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THE FORK AND THE KINASE: A DNA REPLICATION TALE FROM A CHK1 PERSPECTIVE

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Abstract

Replication fork progression is being continuously hampered by exogenously introduced and naturally occurring DNA lesions and other physical obstacles. The checkpoint kinase 1 (Chk1) is activated at replication forks that encounter damaged-DNA. Chk1 inhibits the initiation of new replication factories and stimulates the firing of dormant origins (those in the vicinity of stalled forks). Chk1 also avoids fork collapse into DSBs (double strand breaks) and promotes fork elongation. At the molecular level, the current model considers stalled forks as the site of Chk1 activation and the nucleoplasm as the location where Chk1 phosphorylates target proteins. This model certainly serves to explain how Chk1 modulates origin firing, but how Chk1 controls the fate of stalled forks is less clear. Interestingly, recent reports demonstrating that Chk1 phosphorylates chromatin-bound proteins and even holds kinase-independent functions might shed light on how Chk1 contributes to the elongation of damaged DNA. Such findings unveil a puzzling connection between Chk1 and DNA-lesion bypass, which might be central to promoting fork elongation and checkpoint attenuation. In summary, the multifaceted and versatile functions of Chk1 at ongoing forks and replication origins determine the extent and quality of the cellular response to replication stress.

Keywords

Checkpoint Kinase 1 (Chk1); DNA replication; Translesion Synthesis (TLS); DNA damage

1. CHECKPOINT SIGNALS DURING THE S PHASE AND THE MAINTENANCE OF GENOMIC STABILITY

Cell cycle checkpoints constitute key signaling networks that counteract the continuous threats that both internal and external factors pose to DNA. Checkpoints primary function is

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to inhibit cell cycle progression before entry into S phase (G1/S checkpoint), throughout S phase (S-phase checkpoint), before mitotic entry (G2/M checkpoint) or before entry into anaphase (mitotic spindle checkpoint) (Jackson and Bartek, 2009). By controlling the start and/or progression of DNA replication, the S-phase checkpoint creates a time window to repair damaged DNA. In case of excessive or persistent DNA damage, checkpoint signals may also trigger apoptosis to avoid the propagation of aberrant genomes (Roos and Kaina, 2013). Therefore, checkpoint signaling contributes to the maintenance of genome integrity and avoids the development of diseases associated with genomic instability, such as cancer.

This review focuses on Checkpoint kinase 1 (Chk1), a conserved serine/threonine protein kinase with a pivotal role in the S-phase checkpoint. Importantly, Chk1 regulates S phase progression not only after genotoxic stress, when DNA damage increases, but also during unperturbed replication (in the absence of exogenous damage). As we will discuss herein, different lines of evidence indicate that Chk1 regulates replication initiation (Ge and Blow, 2010; Maya-Mendoza et al., 2007; Petermann et al., 2010), stabilizes replication forks (Smith-Roe et al., 2013; Syljuasen et al., 2005) and promotes lesion bypass (Speroni et al., 2012; Yamada et al., 2013; Yang et al., 2008). These Chk1-mediated mechanisms might prevent the collapse of ongoing forks and promote the proper resumption of DNA synthesis when the stalling signal is removed. Although not discussed in this review, Chk1 function exceeds the control of DNA synthesis. Particularly, solid evidence shows that Chk1 fulfils prominent roles in the G2/M and mitotic spindle checkpoints and in apoptotic signaling (Lam et al., 2004; Myers et al., 2009; Sidi et al., 2008; Zachos et al., 2007).

To analyze the contribution of Chk1 to DNA replication, we divided this review in 5 sections, including this one. The two following sections will concentrate on the molecular signals triggering Chk1 activation and modulating its localization; the subsequent one will focus on the function of Chk1 during DNA replication; and in the last section we will discuss how checkpoint signaling is attenuated, laying special emphasis on the molecular events that might allow forks “in check” to restart DNA replication.

2. STRUCTURES AT THE REPLICATION FORK THAT ACTIVATE CHK1

In eukaryotic cells DNA replication starts at multiple sites called replication origins. Each origin initiates a pair of replication forks, each one moving bi-directionally away from the origin, so that DNA replication terminates when forks that initiated from adjacent origins converge. Each replication fork is associated with a replisome, a multi-component protein complex including the helicase, the polymerases and accessory factors such as the sliding clamp proliferating cell nuclear antigen (PCNA) and its loader the replication factor C (RFC). Importantly, each replication fork consists of a leading and a lagging strand, elongated by the replicative polymerases ε and δ, respectively. Synthesis of the leading strand is continuous, whereas that one of the lagging strand involves the elongation and subsequent ligation of primers (Okazaki fragments) (Fig. 1) (Branzei and Foiani, 2010). Many other components are present at the replication fork, but because they are not the focus of this review, we refer our readers to (Aves et al., 2012; Jones and Petermann, 2012), where they can find in-depth discussions about the factors operating during DNA replication.

During replication cells need to deal with aberrant replication fork structures that challenge genomic stability (Zeman and Cimprich, 2014). Replication stress is caused by DNA lesions, which amount to 10^5 per cell per day in unstressed cells (Hoeijmakers, 2009), and physical barriers like naturally occurring difficult-to-replicate genomic regions (e.g. common fragile sites) (Zeman and Cimprich, 2014). How are physical replication barriers and DNA lesions sensed to induce checkpoint activation? Both kinds of obstacles stall the polymerase while the helicase continues to unwind the parental DNA (Byun et al., 2005; Walter and Newport, 2000). Such helicase-polymerase uncoupling favors the extension of single stranded DNA (ssDNA) tracks (Byun et al., 2005) (Fig. 1). Consistent with this model, excessive ssDNA accumulates when cells are treated with DNA-damaging agents such as hydroxyurea (HU), aphidicolin (APH) and ultraviolet (UV) irradiation (Lupardus et al., 2002; Michael et al., 2000; Sogo et al., 2002). ssDNA tracks are immediately coated by replication protein A (RPA). RPA not only protects the ssDNA but also contributes to checkpoint activation (Oakley and Patrick, 2010; Zou and Elledge, 2003). Indeed, RPA-coated ssDNA was the first DNA structure proposed as a checkpoint-activating structure (Zou and Elledge, 2003). In agreement, the amount of RPA-ssDNA correlates with checkpoint activation (Shechter et al., 2004). However, checkpoint activation requires not only the accumulation of RPA-ssDNA but also that of 5’ single stranded/double stranded DNA junctions (Byun et al., 2005; MacDougall et al., 2007; Zeman and Cimprich, 2014). In fact, even at stalled forks, DNA synthesis continues at a slow rate to form primers on ssDNA templates, and these primers are indispensable for strong checkpoint activation (Van et al., 2010). Chk1 activation by RPA-ssDNA adjacent to primer-template junctions at stalled forks is preceded by ATR (Ataxia-telangiectasia mutated- and Rad3-related) activation, as we discuss below (Fig. 1).

2.1. ATR-ATRIP activation

RPA-ssDNA—The Chk1-activating kinase ATR is a member of the phosphoinositide 3-kinase-related kinase (PIKK) family. ATR exists in a stable heterodimeric complex with ATR-interacting protein (ATRIP) (Cimprich and Cortez, 2008; Cortez et al., 2001). Because ATR-ATRIP is recruited by RPA-ssDNA to sites of replication stress or DNA damage processing, this complex acts as a sensor of genotoxic stress (Zou and Elledge, 2003). Although ATRIP binds RPA directly, additional yet unidentified mechanisms might also ensure the recruitment of ATR-ATRIP to ssDNA. The latter notion is supported by the fact that efficient Chk1 phosphorylation is still observed after disruption of the ATRIP-RPA interaction (Ball et al., 2005) and in conditions in which RPA does not nucleate onto ssDNA or its concentration is low (Dodson et al., 2004; Recolin et al., 2012). So other ssDNA-binding proteins (SSBs) may contribute to checkpoint activation. Indeed, human SSB1 was recently shown to promote Chk1 phosphorylation (Bolderson et al., 2014).

TopBP1—In vitro activation of the ATR-ATRIP complex does not require DNA, yet it needs the BRCT-containing protein DNA topoisomerase II binding protein 1 (TopBP1) (Choi et al., 2010). Furthermore, over-expression of the ATR-activating domain of human TopBP1 fused to a nuclear localization signal triggers phosphorylation of an ATR target independently of DNA damage (Kumagai et al., 2006). These results imply that RPA-ssDNA serves to recruit ATR-ATRIP to sites of damage, whereas the actual activation of

ATR relies on TopBP1. In metazoans, TopBP1 was recently shown to constitute the major if not the sole activator of ATR (Zhou et al., 2013).

Rad17/911 complex—The PCNA-related Rad9-Hus1-Rad1 (9-1-1) heterotrimer and its loader, the Rad17/RFC complex, are required to enhance ATR-dependent Chk1 phosphorylation in vivo (Kobayashi et al., 2004; Weiss et al., 2002). Rad17/RFC loads the 9-1-1 clamp onto the 5' ends of primer-template junctions that accumulate at stalled forks (Majka et al., 2006). Rad17/RFC-mediated 9-1-1 recruitment to chromatin is a process largely independent of ATR-ATRIP recruitment to sites of damage (Zou et al., 2002). Both complexes thus meet at sites of DNA damage and are bridged by TopBP1, whose interactions with ATRIP and the 9-1-1 clamp are necessary for TopBP1-ATR interaction and subsequent ATR activation (Lee et al., 2007; Mordes et al., 2008). Even if the in vitro recruitment of TopBP1 to ssDNA relies on TopBP1 interaction with ATRIP (Choi et al., 2010), the Rad17/9-1-1 complex is also largely responsible for such recruitment in vivo (Delacroix et al., 2007; Lee and Dunphy, 2010). However, others have shown that TopBP1 is initially localized to sites of DNA damage to then control the loading of 9-1-1 onto stalled replication forks (Yan and Michael, 2009).

Pol κ—The primer-template DNA junctions required for the loading of the 9-1-1 clamp were previously thought to be present mainly at the lagging strand (Van et al., 2010). The relevance of the leading strand to the accumulation of primer-template DNA junctions was unclear until recently, when the alternative DNA polymerase Pol κ was shown to be involved in the accumulation of small nascent DNA at stalled forks (Betous et al., 2013). Checkpoint signaling might therefore initiate from both the leading and lagging strands (Betous et al., 2013) (Fig. 1). Importantly, a mutator phenotype was reported in Pol κ-deficient mouse models (Burr et al., 2006; Stancel et al., 2009). This phenotype led (Betous et al., 2013) to propose that the novel checkpoint function of Pol κ might be mechanistically linked to the regulation of genomic stability under normal growth conditions.

2.2. ATR-mediated Chk1 phosphorylation

After ATR is activated, phosphorylation of Chk1 constitutes a major event in the response to stalled replication. Although preliminary evidence places multiple factors upstream of Chk1 activation (Dai and Grant, 2010), we will focus on Claspin and Poly(ADP-ribose) polymerase 1 (PARP1), which have been extensively or rigorously characterized as factors that stimulate Chk1 activity.

Claspin—After replication stress, phosphorylation of Claspin within its Chk1-binding domain by casein kinase 1 γ1 (CK1γ1) (Meng et al., 2011) allows association of Claspin with Chk1 (Chini et al., 2006; Kumagai and Dunphy, 2003). Such interaction enhances Chk1 activation because ATR shows higher affinity for the Chk1-Claspin complex than for Chk1 alone (Lindsey-Boltz et al., 2009). Accordingly, deficiency of Claspin results in reduced replication stress-induced Chk1 phosphorylation (Errico et al., 2007; Kemp et al., 2010; Kumagai and Dunphy, 2000) and a compromised S-phase checkpoint (Chini and Chen, 2003; Chou and Elledge, 2006).

PARP1—Poly(ADP-ribosyl)ation (PARylation) is a posttranslational modification in which ADP(ribose) units are attached to target proteins by members of the PARP family. In response to DNA damage, PARP1 catalyzes PARylation of itself and other substrates, thereby controlling diverse aspects of the DNA damage response (Gibson and Kraus, 2012). For instance, the long PAR chains formed onto PARP1 at stalled forks are bound by a specific motif of Chk1, in a Claspin- and ATR-independent manner. Therefore, by recruiting Chk1 to sites of damage, PARP1 allows Chk1 full activation after UV or HU treatment (Min et al., 2013).

In summary, the current model for ATR-mediated Chk1 phosphorylation upon replication stress goes as follows. First, the ATR-ATRIP and Rad17/RFC complexes are recruited in parallel to RPA-ssDNA and primer-template junctions, respectively. Subsequently, Rad17/RFC loads the 9-1-1 clamp onto such junctions, to where TopBP1 is also recruited. TopBP1 then interacts with ATR-ATRIP to promote ATR activation. Finally, ATR phosphorylates Chk1, preferentially when Chk1 forms a complex with Claspin. Additional factors like PARP1-dependent PARylation modulate Chk1 activation at the fork.

3. CHK1 ACTIVATION AND FUNCTION ARE TIGHTLY LINKED TO ITS SUBCELLULAR LOCALIZATION

ATR phosphorylates serines 317 and 345 within the C-terminal regulatory domain of Chk1 (Walker et al., 2009; Zhao and Piwnica-Worms, 2001). Both phosphorylation events stimulate the latent kinase activity of Chk1 (Kasahara et al., 2010; Katsuragi and Sagata, 2004; Ng et al., 2004; Walker et al., 2009), although it is not clear how. The individual contributions of each phosphorylation event to Chk1 activation and a functional checkpoint response also remain obscure. Although there is consensus that S345 phosphorylation is essential for checkpoint proficiency and even cell survival, the contribution of S317 phosphorylation to such processes remains controversial (Niida et al., 2007; Walker et al., 2009; Wilsker et al., 2008). Still, solid evidence does demonstrate that S317 phosphorylation is a pre-requisite for strong ATR-dependent S345 phosphorylation (Niida et al., 2007; Wilsker et al., 2008).

After undergoing ATR-induced phosphorylation and activation at sites of DNA damage (Jiang et al., 2003; Smits et al., 2006), Chk1 is released to the nucleoplasm (Smits et al., 2006), where it phosphorylates itself on S296 (Kasahara et al., 2010) (Fig. 2). This auto-phosphorylation event is required for Chk1-dependent block of premature mitotic entry after UV irradiation (Kasahara et al., 2010), but the relevance of S296 phosphorylation to Chk1 role in DNA replication remains undetermined. It is of note however that auto-phosphorylation of S296 is followed by dephosphorylation of the ATR sites S317 and S345 (Kasahara et al., 2010; Leung-Pineda et al., 2006; Smits et al., 2006).

3.1. Chk1 activation takes place in the nucleus

Chk1 constantly shuttles between nucleus and cytoplasm but is localized predominantly in the nucleus during the interphase (Enomoto et al., 2009). Such nuclear retention relies on the phosphorylation of S280 in Chk1 by MAP kinase-activated p90 ribosomal S6 kinase (p90

RSK), which takes place in response to serum stimulation in the cytoplasm (Li et al., 2012). Consistent with the fact that ATR activates Chk1 in the nucleus (Jiang et al., 2003; Smits et al., 2006; Wang et al., 2012), the phosphorylation of S280 is stimulated after UV irradiation and required for efficient UV-induced Chk1 activation (Li et al., 2012) (Fig. 2). Thus, nuclear accumulation of Chk1 precedes ATR-dependent phosphorylation of Chk1. Active Chk1 is then restrained to the nucleus by a mechanism that probably involves the masking of a nuclear export signal by the phosphorylation of S345 (Jiang et al., 2003; Wang et al., 2012).

3.2. Signals that regulate Chk1 shuttling between chromatin and nucleoplasm

There is a strong inverse correlation between the amount of chromatin-bound Chk1 and the extent of DNA damage. In unperturbed cells, around 20% of the total cellular Chk1 pool is estimated to be chromatin-associated (Smits et al., 2006), enriched at nascent DNA (Min et al., 2013). But following HU or UV treatment, Chk1 is fully redirected to the nucleoplasm (Kasahara et al., 2010; Min et al., 2013; Smits et al., 2006). This rapid DNA damage-dependent release of Chk1 from chromatin is independent of Chk1 kinase activity but promoted by ATR-dependent phosphorylation of Chk1 on S317 and S345 (Smits et al., 2006). Importantly however, mutation of these residues only partially abolishes DNA damage-induced Chk1 chromatin dissociation (Smits et al., 2006), suggesting that ATR-independent mechanisms contribute to the nucleoplasmic spreading of Chk1. Such mechanisms might depend on the PAR-binding regulatory motif (PbR) and the PCNA-interacting protein (PIP) box motif in Chk1, given that both motives modulate Chk1 association with chromatin (Min et al., 2013; Scorah et al., 2008) (Fig. 2). Interestingly, ATR-independent modes of regulating the levels of chromatin-bound Chk1 raise the possibility that Chk1 release into the nucleoplasm is not an immediate consequence of Chk1 phosphorylation. But the consequences of a potential extended retention of active Chk1 at stalled forks are currently unknown.

3.3. Targets of Chk1 in the nucleoplasm and on chromatin

Although a recent phospho-proteomic screen identified several Chk1 substrates (Blasius et al., 2011), little is known about their function in checkpoint signaling. Hence, the exact mechanisms by which Chk1 controls DNA replication and the response to DNA damage remain unclear. The best characterized Chk1 substrates are the cell division control 25 (Cdc25) phosphatases, which activate Cdk2 (cyclin-dependent kinase 2) by removing an inhibitory phosphate from tyrosine 15 in Cdk2 (Gu et al., 1992; Mailand et al., 2000). Aided by 14-3-3γ(Kasahara et al., 2010), nucleoplasmic Chk1 phosphorylates Cdc25A to promote its degradation by the Skp1/Cullin/F-box protein$^{\beta TrCP}$ ($SCF^{\beta TrCP}$) E3 ubiquitin ligase (Busino et al., 2003; Mailand et al., 2000; Sorensen et al., 2003). Chk1-dependent Cdc25 degradation is followed by Cdk2 inactivation, inhibition of origin firing and cell cycle arrest (Sorensen and Syljuasen, 2012). Importantly, besides modulating Cdc25A levels upon genotoxic stress, Chk1 activity maintains the basal turnover of Cdc25A (Sorensen et al., 2003) (Figs. 2–3).

Apart from phosphorylating Cdc25A in the nucleoplasm, physiologically active Chk1 phosphorylates chromatin-bound proteins, such as H3 on tyrosine 11 (Shimada et al., 2008).

According to the model proposed by (Shimada et al., 2008), Chk1-mediated phosphorylation of H3 promotes H3 acetylation at the promoters of cdk1 and cyclin B1, thus activating their transcription under unperturbed conditions (Shimada et al., 2008) (Fig. 2). In agreement, the expression of these cell-cycle control genes in mouse embryonic fibroblasts (MEFs) requires Chk1 activity (Shimada et al., 2008). Therefore, by controlling transcription, Chk1 maintains timely mitotic transitions in unperturbed cells. Moreover, because Chk1 release from chromatin is stimulated by DNA damage, the report by (Shimada et al., 2008) provided a mechanistic link between Chk1 and the repression of transcription following genotoxic stress. Strengthening the link between Chk1 and transcriptional regulation, a recent study identified and subsequently validated the transcriptional co-repressor KAP1 as a novel target of DNA damage-activated Chk1 (Blasius et al., 2011).

Chk1 targets DNA-bound proteins not only in unperturbed conditions but also when activated by exogenous replication stress. A recently identified chromatin-bound stress-dependent Chk1 target is the ubiquitin ligase anaphase-promoting complex/cyclosome^{Cdh1} (APC/C^{Cdh1}) (Fig. 2). After replication stress caused by HU or cisplatin treatments Chk1 triggers auto-degradation of Cdh1, which most likely results from Chk1-dependent phosphorylation of components of the APC/C^{Cdh1} complex, including Cdh1 itself (Yamada et al., 2013). This model is in line with the identification of APC1 (the largest subunit of the APC/C complex) as a Chk1 substrate in a recent screening (Blasius et al., 2011). Such novel function of Chk1 could contribute to maintain the low levels of APC/C activity observed in S/G2 (Pines, 2011). More interestingly, the biological significance of Chk1-triggered APC/C^{Cdh1} destruction also relates to the facilitation of DNA replication across replication barriers (Yamada et al., 2013), as we will discuss in detail in section 4.3. Other proteins phosphorylated at chromatin by stress-activated Chk1 include the homologous recombination (HR) factors Rad51 and BRCA2 (see section 4.4; Fig. 2).

Altogether, although basal and stress-induced activation of Chk1 are controlled by identical phosphorylation patterns, target selectivity before and after stress apparently exists. Some Chk1 targets (e.g. H3) might be selectively phosphorylated by the basal activity of Chk1 at chromatin. Others (e.g. Cdc25A) might be modified by both low and high levels of activated Chk1. A third group of proteins (e.g. HR factors) might be more obviously subjected to Chk1 regulation only when Chk1 activity is high (Fig.2).

4. CHK1 FUNCTION DURING DNA REPLICATION

In correlation with the accumulation of its mRNA and protein levels, Chk1 becomes activated and localizes to the nucleus at every physiological S-to-M transition, irrespective of any external DNA-damaging agent (Kaneko et al., 1999; Sorensen et al., 2003). Genotoxins further increase the amount of active Chk1, as evidenced, for example, by augmented ATR-mediated phosphorylation of Chk1 in response to HU (Zhao and Piwnica-Worms, 2001). After checkpoint initiation by replication stress, Chk1 a) controls origin firing so as to reduce the number of forks that encounter DNA lesions while avoiding under-replication, b) stabilizes elongating forks so that they retain the ability to restart replication after the removal of replication blocks. We will now debate the evidence linking Chk1 to the

control of origin firing, fork stability and elongation. We will also discuss how Chk1 function during DNA replication may contribute to cell survival.

4.1. The control of origin firing by Chk1

Replication origins are organized into clusters, so that several adjacent origins that fire simultaneously constitute one replication factory. These clusters are activated in a timely regulated fashion, so that factories are classified into early- or late-replicating (Masai et al., 2010; Zink et al., 1999). Importantly, active origins within a cluster are surrounded by dormant origins, which normally remain inactive (Blow et al., 2011). The activation of dormant origins is required to ensure complete genome duplication in cells exposed to replication inhibitors (Ge et al., 2007; Ibarra et al., 2008; Woodward et al., 2006).

Early studies showed that Chk1 negatively affects DNA replication after genotoxic stress. First, (Feijoo et al., 2001) showed that Chk1 inhibits the initiation of new replication factories and the transition from early to late S phase in the presence of APH. Second, by means of velocity sedimentation analysis of nascent DNA molecules, a strong Chk1-dependent reduction in replication initiation of UV-treated human fibroblasts was revealed (Heffernan et al., 2002). Importantly, Chk1 probably targets mostly clusters that fire late in S phase because DNA replication in early clusters must be initiated to elicit checkpoint activation (Zegerman and Diffley, 2009). More insights into the effect of Chk1 on the dynamics of DNA replication were obtained by DNA fiber analysis. This technique allows the study of individual replication forks, providing information such as inter-origin distance and origin density (Conti et al., 2007). With this technology, it was shown that camptothecin (CPT) treatment causes a Chk1-dependent reduction in the number of origins that fire (Seiler et al., 2007). Similarly, in the absence of external damage, Chk1 deletion or inhibition results in aberrantly increased activation of origins (Maya-Mendoza et al., 2007; Miao et al., 2003; Syljuasen et al., 2005). Thus, Chk1 inhibitory effect on global DNA synthesis could depend on its negative effect on origin firing (Fig. 3).

Interestingly, low doses of replicative stress inhibit new factory activation but at the same time stimulate the firing of origins adjacent to stalled forks (Blow et al., 2011; Ge et al., 2007; Ibarra et al., 2008; Woodward et al., 2006). This phenomenon is in part explained by the fact that Chk1 preferentially inhibits new factory activation, redirecting replication resources to chromosomal regions already engaged in DNA synthesis (Ge and Blow, 2010). How Chk1 preferentially inhibits new factory activation and selectively allows dormant origin firing during moderate replication stress is yet unknown, and only speculative models have been discussed (Ge and Blow, 2010). We suggest that as-yet undescribed functions of Chk1, which exceed those performed by the fraction of activated, nucleoplasmic Chk1, explain such differential effect of Chk1 on origins that are close to stalled/damaged replication forks vs. origins that are far from forks encountering DNA damage. Local ATR-dependent Chk1 phosphorylation results in Chk1 dissociation from sites of DNA damage (Jiang et al., 2003; Smits et al., 2006). We reason, that if chromatin-bound Chk1 could locally inhibit origin firing, Chk1 release from chromatin might selectively promote activation of dormant origins in the vicinity of stalled forks (that lack Chk1). In contrast, the high amounts of chromatin-bound Chk1 at undamaged sites would repress new factory

activation. Indeed, during unperturbed replication in Xenopus extracts, when Chk1 sits on chromatin, origins remain dormant due to the action of a pathway sensitive to the ATR inhibitor caffeine (Woodward et al., 2006). It would be thus interesting to check which Chk1 pools are important for the execution of such pathway. When the checkpoint becomes activated over a certain threshold, and no Chk1 remains on chromatin, origin firing could be inhibited at both active and new factories by high levels of nucleoplasmic Chk1.

4.2. Molecular mechanisms involved in Chk1-dependent control of replication origins

During late mitosis origins are licensed by the loading of the minichromosome maintenance (Mcm) helicase complex, which consists of six subunits (Mcm2-7), and part of the pre-replication (pre-RC) complex. During S phase phosphorylation of the pre-RC complex by Cyclin E-Cdk2 (cyclin-dependent kinase 2) and Ddk (Dbf4-Drf1-dependent Cdc7 kinase) promotes the binding of Cdc45 and the GINS (go-ichi-ni-san) complex to chromatin, resulting in the formation of the replicative helicase CMG (Cdc45-Mcm2-7-GINS) (Costa et al., 2013). Cdc45 is considered a limiting factor in this process (Wong et al., 2011), and, consistently, two reports showed that the checkpoint affects initiation prior to Cdc45 loading, preventing helicase activation and polymerase loading (Karnani and Dutta, 2011; Liu et al., 2006). Also in support of such model, Chk1-dependent reduction in Cdc45 binding to chromatin has been observed previously (Falck et al., 2002; Liu et al., 2006; Syljuasen et al., 2005). The mechanism by which Chk1 prevents Cdc45 loading onto origins apparently involves different levels of regulation, as we discuss below (Fig. 3).

Cdk2-dependent control of Cdc45 loading—Cdc45 loading requires Cdk2 activation, which is prevented by Chk1-dependent degradation of Cdc25A (see section 3.3). Such pathway was described in unperturbed cells (Scorah and McGowan, 2009; Sorensen et al., 2003; Sorensen et al., 2004; Zhao et al., 2002) and in cells exposed to γ-irradiation (γ IR) (Falck et al., 2002) or treated with APH (Scorah and McGowan, 2009) (Fig. 3). In contrast, low doses of UV irradiation or Benzo(a)pyrene diolepoxide (BPDE) prevent Cdc45 loading independently of Cdc25 degradation and Cdk2 inactivation, yet in a Chk1-dependent manner (Heffernan et al., 2007; Liu et al., 2006). Apart from giving a hint of a Cdk2-independent mechanism that regulates Cdc45 loading, these data imply that Chk1 dissociation from chromatin, which takes place after γ IR, low doses of UV and probably also after treatment with APH or BPDE (Smits et al., 2006), is not sufficient to trigger Cdc25 degradation. Comparing the difference between unperturbed conditions and low doses of UV or BPDE, we speculate that alternative pathways (see below), which may become activated only after exogenously introduced lesions, might prevail over the Cdc25A-dependent Cdk2 inactivation pathway, which is dominant during unperturbed replication (Sorensen et al., 2003). However, the latter model would not explain why other stress signals, such as γ IR, switch the control of Cdc45 loading back to a pathway dependent on Cdc25A degradation. The response to γ IR requires ATM, a kinase related to ATR that responds to double strand breaks (DSBs) (Falck et al., 2002), whereas S-phase checkpoint activation by low doses of UV is ATM-independent (Heffernan et al., 2002). Stress-activated Chk1 may thus need to act in concert with ATM or other pathway activated by high levels of genotoxins to trigger Cdc25 degradation (Heffernan et al., 2007).

Cdc7-regulated loading of Cdc45—The Dbf4-regulated Cdc7 kinase also promotes the loading of Cdc45 onto chromatin, so (Heffernan et al., 2007) hypothesized that Cdc7 constitutes a link between Chk1 and Cdk-independent inhibition of replication initiation (Fig. 3). Dbf4 levels are in fact rate-limiting for ATR-dependent inhibition of replication initiation in Xenopus extracts (Costanzo et al., 2003). In human cells, over-expression of Dbf4 does not affect the BPDE-induced S-phase checkpoint (Liu et al., 2006) but attenuates the UV-C-triggered reduction in origin firing (Heffernan et al., 2007) and overrides the inhibition of DNA replication and cell cycle progression in response to the DNA topoisomerase II inhibitor etoposide (Tsuji et al., 2008). In addition, a link between Chk1 and HU- or cisplatin-induced chromatin retention of Dbf4-Cdc7 after replication stress was recently established (Yamada et al., 2013). While this manuscript suggests that such novel Chk1-Dbf4 pathway is more relevant to the control of fork elongation than to that of origin firing (see section 4.3), it undoubtedly shows that Chk1 regulates at least some aspects of Cdc7 activation (Yamada et al., 2013). The above-mentioned information however requires careful interpretation because others have reported that Dbf4 over-expression is accompanied by reduced Chk1 phosphorylation (Tsuji et al., 2008). Thus, the Dbf4-Cdc7 kinase complex, which is central for the firing of origins, both regulates and is regulated by the ATR-Chk1 pathway.

TopBP1/Treslin interaction and the control of Cdc45 loading—DNA replication initiation is also controlled by TopBP1 interaction with Treslin, a factor that promotes the assembly of the CMG replicative helicase. Disruption of TopBP1/Treslin interaction by Chk1 leads to impaired Cdc45 loading in HU-treated human cells (Boos et al., 2011) (Fig. 3). Although Treslin is regulated by Cdk-mediated phosphorylation, whether Chk1 lies upstream of Cdk in such pathway or the latter operates in parallel to that of Chk1-Treslin is presently unknown (Boos et al., 2011). So, Chk1 might regulate origin firing by interfering with Treslin-TopBP1 interaction in a Cdk- and Ddk-independent manner.

4.3. The control of replication fork stability by Chk1

Failure to stabilize stalled forks leads to their collapse, an event characterized by the accumulation of aberrant DNA structures and damage (Paulsen and Cimprich, 2007). Several lines of evidence support the hypothesis that Chk1 avoids the collapse of ongoing forks: i) Chk1 pharmacological inhibition or depletion by siRNA lead to increased accumulation of replication-associated DSBs (Forment et al., 2011; Murfuni et al., 2013; Sorensen et al., 2005; Syljuasen et al., 2005); ii) CHOC cells treated with the Chk1 inhibitor UCN-01 and Chk1−/− avian B-lymphoma cells show reduced ability to resume replication after an APH-induced arrest (Feijoo et al., 2001; Zachos et al., 2003); iii) Chk1 inactivation reduces the rate of replication fork progression in unperturbed, UV-exposed and CPT-treated cells (Petermann and Caldecott, 2006; Seiler et al., 2007; Speroni et al., 2012). Although these results suggest that Chk1 stabilizes replication forks, impaired fork elongation in Chk1-deficient backgrounds may represent an indirect effect of elevated origin firing (Petermann et al., 2010). This latter notion is supported by the fact that the slow fork speeds in Chk1-inhibited or -depleted unperturbed cells can be reversed if origin firing is simultaneously down-regulated by Cdk inhibition or Cdc7 depletion (Petermann et al., 2010). However, the inhibition of Cdks in Chk1-deficient cells does not completely reverse

the slow speed of forks caused by Chk1 inhibition alone (Petermann et al., 2010), so Chk1-mediated mechanisms distinct from origin firing inhibition could directly promote fork progression. Indeed, in cells harboring a modified version of Chk1 (Chk1S317A), slower fork progression occurs independently of an origin firing phenotype (Wilsker et al., 2008). Thus, the control of fork elongation by Chk1 could be, at least in part, independent of the control of origin firing. The molecular mechanisms underlying Chk1 function in fork stabilization and elongation are just starting to become elucidated, as we will discuss below.

Prevention of replisome dissociation to ensure fork stability—In Xenopus extracts ATR promotes the re-loading of DNA polymerase ε at forks that collapse due to treatment with mytomicin C or CPT (Trenz et al., 2006). While no information from human or mouse studies supports the link between Chk1 and the maintenance of replisome stability at stalled forks, yeast data have contributed to a model in which checkpoint proteins are required to keep polymerases and the helicase loaded on stalled forks (Cobb et al., 2003; Katou et al., 2003). However, this long-standing model has been recently challenged by a genome-wide high-resolution study of replisome progression, which has conclusively demonstrated that checkpoint signaling modulates the activity (e.g. phosphorylation of a subunit of the CMG helicase at forks) rather than the recruitment of the different components of the replisome (De Piccoli et al., 2012).

Chk1 promotes TLS—TLS is a process in which alternative DNA polymerases that can accommodate distorted bases in their active sites replicate across damaged DNA (Prakash et al., 2005). Pol η is an alternative DNA polymerase that bypasses UV-induced cyclobutane pyrimidine dimers (CPDs) with a very low error rate (Lehmann et al., 1975; Yoon et al., 2009). Because CPDs constitute the major photoproduct provoked by UV, Pol η has a prominent role in UV-induced TLS. Besides, Pol η loss in humans has been linked to the DNA replication defects that cause a cancer prone syndrome known as Xeroderma Pigmentosum variant (XPV) (Masutani et al., 1999). A well-studied mechanism that initiates TLS is the mono-ubiquitination of PCNA (Yang et al., 2013), which is catalyzed by the Rad6/Rad18 ubiquitin ligase (Hoege et al., 2002). Interestingly, a factor that recruits Rad18 to DNA-bound PCNA is Pol η itself (Durando et al., 2013).

Multiple lines of evidences suggest a link between TLS and Chk1. First, replication stress caused by HU or cisplatin promotes ATR- and Chk1-dependent recruitment of Rad18 and Pol η to chromatin (Yamada et al., 2013). Interestingly, such novel function of Chk1 requires the phosphorylation of APC/C^{Cdh1} by Chk1, which results in the recruitment to chromatin of the Dbf4-Cdc7 complex, followed by that of Rad18 (Yamada et al., 2013). Second, Chk1 potentiates UV-induced PCNA mono-ubiquitination (Yang et al., 2008). Third, Chk1 contributes to the recruitment of Pol η to replication factories after UV irradiation (Speroni et al., 2012). Fourth, the depletion of Chk1 impairs DNA elongation after UV-irradiation (Speroni et al., 2012), a phenotype reminiscent of that of UV-irradiated XPV fibroblasts (Quinet et al., 2014). Intriguingly, the fork elongation and Pol η foci phenotypes reported by (Speroni et al., 2012) do not correlate with reduced PCNA ubiquitination. Indeed, PCNA ubiquitination is not required to maintain fork progression after UV but is essential for post-replicative filling of UV-provoked gaps (Edmunds et al.,

2008; Lehmann and Fuchs, 2006). In conclusion, Chk1 could regulate Pol η-mediated post-replicative gap filling by promoting PCNA ubiquitination and Dbf4-Cdc7 stabilization, while controlling replicative TLS by currently unknown mechanisms.

Although the evidence described above unveil a clear link between Chk1 and UV-induced Pol η-mediated TLS, other alternative polymerases such as PrimPol and Rev1, which also promote fork elongation after UV irradiation (Jansen et al., 2005; Mouron et al., 2013), could be regulated by UV-activated Chk1 as well. Regarding normal S phase, MAP kinase-activated protein kinase 2 reverts the negative effect that Chk1 inhibition has on fork elongation in a manner that might depend on the recruitment of alternative DNA polymerases to elongating forks (Kopper et al., 2013). Altogether, Chk1 might avoid unnecessary or prolonged stalling of replication forks by positively regulating TLS in unperturbed cells (Kopper et al., 2013) and in UV-exposed cells (Speroni et al., 2012).

Chk1-mediated control of DSB formation and repair (Fig. 3)—Irreversible fork stalling eventually triggers DSB formation. Chk1 promotes the repair of such lesions by HR, an error-free process involving the recombinase Rad51 and its loading factor BRCA2. Particularly, Chk1 phosphorylates Rad51 and BRCA2 to stimulate the recruitment of Rad51 to sites of DNA damage (Bahassi et al., 2008; Sorensen et al., 2005). Nuclease-mediated accumulation of DSBs after Chk1 inhibition or depletion reveals that, apart from promoting HR-dependent processing of DSBs, Chk1 prevents the accumulation of DSBs in the first place (Forment et al., 2011; Murfuni et al., 2013; Syljuasen et al., 2005). Specifically, DSB formation in Chk1-deficient cells is catalyzed by the Mus81 endonuclease (Forment et al., 2011; Murfuni et al., 2013) and the MRE11 nuclease (Thompson et al., 2012). Moreover, nucleases such as SLX4 and GEN1 cause DSB formation in the absence of both Chk1 and Mus81 (Murfuni et al., 2013). Together, these data suggest that Chk1 prevents fork collapse by inhibiting the activation of different nucleases at stalled forks. If DSB formation cannot be avoided, Chk1 promotes HR-mediated repair.

4.4. Chk1-dependent regulation of DNA replication and cell survival

Deletion of Chk1 in mice results in serious proliferation defects and embryonic lethality (Liu et al., 2000; Takai et al., 2000). Likewise, depletion of Chk1 in mice breast tissues triggers apoptosis (Lam et al., 2004). Thus, Chk1 is essential for mice proliferating somatic and embryonic cells. Conversely, Chk1-deficient avian B-lymphoma cells are viable but do proliferate more slowly and present higher levels of apoptosis in comparison with control cells (Zachos et al., 2003). Altogether, these results highlight Chk1 key role in unperturbed cell cycle progression. Because Chk1 has emerged as a central component of eukaryotic DNA replication, aberrant DNA replication could be the underlying cause of the death observed in cells lacking Chk1.

The death pathway activated in cells lacking Chk1 is probably a consequence of increased genomic instability, given that Chk1 inhibition or deficiency is associated with the accumulation of DNA lesions (Lam et al., 2004; Syljuasen et al., 2005). Importantly, the loss of viability of Chk1-null embryonic mouse stem cells can be rescued by the expression of Chk1S317A (a version of Chk1 unable to get phosphorylated at S317; A stands for

Alanine), but not by that of Chk1S345A (Niida et al., 2007), being the first phosphorylation site linked to Chk1 role as a regulator of replication fork elongation and the second one to Chk1 function during the G2-to-M transition (Wilsker et al., 2008). In agreement, the substitution of endogenous Chk1 for Chk1S345A in human colorectal cancer cells is lethal, whereas the phenotype of cells expressing Chk1S317A is much milder and restricted to reduced replication fork progression (Wilsker et al., 2008). These data suggest that unscheduled mitotic entry and not aberrant DNA replication result in the poor viability of Chk1-null cells. Notwithstanding this, the possibility that misregulated replication contributes to the lethality of Chk1-null mice cannot be excluded. Indeed, the accumulation of replication-associated DNA lesions and the low proliferation rates caused by Chk1 depletion can be partially rescued by co-depleting Cdc25A (Beck et al., 2010), a downstream effector of Chk1 that prevents unscheduled origin firing and the accumulation of DNA damage during unperturbed S phase (Syljuasen et al., 2005). Besides, the depletion of the endonuclease Mus81 largely prevents replication-associated DSB formation in human U2OS and GM01604 cells lacking active Chk1 (Forment et al., 2011; Murfuni et al., 2013). However, although Mus81 compromises cell survival of human U2OS cells treated with the Chk1 inhibitor AZD7762 (Forment et al., 2011), this nuclease is rather required to enhance the survival of GM01604 cells treated with the Chk1 inhibitor UCN-01 (Murfuni et al., 2013). Further analysis is therefore needed to strengthen or discard the link between DSB formation caused by aberrant replication and subsequent cell death in Chk1-deficient cells.

5. ATTENUATION OF CHK1 SIGNALS

Once DNA damage is removed, checkpoint signaling should be attenuated to allow the resumption of DNA replication and avoid cell death resulting from irreversible trapping in S phase. Chk1 is inactivated directly by dephosphorylation and proteolytic degradation or indirectly by destruction of Claspin, an adaptor protein required for ATR-mediated Chk1 phosphorylation (Leung-Pineda et al., 2006; Lu et al., 2005; Mailand et al., 2006; Peschiaroli et al., 2006; Zhang et al., 2005). While these aspects of checkpoint attenuation are being profoundly explored, the recovery of the forks that initiate checkpoint signaling has received less attention. We speculate that such recovery requires not only a steep reduction in Chk1 levels but also the removal of Chk1 activators from those forks, as we will discuss at the end of this section.

5.1. Active Chk1 is preferentially degraded

Chk1 gene is subject to p53-mediated transcriptional repression (Gottifredi et al., 2001). With respect to post-translational regulation, a severe reduction in the levels of Chk1 protein is observed after genotoxic stress in several human cells lines (Zhang et al., 2005). Furthermore, the addition of proteasome inhibitors results in the accumulation of Chk1 protein in normal cycling cells, indicative of constant degradation (Zhang et al., 2005). A C-terminal degron-like region that marks Chk1 for ubiquitination and degradation is largely responsible for the regulation of Chk1 protein levels (Zhang et al., 2009; Zhang et al., 2005). The Cullin-RING E3 ubiquitin ligases (CRLs) containing the Cullin proteins Cul1 and Cul4A mediate Chk1 ubiquitination (Zhang et al., 2005). The F box protein FBX6 and the DDB1- and CUL4-associated factor (DCAF) CDT2 recruit Chk1 to CRL1 and CRL4,

respectively. Interestingly, $CRL1^{FBX6}$ ubiquitinates Chk1 in the cytoplasm, whereas $CRL4^{CDT2}$ destroys activated Chk1 in the nucleoplasm (Huh and Piwnica-Worms, 2013; Zhang et al., 2009).

A further interesting feature of Chk1 protein regulation is that its ubiquitination and concomitant degradation require phosphorylation of S345 by ATR (but intriguingly not of S317) (Huh and Piwnica-Worms, 2013; Zhang et al., 2005). To explain this result, a model was proposed in which the afore-mentioned degron becomes accessible after the transition of Chk1 from a closed to an open configuration, an event known to be promoted by ATR-mediated phosphorylation (Zhang et al., 2009). Indeed, a constitutively open Chk1 mutant shows an increased turnover rate of the protein and a faster recovery from a checkpoint-induced arrest (Zhang et al., 2009). Even if the phosphomimetic mutant Chk1S345E (E stands for glutamic acid) is more unstable than wild type Chk1, other modes of regulation must exist, because the degradation of Chk1S345E is further enhanced upon CPT exposure (Zhang et al., 2005). In line with these experiments, although S345 phosphorylation contributes to the interactions between Chk1 and Cul4A/DDB1 or FBX6, the abrogation of S345 phosphorylation does not abolish them (Leung-Pineda et al., 2009; Zhang et al., 2009).

5.2. Chk1 dephosphorylation

The extinction of active, S317/S345-phosphorylated Chk1 is promoted by the destruction of Claspin, which is marked for degradation by Polo-like kinase 1 (Plk1)-mediated phosphorylation (Mailand et al., 2006; Peschiaroli et al., 2006). In addition, direct Chk1 dephosphorylation occurs in normal cycling cells and in cells exposed to genotoxic stress. The serine/threonine phosphatase PP2A (protein phosphatase 2A) dephosphorylates S317 and S345 in Chk1 in unperturbed HeLa cells. Importantly, although desphosphorylation of Chk1 requires Chk1 kinase activity in undamaged cells, during genotoxic stress, when Chk1 activity is higher, Chk1 is relieved from PP2A-mediated dephosphorylation (Leung-Pineda et al., 2006).

Future experiments should uncover the differences between the Chk1-containing complexes from unstressed and stressed cells that alter PP2A specificity towards Chk1. A second phosphatase involved in S-phase checkpoint recovery is the p53-regulated PP2C serine/threonine protein phosphatase Wip1 (PPM1D) (Lu et al., 2005). PPM1D contributes to Chk1 dephosphorylation and inactivation in vitro but does not seem to play a role in Chk1 destruction during unperturbed replication (Lu et al., 2005). Conversely, and consistent with the fact that PPM1D is induced by several genotoxins (Fiscella et al., 1997; Takekawa et al., 2000), its knock-down by siRNA in UV-exposed U20S cells results in a dramatic increase in phospho-S345-Chk1 levels (Lu et al., 2005). Notwithstanding this, the absence of PPM1D only modestly affects the levels of phospho-S317-Chk1, implying that PPM1D targets preferentially S345.

Chk1 is evidently subject to strict regulation. But the molecular events that coordinate the recycling of Chk1 by dephosphorylation and degradation remain unexplored. We presume that fast dephosphorylation prevents Chk1degradation, whereas delayed dephosphorylation promotes Chk1 destruction. But the emerging picture seems more complicated: the accumulation of phospho-Chk1 correlates with that of Chk1 protein in PPM1D-deficient

UV-exposed cells (Lu et al., 2005). These results highlight the need for further research on the regulatory mechanisms impinging on Chk1 stability.

5.3. Checkpoint recovery beyond Chk1

After checkpoint activation, the stalled forks bound by ATR could keep signaling downstream. So even if Chk1 is efficiently inactivated and degraded, we speculate that the removal of the checkpoint machinery from stalled forks represents a key mechanism to attenuate checkpoint signaling and enable DNA replication resumption at those forks. We hypothesize that the stalled forks that initiate checkpoint signaling can take at least two non-exclusive options: i) tolerate the lesions that induced stalling and checkpoint activation; ii) collapse and subsequently repair those lesions. We and others have recently found evidence of Chk1-induced TLS, supporting the first option (Speroni et al., 2012; Yamada et al., 2013; Yang et al., 2008). On the basis of such results, we speculate that, once ATR achieves efficient Chk1 activation, Chk1 release from a given stalled fork promotes TLS-coupled checkpoint attenuation and DNA replication restart at that fork. In this way, forks that encounter replication barriers might generate global nucleoplasmic signals, which prevent origin firing, without risking fork collapse triggered by extended stalling (Fig. 4). The coordination between TLS-dependent recovery of stalled forks and increased origin firing might be central to adequately responding to replication stress. But how crucial such balance is to cell survival awaits testing.

An important question is how Chk1 promotes TLS in response to UV irradiation. Because Pol η and Chk1 bind PCNA (Kannouche et al., 2004; Scorah et al., 2008), one possibility is that UV-dependent Chk1 release from chromatin allows the binding of Pol η to mono-ubiquitinated PCNA. In fact, forced retention of Chk1 at chromatin impedes TLS activation by UV irradiation (Speroni et al., 2012). Likewise, another PCNA binding partner, the cyclin kinase inhibitor p21, is degraded upon UV to allow the recruitment of TLS polymerases to replication foci (Mansilla et al., 2013). However, this competition model does not explain why the depletion of Chk1 by siRNA negatively affects UV-induced Pol η focal organization (Speroni et al., 2012). Moreover, it has been shown that only proteins containing PIP degrons and not just PIP boxes (as the one in Chk1) interfere with the binding between PCNA and TLS polymerases (Tsanov et al., 2014). We thus believe that Chk1 actively promotes TLS activation by an unknown mechanism; however, we cannot exclude that UV-induced Chk1 release from PCNA facilitates the recruitment of TLS to sites of DNA damage.

6. CONCLUDING REMARKS

During the last two decades we have been confronted with a huge amount of information on checkpoint signaling. But still too many basic questions remain unanswered. From the many unsolved aspects of checkpoint research, we will now focus on two issues that intrigue us: Is there any function of the Chk1 kinase that takes place at the replication fork before Chk1 is released from chromatin? How independent is the checkpoint response from other signals originated at the replication fork?

The most widespread checkpoint activation model regards Chk1 as a kinase that, after being activated at replication forks, functions in the nucleoplasm to indirectly regulate Cdk activity. Nonetheless, recent data add new layers of complexity to this model. To begin with, Chk1 phosphorylates proteins that sit on chromatin during unperturbed and perturbed replication (Fig. 2; see section 3.3). Even if the work by (Yamada et al., 2013) suggests that soluble Chk1 phosphorylates targets at chromatin, newly activated Chk1 may phosphorylate proteins prior to its release from stalled forks. Such an scenario would imply that the fate of stalled forks is determined not only by the final levels of active Chk1 at chromatin but also by the kinetics of Chk1 release from stalled forks. Furthermore, the fate of stalled forks might be controlled by kinase-independent functions of Chk1. In support of such speculation, four lines of evidence suggest that Chk1 might have ATR-, kinase-independent functions during S phase. First, the novel role of Chk1 in the nuclease-dependent processing of replication intermediates is only partially dependent on its kinase activity (Murfuni et al., 2013). Second, APH-triggered Chk1-mediated regulation of origin firing is independent of Claspin (Scorah and McGowan, 2009), suggesting that Chk1 can modulate Cdc25 independently of its kinase activity. But this result requires careful interpretation because Chk1 could become activated by Claspin-independent mechanisms in the presence of excessive replication blocks. Third, inhibition of DNA synthesis, chromosomal instability and cell viability in Chk1-depleted human cells are not phenocopied by ATR deletion. For example, severe inhibition of DNA synthesis was observed when human fibroblasts were depleted of Chk1 but not when cells lacked ATR (Smith-Roe et al., 2013). Fourth, PCNA ubiquitination, Pol η recruitment to active replication factories and DNA elongation after UV irradiation require neither ATR nor the kinase activity of Chk1 (Speroni et al., 2012; Yang et al., 2008). Interestingly however, Rad18 and Pol η recruitment to chromatin do require Chk1 kinase activity (Yamada et al., 2013). Thus, the kinase activity of Chk1 promotes certain aspects of TLS signaling but is dispensable for others. Altogether, kinase-independent roles of Chk1 emerge as a new feature of the S-phase checkpoint, and we speculate that such roles are related to the modulation of protein-protein interactions. We believe that experiments devoted to analyzing protein complex formation/dissociation at ongoing forks will certainly shed light on this novel aspect of Chk1 signaling.

DNA replication is a complex process that requires the concerted action of a myriad of proteins. Chk1 is one of them, but its function has been studied rather in isolation. For example, TLS and checkpoint pathways were supposed to take place as independent events, and the down-regulation of one of these pathways was expected to up-regulate the other. In fact, Pol η depletion unleashes Chk1 activation allowing cell survival (Bomgarden et al., 2006; Despras et al., 2010). However, current research indicates that the TLS and checkpoint pathways are not mutually exclusive choices for a replication fork that stalls. Instead, mounting evidence demonstrates that they work in tight coordination. Pol η is a substrate of ATR (Gohler et al., 2011) and Pol κ prompts checkpoint activation by increasing the density of primer-template junctions at stalled forks (Betous et al., 2013). Moreover, we and others believe that checkpoint-induced TLS is required to prevent excessive collapse of replication forks (Speroni et al., 2012; Yamada et al., 2013). After a fork that stalls at a DNA lesion activates checkpoint signaling, this fork could restart DNA synthesis by undergoing TLS. Thus, to preserve their integrity, stalled forks might

participate in checkpoint activation only transiently (Fig. 4). Although more experimentation is needed to understand the complex interplay between DNA replication, checkpoint and TLS, the discoveries made by (Speroni et al., 2012; Yamada et al., 2013; Yang et al., 2008) highlight the biological significance of the coordination between pathways that, until recently, were accepted as independent responses to stress.

Also interesting would be to gain further understanding into how Chk1 interacts with other factors equally important for replication. Insightful studies have already provided evidence that Chk1 is linked to HR and PARP1-dependent PAR formation (Bahassi et al., 2008; Min et al., 2013; Murfuni et al., 2013; Sorensen et al., 2005). But a lot more proteins are present at the replication fork, and we are just beginning to understand the intricate network they belong to (Kohn et al., 2009). The coordination of different DNA damage-induced pathways is certainly crucial to the delicate balance between viability and genomic stability and thus of utmost relevance to the prevention of oncogenesis and to cancer cells escaping from genotoxic treatments.

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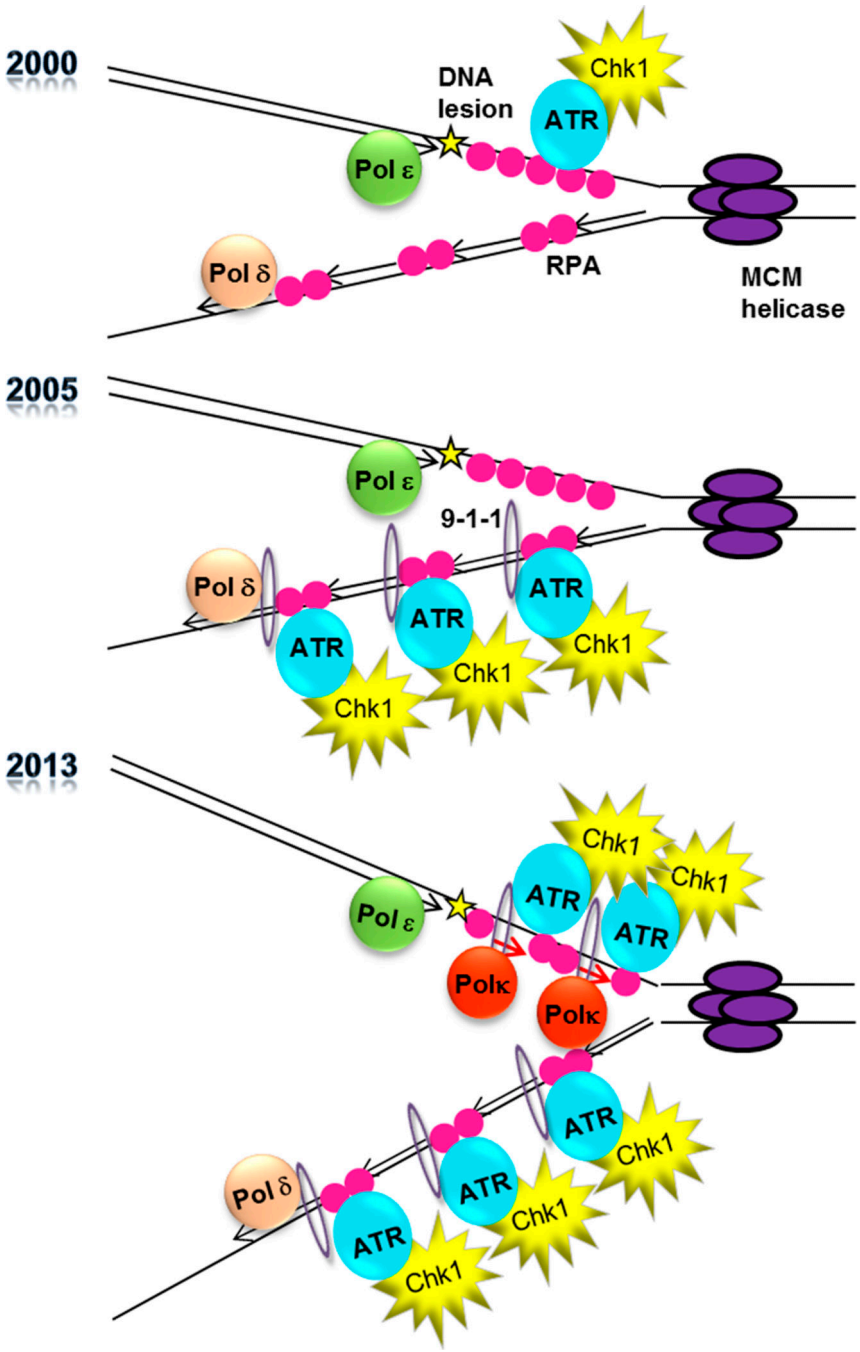

Figure 1. Chk1 activation models over one decade

Year 2000: The uncoupling of the helicase and the replicative polymerase Pol ε generates long stretches of RPA-ssDNA, a pre-requisite to recruit and activate ATR. ATR then phosphorylates Chk1, resulting in Chk1 activation. Note that in this model only the leading strand is able to initiate checkpoint signaling.

Year 2005: ATR activation is preceded by the loading of the 9-1-1 clamp onto the 5 junction of an Okazaki fragment elongated by Pol δ. Note that in this model only the lagging strand is able to initiate checkpoint signaling.

Year 2013: Current model for checkpoint activation (for simplification not all checkpoint components are shown). The TLS polymerase Pol κ elongates primers at the leading strand. Therefore, primer-template junctions are available at both strands, which equally allow the loading of the 9-1-1 clamp. Therefore, ATR-dependent Chk1 activation originates from the leading and lagging strands.

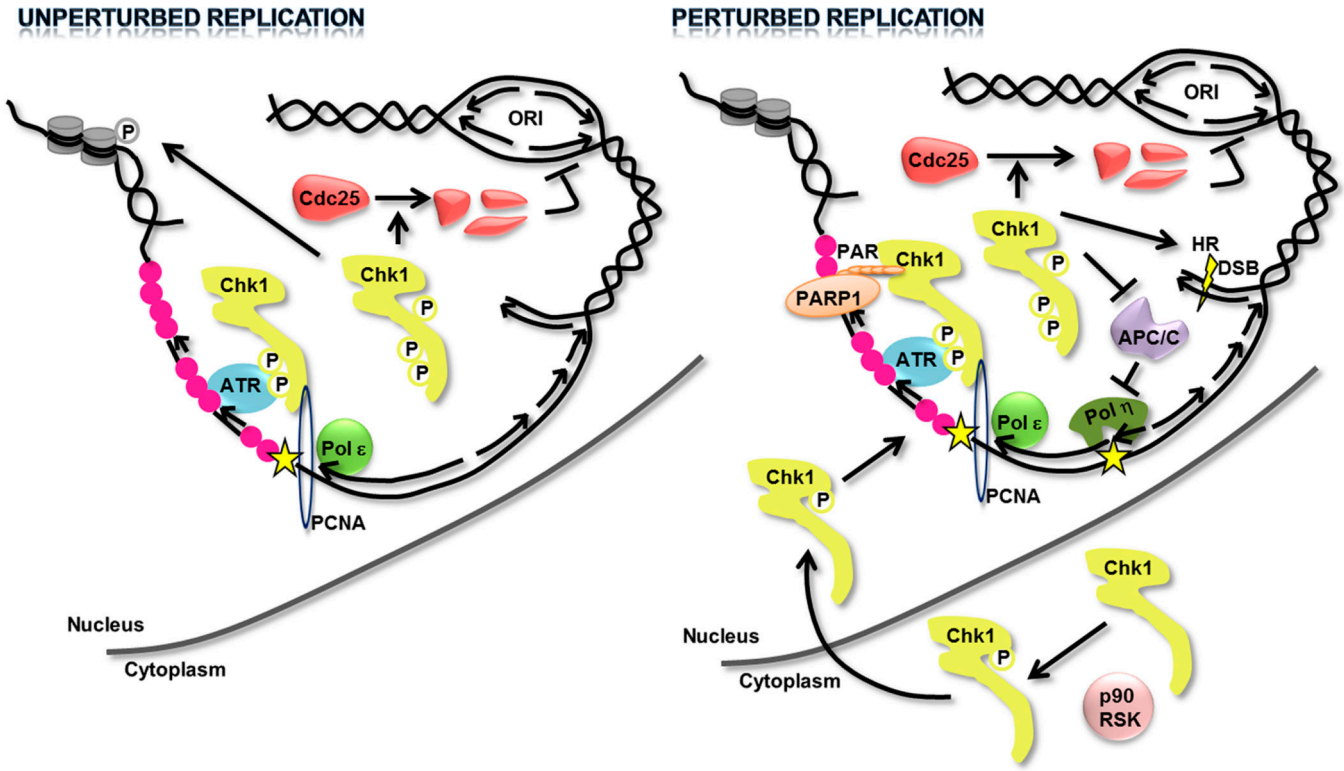

Figure 2. Proper Chk1 activation and function require a dynamic, tightly regulated balance of its subcellular localization

A fork initiating checkpoint signaling is shown (for simplification one of the parental strands is not shown). Under unperturbed conditions, a fraction of Chk1 is recruited to chromatin, in part through its C-terminal PIP motif that binds PCNA. ATR then phosphorylates Chk1 on serines 317 and 345, and ATR-activated Chk1 engages in auto-phosphorylation of serine 296 (represented as the third P in the soluble Chk1). Upon genotoxic stress, at least two mechanisms contribute to Chk1 accumulation at sites of DNA damage: a) cytoplasmic, p90 RSK-dependent phosphorylation of S280 serves to accumulate Chk1 in the nucleus (whether this modification is lost or kept upon nuclear entry is not known); b) the N-terminal PbR motif in Chk1 binds PAR chains synthetized by and attached to PARP1. As a result of these two mechanisms and of increased loading of ATR onto stalled forks (Fig. 1), more Chk1 molecules get activated under perturbed in comparison to unperturbed conditions. Physiologically active and stress-activated Chk1 phosphorylate proteins both at chromatin and in the nucleoplasm (see text for details).

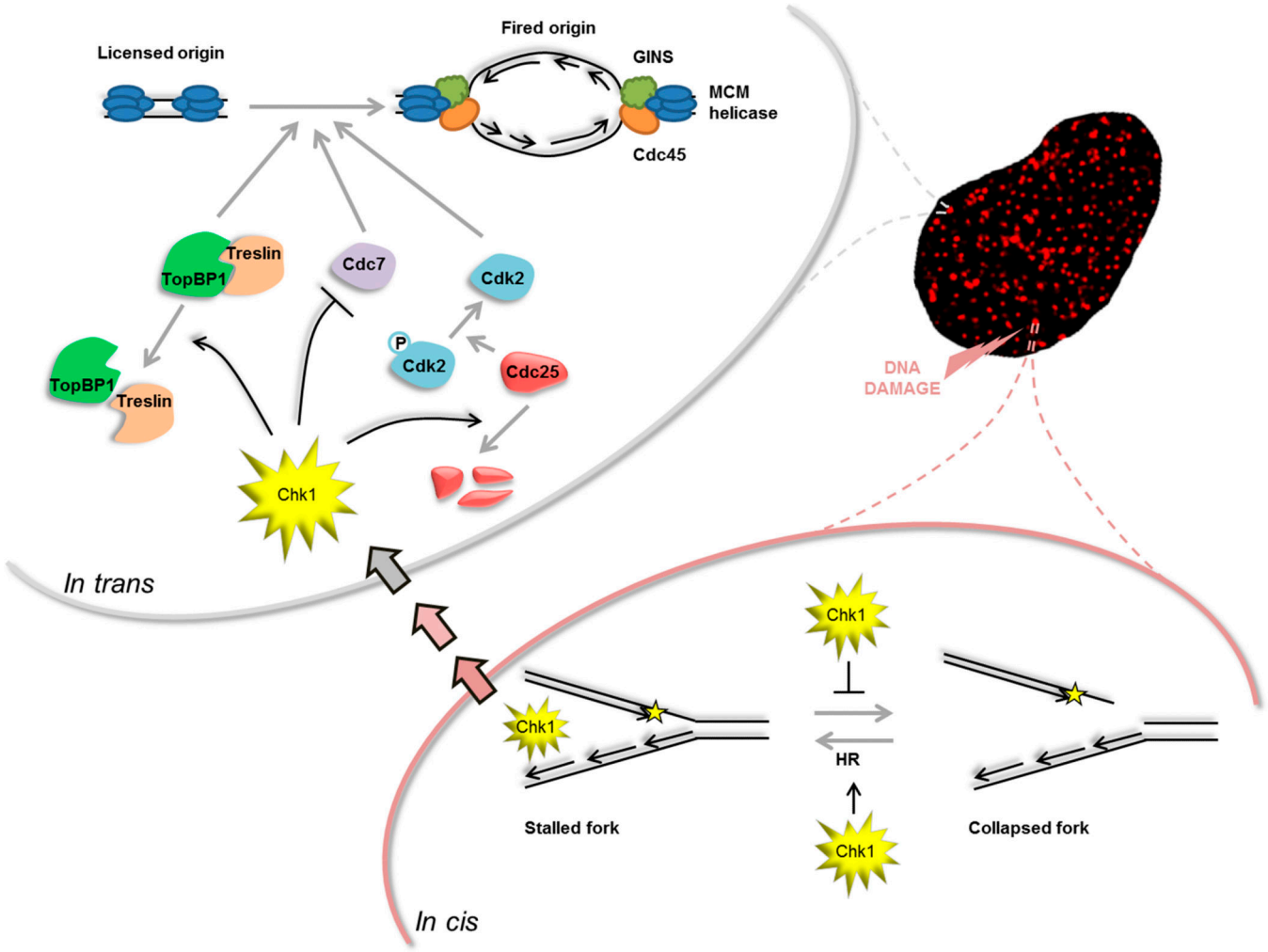

Figure 3. Chk1 functions in DNA replication

A nucleus in S phase with replication factories foci is shown. The arrow represents the incidence of DNA damage upon a given factory. Note that in unperturbed conditions endogenous damage can account for Chk1 activation. Active Chk1 performs its function at the same replication fork that activates it (in cis) or, after being released to the nucleoplasm, at other replication factories (in trans).

In trans Chk1 inhibits origin firing by preventing Cdc45 loading. In unperturbed conditions this is achieved by Chk1-dependent Cdc25A degradation. Upon genotoxic stress, apart from impinging on Cdk2 activity, Chk1 might also inactivate Cdc7 and disrupt the interaction between TopBP1 and treslin; both mechanisms lead to impaired loading of the Cdc45-MCM2-7-GINS replicative helicase.

In cis Chk1 prevents DSB formation by unknown mechanisms, thereby stabilizing forks. Chk1 also aids HR repair of collapsed forks by positively regulating the recruitment of Rad51 and limits genomic instability by restraining the activity of Mus81 and other nucleases. In addition, Chk1 might promote the elongation of forks that have stalled and allow dormant origin firing to ensure that DNA replication is completed (not represented).

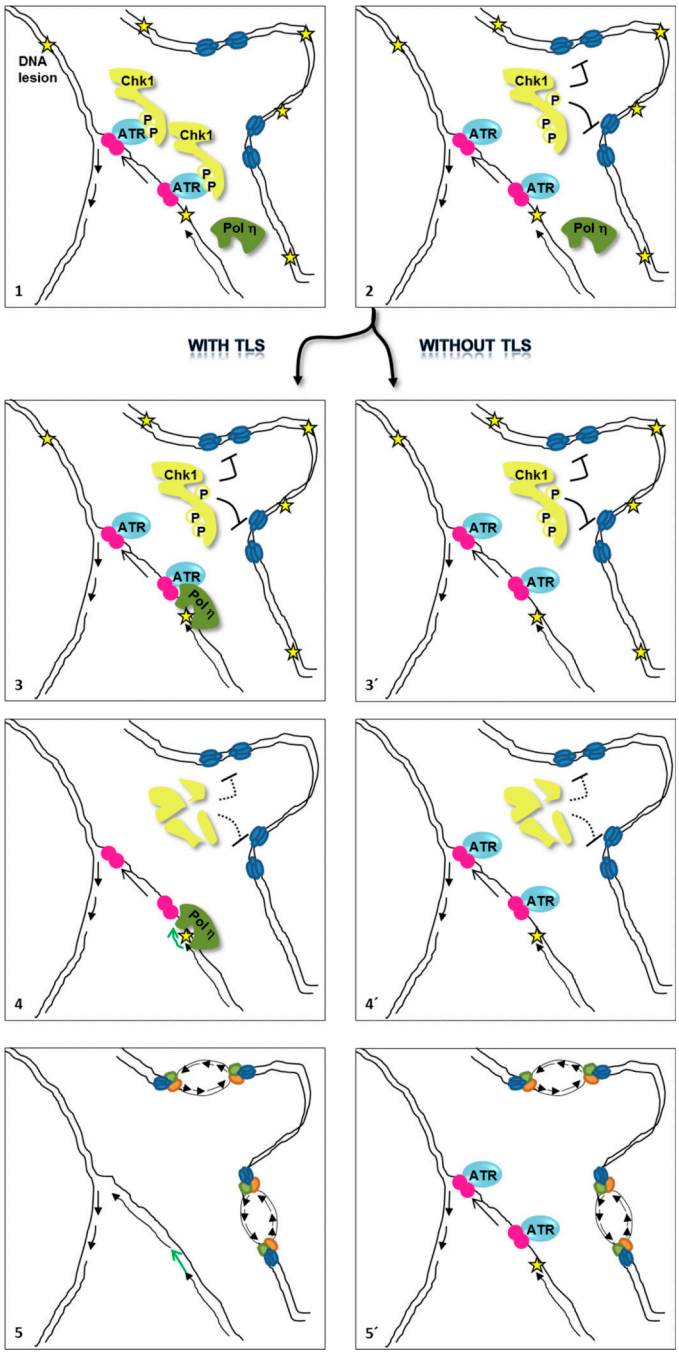

Figure 4. Reversible checkpoint activation requires TLS

A) Isolated model for checkpoint activation: DNA lesions accumulate randomly in the DNA. A fork that encounters a lesion triggers ATR and Chk1 activation (1). Active Chk1 dissociates from chromatin, inhibiting origin firing and creating a time window for DNA repair (2). DNA repair removes DNA lesions (4'). Chk1 signal is attenuated in the nucleoplasm by multiple mechanisms, including degradation of active Chk1. As a consequence, DNA replication is resumed at inhibited origins (4') but not at forks in check (5').

B) A checkpoint and TLS coordination model: when Chk1 is released to control origin firing, it promotes the loading and activation of Pol η at the fork in check (3). As in A), origins are no longer inhibited after active Chk1 is degraded. In contrast to A), Chk1 activation and its release from chromatin enable a TLS-dependent restoration of DNA elongation at forks in check (4). DNA replication is thus resumed after efficient lesion removal, thereby avoiding the collapse of stalled forks (5).