf4 overexpression makes it very difficult to compare and interpret different studies. Further investigation is needed to establish the role of the ATR-DDK axis in the replication checkpoint. With recent observations that the PP1 family of phosphatases contribute to reversing Cdc7-dependent phosphorylations at dormant origins [100, 101], regulation of these phosphatases in response to DNA damage should also be investigated.

Conclusions and future directions

The initiation of DNA replication in humans is an extremely complex process, and caution is necessary while attempting to translate findings from relatively simple yeast and *Xenopus* extract systems into mammalian cells. Major gaps in knowledge about origin firing in human cells include the mechanism through which the GINS complex and DNA polymerase epsilon are recruited to the MCM helicase and mechanisms that regulate DDK after DNA damage. Also, while the requirement for Cdk2 and Cdc7 activities for certain steps in replication initiation is clear, the targets of these kinases and the functions of the phosphorylated targets are yet to be elucidated in detail. It is very likely that kinase-phosphatase gradients play critical roles in the regulation of the initiation of DNA replication, and that balancing Cdc7- Cdk2- Chk1 and ATR-dependent phosphorylation systems determines the spatiotemporal pattern of origin firing that is conserved from one cell division to the next. Identifying the mechanisms that underlie these gradients is of fundamental and translational interest.

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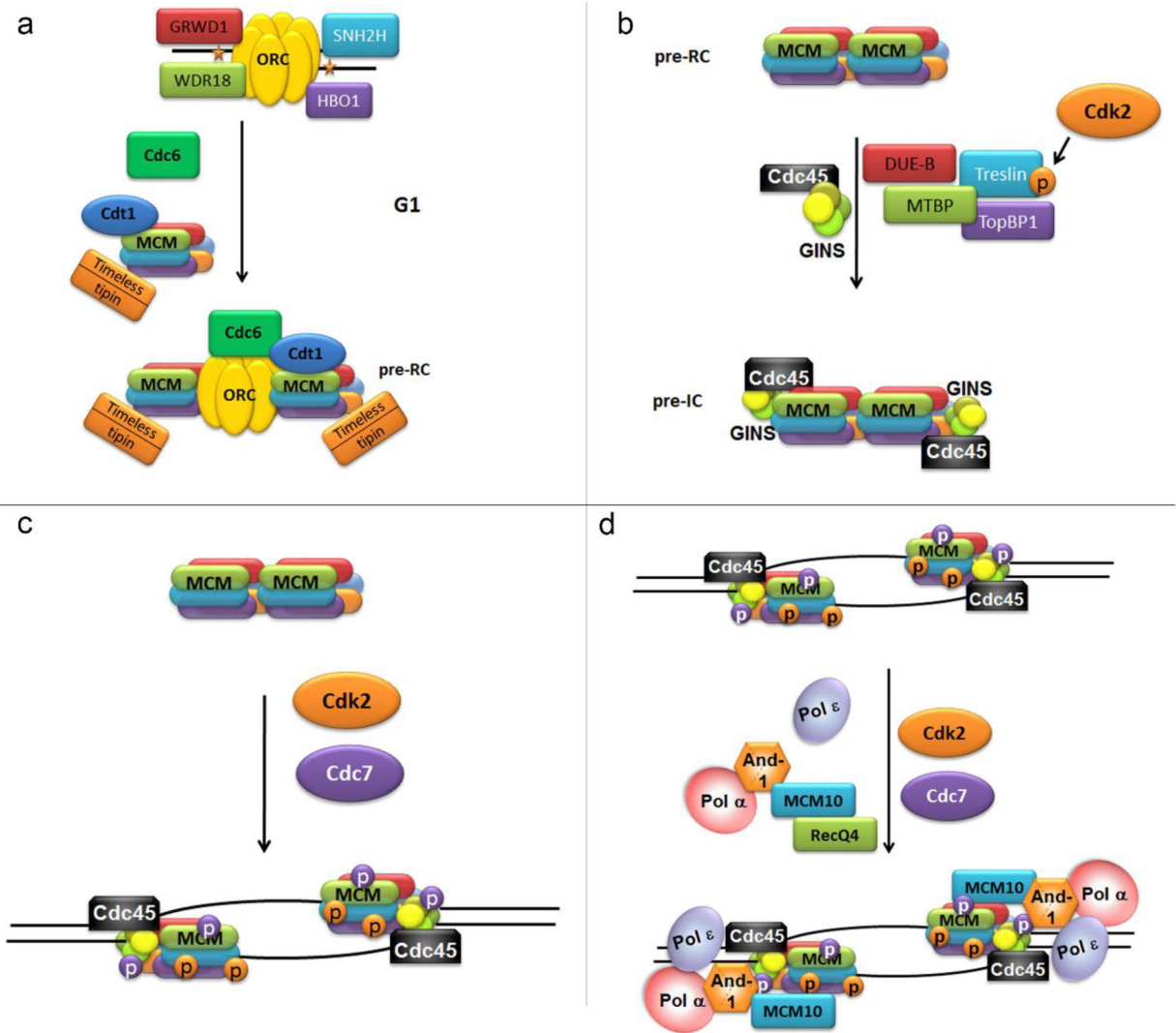


Figure 1. Replication initiation in human cells. 1a. MCM loading; 1b. CMG assembly; 1c. CMG activation. 1d. Full replication complex

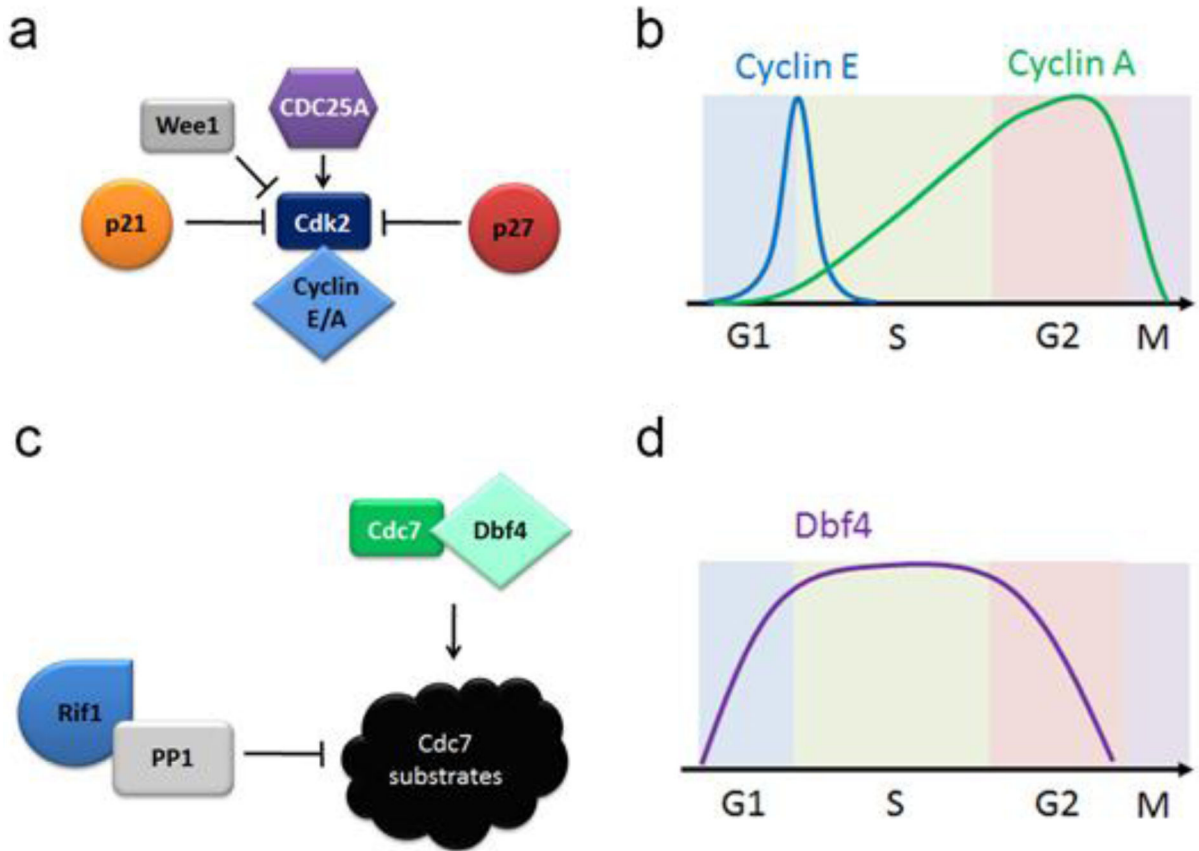


Figure 2. Replication initiation signaling. 2a. Cdk2 regulation; 2b. Cyclins' regulation. 2c. Cdc7 regulation. 2d. Dbf4 regulation.

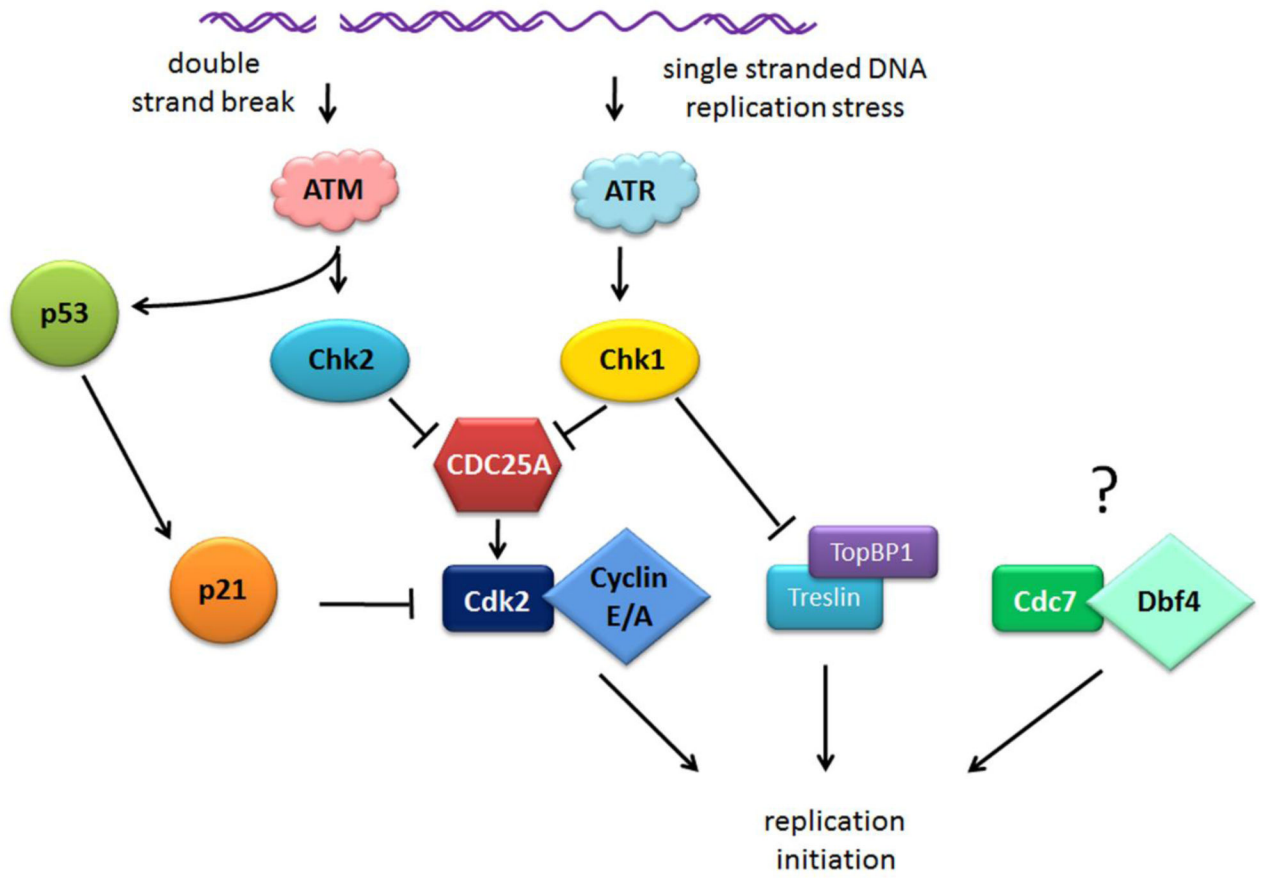


Figure 3.
Regulation of DNA replication initiation after DNA damage

Table 1:

Key players of replication initiation in various organisms:

| Step | <i>S. cerevisiae</i> | <i>Xenopus laevis</i> | <i>Homo sapiens</i> |
|---------------------------|--|---|---|
| MCM loading | MCM2-7; Cdt1; Cdc6; ORC1-6 | MCM2-7; Cdt1; Cdc6; ORC1-6 | MCM2-7; Cdt1; Cdc6; ORC1-6; HBO1; SNF2H; GRWD1; WDR18 |
| CMG assembly | Cdc45; GINS 1-4; Sld3; Sld7; polymerase epsilon; CDK; DDK, | Cdc45; GINS 1-4; Treslin; DUE-B; MTBP; TopBP1; GEMC1; Cdk2; DDK | CDC45; GINS 1-4; Treslin; DUE-B; MTBP; TopBP1; Cdk2; DDK |
| CMG activation | DDK | DDK | DDK |
| Replication initiation | Ctf4, MCM10, polymerase alpha | Ctf4, MCM10, polymerase alpha | And-1, MCM10, RecQ4, polymerase alpha, DDK, Cdk2 |

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Table 2.

Modifications, required for origin firing in various organisms (X – unidentified substrate)

| Step | <i>S. cerevisiae</i> | <i>Xenopus laevis</i> | <i>Homo sapiens</i> |
|------------------------|---|-----------------------|---|
| MCM loading | | | Acetyl-H3K14 Acetyl-H4 |
| CMG assembly | Phospho-S1d3 (CDK) Phospho-MCM (DDK) | Phospho-Treslin (CDK) | Phospho-Treslin (Cdk2) Phospho-Due-B (DDK) |
| CMG activation | Phospho-MCM (DDK) | Phospho-MCM (DDK) | Phospho-MCM (DDK) |
| Replication initiation | | Phospho-MCM10(CDK) | Phospho-GIN S4(DDK) Phospho-X (Cdk2) |

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