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REVIEW ARTICLE

Roles of Claspin in regulation of DNA replication, replication stress responses and oncogenesis in human cells

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Abstract

Human cells need to cope with the stalling of DNA replication to complete replication of the entire genome to minimize genome instability. They respond to "replication stress" by activating the conserved ATR-Claspin-Chk1 replication checkpoint pathway. The stalled replication fork is detected and stabilized by the checkpoint proteins to prevent disintegration of the replication fork, to remove the lesion or problems that are causing fork block, and to facilitate the continuation of fork progression. Claspin, a factor conserved from yeasts to human, plays a crucial role as a mediator that transmits the replication fork arrest signal from the sensor kinase, ataxia telangiectasia and Rad3-related (ATR), to the effector kinase, Checkpoint kinase 1 (Chk1). Claspin interacts with multiple kinases and replication factors and facilitates efficient replication fork progression and initiation during the normal course of DNA replication as well. It interacts with Cdc7 kinase through the acidic patch segment near the C-terminus and this interaction is critical for efficient phosphorylation of Mcm in non-cancer cells and also for checkpoint activation. Phosphorylation of Claspin by Cdc7, recruited to the acidic patch, regulates the conformation of Claspin through affecting the intramolecular interaction between the N- and C-terminal segments of Claspin. Abundance of Claspin is regulated at both mRNA and protein levels (post-transcriptional regulation and protein stability) and affects the extent of replication checkpoint. In this article, we will discuss how the ATR-Claspin-Chk1 regulates normal and stressed DNA replication and provide insight into the therapeutic potential of targeting replication checkpoint for efficient cancer cell death.

Keywords ATR-Claspin-Chk1 · DNA replication · Replication stress · Replication checkpoint control · Oncogenesis · Biological stresses

Introduction

Cancer incidence is a prevalent cause for human death in developed countries with aging society. How to effectively inhibit cancer cells from progression, metastasis and even relapse is one of the key medical issues that are being studied in different disciplines from various points of views (Klein, 2020). Cancers can arise from accumulation of genetic alterations that may be generated during chromosome replication and inheritance (Andor et al., 2017). Accordingly, investigation of molecular mechanisms of maintenance of genome integrity has been a mainstay of cancer research.

The major cause of genome instability is the presence of replication stress during DNA replication which stalls replication fork progression and reduces the replication fork rate (Bartkova et al., 2006; Di Micco et al., 2006; Gorgoulis et al., 2005). The sources of replication stress include lesions on DNA (DNA strand breaks, double-strand cross-linking, DNA assaults, and chemical modification of bases, etc.), unusual DNA structures on the template DNA, reduced supply of nucleotide precursors, and collision of replication and transcription. The replication stress can be induced by untimely induction of DNA replication or by dysregulated origin firing (Gaillard et al., 2015).

To preserve the genome integrity during replication stress, the ATR-Claspin-Chk1 replication checkpoint pathways is activated (Gaillard et al., 2015). Here, we will mainly focus on the cellular roles of the ATR-Claspin-Chk1

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pathway in control of DNA replication, replication stress responses and oncogenesis. Readers are also referred to other recent reviews on Claspin (Azenha et al., 2019; Smits et al., 2019).

Functional roles of Claspin during normal DNA replication, replication fork progression and initiation

Although Claspin and its yeast ortholog, Mrc1, were discovered as a regulator of replication checkpoint, they have been shown to regulate origin firing and fork progression during the normal course of DNA replication both in yeast and human cells (Hayano et al., 2011; Petermann et al., 2008; Yeeles et al., 2017). Recently, we reported that Claspin is required also for DNA replication initiation in non-cancer cells (Masai et al., 2017; Yang et al, 2016). Claspin recruits Cdc7 kinase, essential for initiation, to the conserved acidic patch (AP) region (residues 986–1100) near the C-terminus of Claspin. The replacement of the acidic residues in AP with alanine impairs the Cdc7 binding and abolishes the ability of Claspin to recruit Cdc7 kinase, and reduces the phosphorylation of Mcm (Fig. 1A). Accordingly, the rate of DNA synthesis is compromised in the Claspin AP-mutant MEF cells. Cdc7 can also phosphorylate multiple sites on Claspin (Kim et al., 2008; Rainey et al., 2013; Yang et al., 2019). Indeed, DE/A mutant of Claspin is not phosphorylated by Cdc7 kinase in vitro. The AP mutation abrogates the intramolecular looping between the N-terminal (N-ter) and C-terminal (C-ter) regions within Claspin. This intramolecular interaction suppresses the DNA- and PCNA-binding activities of Claspin, and is disrupted by phosphorylation of the N-ter by Cdc7, strongly suggesting that the recruitment of Cdc7 by Claspin plays important roles in initiation not only by facilitating the phosphorylation of Mcm proteins, but also by activating DNA and PCNA binding of Claspin (Masai et al., 2017; Matsumoto et al., 2017; Yang et al, 2016).

To ensure appropriate replication fork progression, Ctf4 (And-1), Mrc1 (Claspin), and Csm3/Tof1 (Tipin/Timeless) together with CMG complexes, constitute replisome progression complexes in yeast (Baretić et al., 2020; Gambus et al., 2006; Fig. 1B). Claspin has been shown to be an integral component of the replisome progression complex, and loss of Claspin leads to reduced replication fork rate in cells.

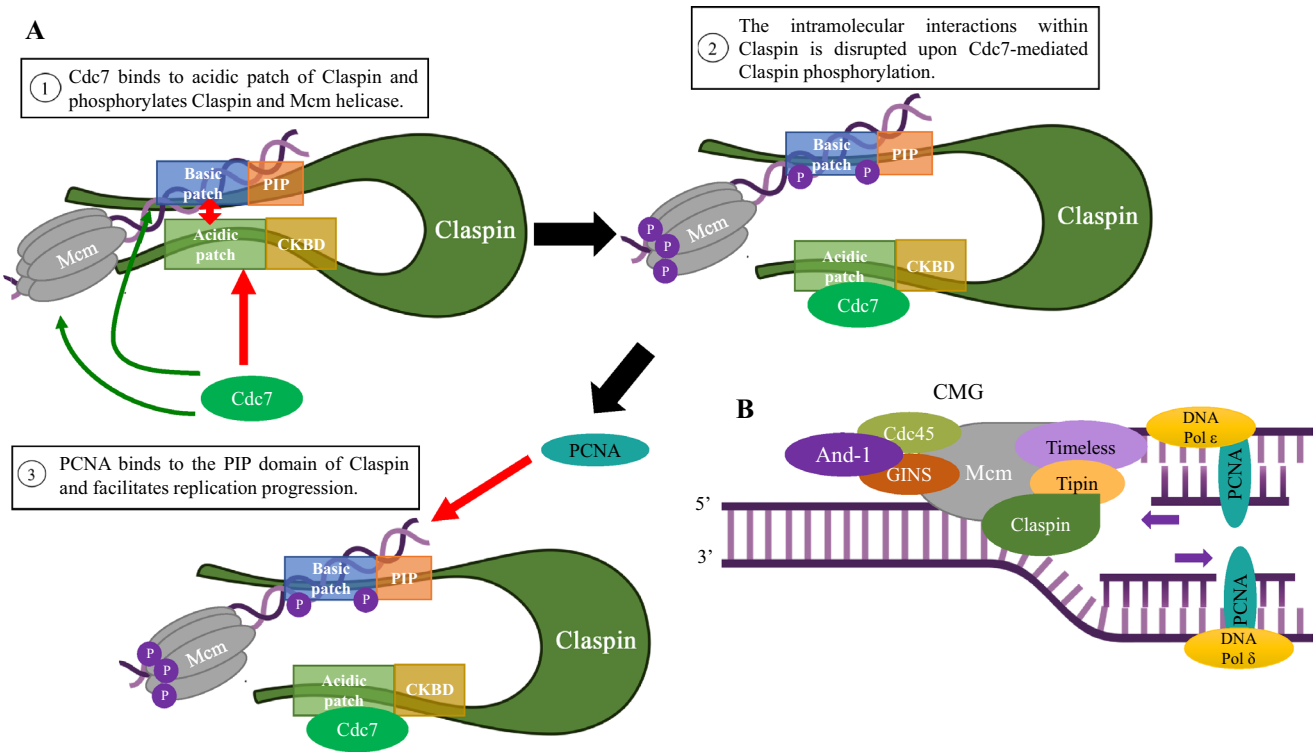

Fig. 1 The roles of Claspin in DNA replication initiation and fork progression. **A** The AP (Acidic Patch) and basic patch segments of Claspin undergo intramolecular interaction. Cdc7 kinase is recruited to AP of Claspin on the chromatin (indicated by a red arrow; ①), facilitating the phosphorylation of Mcm and Claspin itself. (indicated by green arrows) This phosphorylation disrupts the intramolecular interactions within Claspin (②) and facilitates its binding to PCNA (indicated by a red arrow) and DNA (③). All of them contribute to efficient DNA replication initiation. **B** Through direct interactions with Tipin, Timeless, and CMG complex, Claspin stabilizes the replication fork, regulates fork speed, and ensures efficient fork progression

The direct role of Mrc1 in efficient DNA replication fork progression in yeast has been further demonstrated using the in vitro reconstituted DNA replication system (Yeeles et al., 2017). In the reconstituted DNA replication system with a set of origin firing proteins (e.g., Cdc45, DNA polymerase ε, Mcm10, Sld3/7, Sld2, Dpb11, S-CDK, GINS), replication elongation factors (e.g., TopoII, DNA polymerase α, RPA, Ctf4) and fork-associating factors (Mrc1, CMG, FACT, TopoI, Csm3, and Tof1), omission of Mrc1 dramatically decreases DNA synthesis rate, nearly identical to the replication profile exhibited by minimal replisome which is composed of all origin firing proteins along with TopoII and Ctf4. Replication fork rate is much reduced in the absence of Csm3 and Tof1, suggesting Mrc1 in conjunction with Csm3 and Tof1 contributes to replication fork progression at the maximum rate (Yeeles et al., 2017).

It was previously reported that the interaction between Mrc1 and Hsk1 (fission yeast homologue of Cdc7 kinase) regulates origin firing in yeast in a checkpoint-independent fashion (Matsumoto et al., 2017). This interaction relies on the Hsk1 bypass segment (HBS) in Mrc1 to which Hsk1 binds. Similar to Claspin that is phosphorylated by Cdc7, Mrc1 is robustly phosphorylated by Hsk1 kinase through the HBS and this phosphorylation leads to the disruption of intramolecular looping formed between HBS and N-terminal target of HBS (NTHBS) within Mrc1 (Matsumoto et al., 2017). Thus, regulation of Claspin/Mrc1 through an intramolecular interaction is evolutionally conserved.

Furthermore, Claspin (Mrc1 in yeast) in conjunction with TIMELESS (Tof1 in yeast) and TIPIN (Csm3 in yeast) is physically tethered to mini-chromosome maintenance (MCM) DNA helicase subunits and DNA polymerases. This association is important for both efficient fork progression and for fork stabilization under normal and replication stress conditions (Leman & Noguchi, 2012; Leman et al., 2010; Petermann et al., 2008; Yoshizawa-Sugata & Masai, 2007). Recent studies show that Mrc1 forms a complex called MTC or fork protection complex with Tof1 and Csm3 (Lewis et al., 2017; Noguchi et al., 2003). The association of the MTC complex with the fork significantly increases the fork speed and replication progression, shown by DNA stretching assay at the single-molecule level. The transient interaction between MTC and replisome is due to the weak affinity of MTC to replisomes, making the movement of the replisome a highly dynamic process. It has also been reported that Mrc1 in cooperation with Tof1 and Csm3 protects specifically CAG repeats from DNA contractions and breakages. This is indicated by the finding that Mrc1 deletion leads to higher vulnerability of CAG repeats (Gellon et al., 2019). The protective functions of Mrc1 in CAG repeat stability might be due to the fact that the MTC complex can be coupled to the helicases, such as Srs2 and Sgs1, in yeast. Similar roles of Claspin-Timeless-Tipin in stabilization of trinucleotide repeats are observed in human cells (Liu et al., 2012a, 2012b).

Although Mrc1 and Claspin are orthologs and possess functional and mechanical similarities including intramolecular regulation between N- and C-terminal segments, as stated above, there may be some differences in terms of their functional significances (Matsumoto et al., 2017). The N–C interaction in the yeast Mrc1 appears to contribute to the negative regulation of initiation, since its disruption causes precocious initiation specifically at early-firing origins. In mammalian cells, this interaction contributes positively to the initiation in non-cancer cells. However, the effects of the intramolecular interaction differ between cell types in mammalian cells (Hsiao et al. unpublished data), and thus roles of Claspin need to be evaluated in various cell types. Claspin could also negatively regulate the initiation in mammalian cells (see below).

The conserved ATR-Claspin-Chk1 pathway regulates replication checkpoint

The stalled replication forks are quickly detected by the conserved ATR-Claspin and various downstream events are induced, including the suppression of origin firing, slowed replication fork progression and inhibition of mitosis (Tercero et al., 2003). However, stalled replication forks could generate DNA breaks (nicks) if they are not properly attended by the ATR-Claspin-Chk1 checkpoint pathway. In yeast, the DNA replication checkpoint kinase, Cds1, suppresses the endonuclease activity of Mus81/Eme1 during perturbed DNA replication to maintain genome stability (Froget et al., 2008). The Mus81/Eme1 heterodimeric complex can cleave the branched DNAs in a structure-specific manner. When the Cds1 functions improperly or is absent, DNA is cleaved by Mus81/Eme1 and stalled replication forks are at a higher risk of DNA breaks and mutations or more vigorous genomic rearrangements (Froget et al., 2008).

DNA damages are caused by persistent endogenous and exogenous genotoxic insults and activate DNA damage responses (DDRs) (Gaillard et al., 2015; Técher et al., 2017). Replication checkpoint/DNA damage checkpoints further activate subsequent checkpoint signaling pathways for DNA repair and cell cycle arrest. The ATR/Claspin/Chk1 in human or Mec1(Rad3)/Mrc1/Rad53(Cds1) in yeasts, well-conserved in eukaryotes, plays a crucial role in replication checkpoint control in response to replication stress (Bacal et al., 2018; Berens & Toczyski, 2012; Smits et al., 2019; Yang et al., 2019). However, how this axis is precisely controlled is not completely understood.

When replication fork is stalled, ssDNA regions are generated and are coated with replication protein A (RPA), which is then sensed by ATR-interacting protein (ATRIP)

that forms a complex with ATR, triggering ATR autophosphorylation at Thr1989 (Liu et al., 2011). Then, the ATR–ATRIP complex stimulated by TopBP1 and ETAA1 phosphorylates Claspin, resulting in Chk1 phosphorylation at Ser317 and Ser345 which is mediated by ATR (Kim et al., 2008; Liu et al., 2011; Rao et al., 2018; Yang et al., 2019; Zou, 2017). Meanwhile, the stalled replication forks generated by replication stress are protected and stabilized by the complex containing Tim, TIPIN, Claspin and AND-1 (Kemp et al., 2010; Leman & Noguchi, 2012; Leman et al., 2010; Rageul et al., 2020; Yoshizawa-Sugata & Masai, 2007). As previously described, Tim and Tipin are involved in the maintenance of replication forks under both normal replication and replication stress conditions (Leman & Noguchi, 2012; Leman et al., 2010; Yoshizawa-Sugata & Masai, 2007). In the presence of replication stress, Tipin interacts with RPA bound to ssDNA and regulates Claspin-dependent Chk1 phosphorylation (Kemp et al., 2010) (Fig. 2). It has been further shown that SDE2, a PCNA-interacting protein regulating DNA replication fork progression, is involved in Chk1 activation (Rageul et al., 2020). Since the recruitment of Claspin to chromatin is disrupted upon SDE2 or Tim depletion, it has been concluded that both SDE2 and Tim are required for Claspin engagement in the replication fork complex upon replication stress (Rageul et al., 2020).

To sum up, Claspin is tightly associated with successful replication checkpoint activation in response to replication stress through a complex protein regulatory network. Although a number of proteins have been identified to regulate the ATR-Claspin-Chk1 axis, the detailed molecular mechanisms require further investigation.

Regulation of Claspin expression and stability in its functional control

Stabilization and modification of Claspin at both transcriptional and post-translational levels play a crucial role in replication checkpoint signaling in response to replication stress. The Claspin mRNA can be structurally stabilized by tristetraprolin (TTP), mRNA-interacting protein (Lee et al., 2020) (Fig. 3A). The association of 3' untranslated region of Claspin and TTP is required for Claspin to exert its normal functions, including replication fork progression and appropriate replication checkpoint activation. This is indicated by the fact that TTP depletion significantly thwarts normal DNA replication fork progression and diminishes Claspin-dependent Chk1 phosphorylation after replication stress.

At the post-translational level, Claspin can be targeted and ubiquitinated by a series of ubiquitinases for its protein turnover and regulation during cell cycle progression.

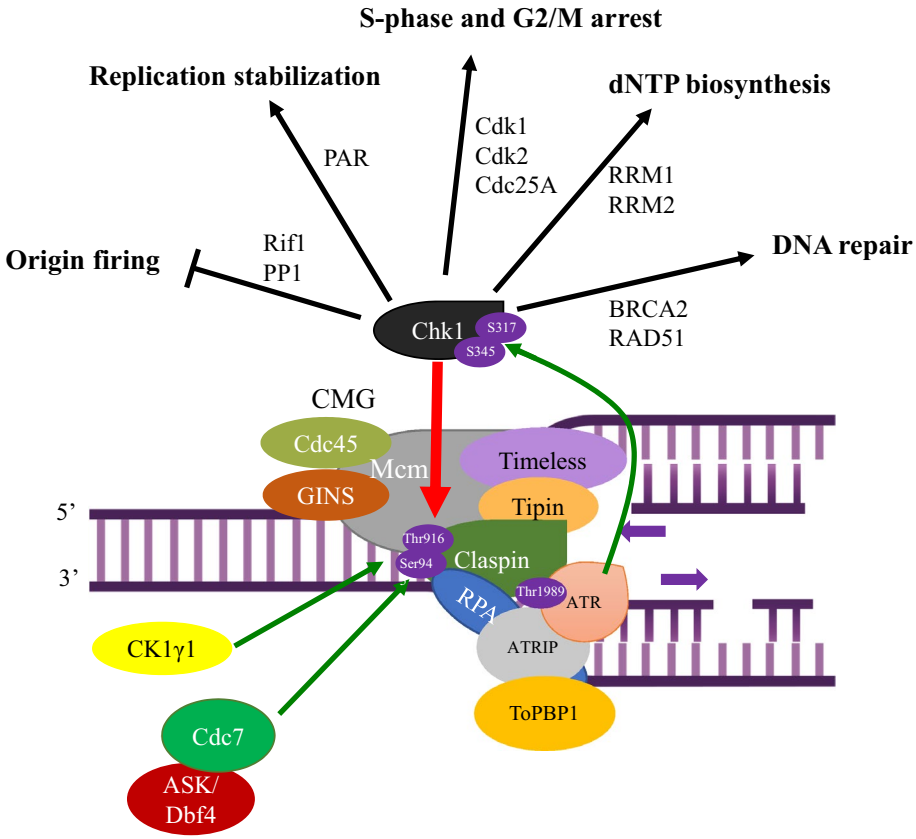

Fig. 2 The signaling cascade of the ATR-Claspin-Chk1 replication checkpoint pathway. Upon fork stall caused by replication stress, ATR is activated by RPA/ATRIP (ATR-interacting protein) and TopBP1. Recruitment of ATR–ATRIP to RPA-coated ssDNA leads to autophosphorylation at Thr-1989 of ATR (Liu et al., 2011). Cdc7 (or CK1γ1) is recruited to Claspin (red arrow), phosphorylating T916 and S945 in CKBD of Claspin (indicated by green arrows), inducing the binding of Chk1, which is phosphorylated by ATR at S317 and S345 (indicated by a green arrow). Phosphorylated Chk1 now regulates origin firing, replication fork progression, cell cycle progression, repair of lesions and removal of the causes of replication stress. See text for more details

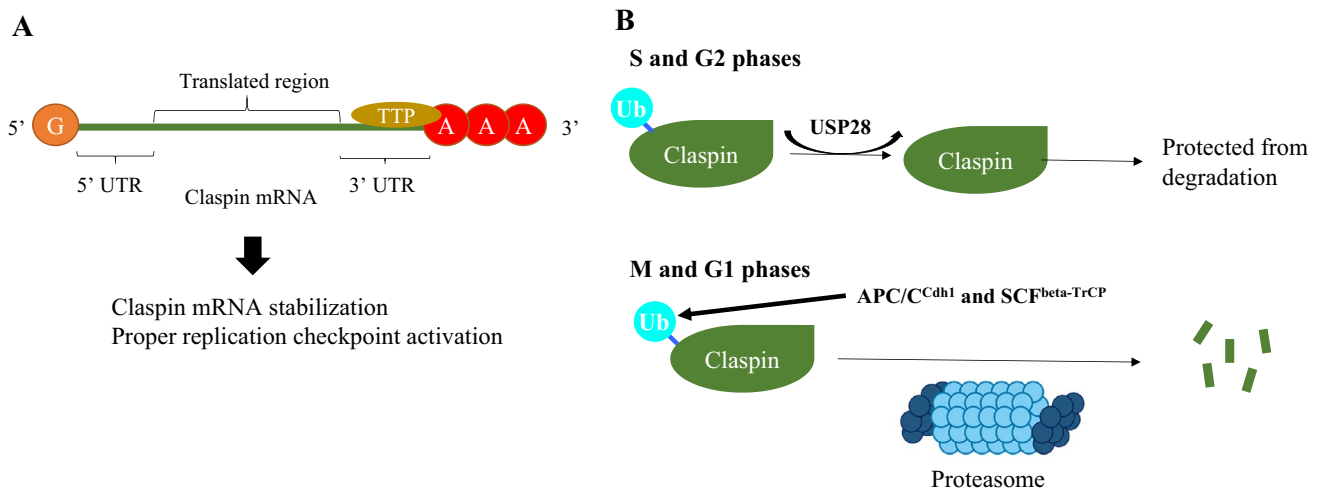

Fig. 3 Claspin stability during cell cycle is regulated at the mRNA and protein levels. **A** Effect of TTP-mediated regulation on the Claspin mRNA during S phase. In S phase, Claspin mRNA can be stabilized via binding of TTP to its 3' UTR. **B** Effect of post-translational modifications on Claspin stability during cell cycle. Claspin can be targeted and regulated by ubiquitin-mediated proteasomal degradation during unperturbed cell cycle progression

However, this can be antagonized by ubiquitin-specific peptidases (USPs), including USP7, USP9X, USP11, USP20, USP28, and USP29 (Faustrup et al., 2009; Ito et al., 2018; Martin et al., 2015; McGarry et al., 2016; Wang et al., 2017; Yuan et al., 2014; Zhao et al., 2021) (Fig. 3B). Depletion of hepatocyte nuclear factor 1-β (HNF-1β) compromises Claspin-dependent replication checkpoint activation, including Chk1 phosphorylation, in response to replication stress (Ito et al., 2018). This is because HNF-1β is needed to stabilize Claspin by stimulating USP28 gene expression and enhancing USP28-mediated deubiquitination. These findings strongly suggest that manipulation of Claspin expression and stability at both mRNA and post-translational levels can be exploited for controlling replication checkpoint activity.

Claspin stability is modulated by MTA1, a chromatin remodeler that drives transcriptional regulation, as well (Li et al., 2010). MTA1 is stabilized by UV which suppresses MTA1 ubiquitination and thus prevents its degradation, and is required for the efficient expression of Chk1 and Claspin. MTA1 interacts with ATR in response to UV, and maintains the expression levels of Claspin and Chk1, and thus is required for efficient replication checkpoint activation.

Protein kinases control replication checkpoint in response to biological stresses

The key step for the replication checkpoint signaling in response to replication stress and fork stalling is the recruitment of multiple protein kinases to the stalled fork. ATR kinase triggers DNA replication checkpoint signaling in response to replication stress. ssDNA, generated at the stalled replication fork, is coated by RPA, which then recruits ATRIP, the activator of ATR. In response to replication stress, Chk1-binding domain (CKBD) in Claspin is phosphorylated. This is conducted primarily by Cdc7 kinase in cancer cells, while CK1γ1 is predominantly responsible in non-cancer cells (Fig. 2; Yang et al., 2019). This phosphorylation is absolutely required for binding of Chk1 to Claspin, a step prerequisite for checkpoint activation.

ATR and Chk1 kinases cooperatively promote replication checkpoint. Chk1 phosphorylation mediated by Claspin and ATR induces Cdc2/cyclin B1-mediated cell cycle arrest and p53-regulated cellular responses to ssDNA breaks, including DNA repair and even apoptosis when the damage is not properly fixed (Chen, 2016; Lanz et al., 2019; Ronco et al., 2017). Cdc25A, a critical factor that promotes G1/S and G2/M transition, is phosphorylated and targeted for proteasomal degradation as a result of Chk1 activation, leading to the delay of S-phase progression and mitotic entry (Goto et al., 2019; Ronco et al., 2017) (Fig. 2). Activation of Chk1 kinase also induces C-terminal acetylation of p53 (Craig et al., 2007; Shieh et al., 2000; Yogosawa & Yoshida, 2018), which enhances p53 DNA-binding affinity, resulting in upregulation of its downstream target genes (Ou et al., 2005).

Furthermore, one recent study illustrates how inhibition of ATR and Chk1 kinase activities leads to enhanced origin firing. Dual inhibition of ATR and Chk1 kinase activities destabilizes the interactions between Rif1 and phosphatase 1 (PP1), and therefore, PP1 fails to counteract the action of Cdc7 and Cdk, required for initiation, leading to increased origin firing. This is caused by phosphorylation of S2205 of Rif1 by Cdk, and authors suggest that

ATR and Chk1 suppress Cdk activity during the unperturbed S phase (Moiseeva et al., 2019) (Fig. 2). Chk1 can also increase replication fork stability in conjunction with poly(ADP-ribose) (PAR) via a C2H2 motif (C8-C6-H8-H) in Chk1 which is conserved in all vertebrates and thus was named as PAR-binding regulatory (PbR) motif (Min et al., 2013). PAR binding to Chk1 also regulates Chk1 kinase activity in an ATR-independent manner, suggesting PAR is also involved in Chk1 activation (Min et al., 2013). Chk1 also affects nucleotide metabolism. RRM1 and RRM2, two subunits of ribonucleotide reductase (RNR) are degraded upon ATR or Chk1 inhibition via increased CDK2 activities (Koppenhafer et al., 2020). At cellular level, as RRM1 and RRM2 are degraded by ATR or Chk1 inhibition, cells enter an apoptotic state due to persistent DNA damages, suggesting a role of ATR and Chk1 in maintenance of nucleotide pools (Koppenhafer et al., 2020). Furthermore, ATR-mediated phosphorylation of Chk1 at Ser-317 and Ser-345 induces its auto-phosphorylation at Ser-296 (Okita et al., 2012). The phosphorylated Ser-296 generates a docking site for 14-3-3γ protein on Chk1 and also enhances interaction between Cdc25A and 14-3-3 protein, promoting the complex formation among Chk1, 14–3-3, and Cdc25A, leading to Cdc25A degradation and preventing mitotic entry (Goto et al., 2014; Kasahara et al., 2010). Chk1 can activate DNA repair pathways by phosphorylating BRCA2 and RAD51 (Bahassi et al., 2008; Enomoto et al., 2009; Ou et al., 2005).

Next, we would like to discuss structural basis on how ATR, Claspin and Chk1 coordinate replication checkpoint. A recent structural analysis demonstrates that dimerized ATR kinase forms a complex with ATR-interacting proteins (ATRIP) (Rao et al., 2018). ATR is composed of N-terminal heat repeats (N-HEAT; residues 1–1383), FAT (FRAP, ATM, TRRAP) domain, a kinase domain (KD), and a C-terminal short fragment referred to as FATC. The structure of ATR–ATRIP complex with a short peptide of Chk1 (residues 343–352) has also been determined. The short Chk1 fragment is modeled into the KD polypeptide and shown to pack against the structure formed by FATC, catalytic loop, and activation loop. Thus, Ser-345 in Chk1 is right in front of the catalytic site of ATR and can be efficiently phosphorylated (Rao et al., 2018). A study on the functional activities of Chk1 phosphorylation sites showed that defective Ser-345 phosphorylation of Chk1 exhibits impaired replication checkpoint and aberrant mitosis, resulting in failure to be localized in the cytoplasm; therefore, Ser-345 phosphorylation is critical for replication checkpoint activation, mitotic progression, and cytoplasmic localization (Niida et al., 2007). On the other hand, Ser-317, another crucial residue for phosphorylation of Chk1, affects replication checkpoint and chromatin binding abilities of Chk1 but not mitotic progression and cytoplasmic localization (Niida et al., 2007), suggesting differential regulation exerted by distinct phosphorylation sites.

Another recent structural study illustrates that Chk1 can recognize phosphorylated Claspin with its Lys-54, Arg-129, Thr-153, and Arg-162 within the kinase domain (Chk1-KD, residues 1–270). These amino acid residues fit to the phosphorylated Ser-945 of Claspin and contribute to the proper interaction between Chk1 and Claspin (Day et al., 2021). It further shows that Chk1-KD can still interact with one of its substrates, Cdc25C, in the presence of bound Claspin, as indicated by fluorescent polarization (FP) assay and NADH-coupled ATPase experiments. These results suggest that Claspin–Chk1 interactions do not have conspicuous impacts on Chk1 kinase activity and that Claspin merely acts as a mediator protein to recruit Chk1 for subsequent replication checkpoint signaling (Day et al., 2021).

Crosstalk between the replication stress checkpoint and general biological stresses

It has been reported that ATR/Claspin/Chk1 or Mec1(Rad3)/Mrc1/Rad53(Cds1) can be activated by various biological stresses (Duch et al., 2013, 2018; Tuul et al., 2013). In budding yeast, heat shock, osmotic stress, hydrogen peroxide (H_2O_2), and nutrient deprivation are shown to induce replication inhibition, in a manner independent of Mec1 and Rad53 (Bennett & Clarke, 2006; Duch et al., 2013, 2018; Tuul et al., 2013) (Fig. 4A). However, it depends on Mrc1, and critical phosphorylation events are identified that occur in response to these stresses. A comprehensive kinase screening reveals that multiple stress-activated protein kinases (SAPKs) are capable of phosphorylating the specific N-terminal target residues in response to specific cellular stress (Duch et al., 2018). For example, Hog1 interacts with and phosphorylates Mrc1 upon osmotic stress, coordinating replication program with replication stress induced by transcription–replication collision (Duch et al., 2013, 2018). The hog1 mutants fail to phosphorylate Mrc1 and does not slow down *S* phase, exhibiting DNA damages. Also, phosphorylated Mrc1 induced by Hog1 promotes Cdc45 unloading and reduces replication fork rate (Duch et al., 2013). Likewise, heat stress, oxidative stress or glucose deprivation induces Mpk1, Psk1 and Snf1, respectively, which phosphorylate the critical N-terminal target residues of Mrc1 (Duch et al., 2018). In mammalian cells, Claspin–Chk1 is activated by Unfolded Protein Response (UPR) induced by stresses such as hypoxia to slow down replication fork and reduce origin firing (Fig. 4B). The inhibition of DNA synthesis depends on UPR effector PERK, and is associated with phosphorylation of Claspin (Cabrera et al., 2017). On the other hand, hypoxia induced Senataxin, an RNA–DNA hybrid helicase, in a PERK-dependent manner. Senataxin decreases

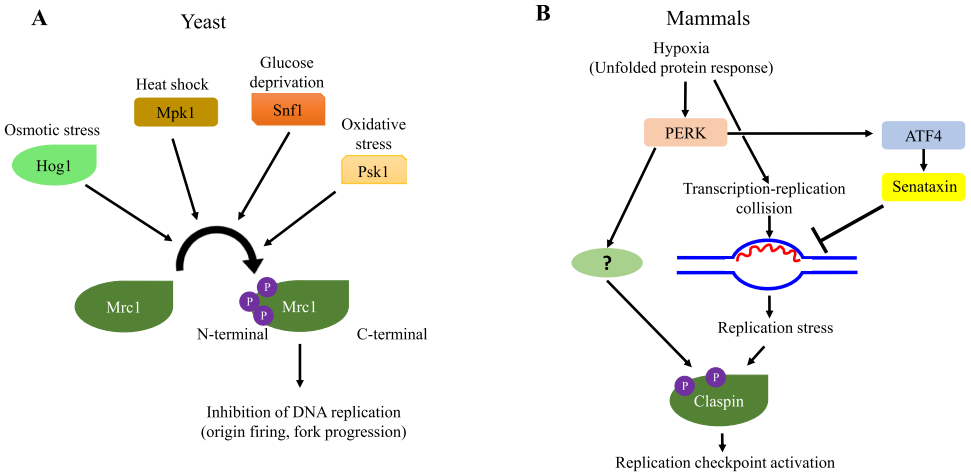

Fig. 4 Claspin/Mrc1 coordinates replication checkpoint signaling in response to various biological stresses. **A** Different cellular stresses can induce stress-specific kinases, which phosphorylate Mrc1, triggering replication checkpoint signaling in budding yeast. Hog1, Mpk1, Snf1, and Psk1 in yeast phosphorylate the N-terminal segment of Mrc1 in response to osmotic stress, heat shock, glucose deprivation, and oxidative stress, respectively (Duch et al., 2013, 2018). This was termed "Mrc1 transcription–replication safeguard mechanism" and was proposed to serve for maintaining genomic integrity in response to various cellular stresses. **B** In mammalian cells, hypoxia induces unfolded protein response through PERK, and this would lead to Claspin phosphorylation and Chk1 activation in a manner dependent on PERK. Hypoxia induces transcription-replication collision, which results in formation of RNA–DNA hybrids (R-loop). This also would lead to replication stress-induced Claspin–Chk1 activation. Hypoxia also induces Senataxin (RNA–DNA hybrid helicase) through PERK-ATF4, which antagonizes the formation of RNA–DNA hybrids and reduces the replication stress

the numbers of DNA–DNA hybrids and protects cells from DNA damages (Ramachandran et al., 2021).

We observe that Chk1 is activated by a spectrum of cellular stresses, including heat, osmotic stress, arsenate, oxidative stress, hypoxic stress, glucose shock and so forth, in mammalian cells, and this activation relies on Claspin (Yang et al., unpublished data). Some of these cellular stresses appear to directly induce replication stress, whereas others may activate Claspin–Chk1 through pathways distinct from replication stress.

In conclusion, Mrc1/Claspin coordinates biological stress signals as a general mediator which may or may not activate the downstream effector kinase (Rad53 or Chk1). Currently, upstream sensor and downstream effector kinases of these stress-induced replication checkpoint are being examined in more detail.

The biological significance of the ATR-Claspin-Chk1 axis in oncogenesis and clinical phenotypes in cancer patients

Genome instability is a major driving force that contributes to cancer development and it would be crucial to understand the molecular basis of genome instability to develop novel therapeutic strategies. Therefore, major efforts have been made on studies of mechanistic links between the impaired replication checkpoint control and genome instability. Claspin plays a central role as a mediator between ATR and Chk1, maintaining replication fork stability and safeguarding the genome (Faustrup et al., 2009; Goto et al., 2019; Ito et al., 2018; Kim et al., 2008; Martin et al., 2015; McGarry et al., 2016; Scorah & McGowan, 2009; Sørensen et al., 2003; Yang et al., 2019; Yuan et al., 2014). The ATR-Claspin-Chk1 pathway ensures genome integrity and potentially prevents oncogenesis by several proposed mechanisms, including inhibition of mitotic progression with incompletely duplicated genome, suppression of initiation at dormant origins under replication stress (e.g., HU treatment), and regulation of dNTP pool (Lecona & Fernandez-Capetillo, 2018; Oakes et al., 2014).

In the following section, we would like to discuss clinical correlation between the ATR-Claspin-Chk1 axis and cancer formation. ATR was shown to be upregulated in adenoid cystic carcinoma (ACC), mediated by MYB, a transcription factor noted to be activated in ACC, and this may affect ATR-Claspin-Chk1 signaling (Andersson et al., 2020). However, general roles of Claspin in oncogenesis are still controversial, as it could either support or inhibit cancer cell growth depending on circumstances (Azenha et al., 2017; Bianco et al., 2019; Cai et al., 2021; Kobayashi et al., 2019, 2020; Wang et al., 2017; Yuan et al., 2014). For instance, a recent report shows that Claspin and Tim are overexpressed in primary lung, colorectal and breast cancer specimens and that Claspin together with Tim protein can enhance cell proliferation in untransformed fibroblasts and HCT116 cells, a colorectal carcinoma cell line (Bianco et al., 2019). This report further shows that reduction of Claspin and

Tim expression decelerates replication fork progression in HCT116 cells and these two proteins promote the tolerance of oncogene-induced replication stress where an oncogenic form of Ras protein is introduced. Overall, higher expression levels of Claspin and Tim are positively associated in some primary cancer cells and cancer cell lines, and protects cancer cells from oncogene-induced replication stress in a checkpoint-independent manner (Bianco et al., 2019). On the other hand, Claspin may also possess tumor suppressive functions. For example, the expression level of Claspin can be stabilized by USP20, a peptidase for ubiquitin that targets Claspin for degradation, in gastric cancer (GC) cells and a recent study shows that USP20 expression is positively correlated with expression levels of Claspin and that a lower expression level of Claspin is intimately linked to poorer survival and prognosis in GC patients. Indeed, Claspin is one of the major substrates targeted by USP20 (Yuan et al., 2014) and both can suppress tumorigenesis (Wang et al., 2017). Hence, the dual functions of Claspin, both positive and negative, in carcinogenesis require further investigation. Furthermore, Chk1 overexpression has also been noted in several malignant cancers, including breast cancer, T-cell acute lymphoblastic leukemia (T-ALL), and neuroblastoma (Ando et al., 2019; Sarmento et al., 2015; Wu et al., 2019). For instance, Chk1 is overexpressed at mRNA level both in T-ALL cell lines and clinical specimens of T-ALL patients (Sarmento et al., 2015). Chk1 inhibition in T-ALL cell lines causes premature occurrence of DNA replication and induces significantly higher levels of DNA damage and cancer cell death. In T-ALL xenograft models, Chk1 inhibition also retards tumor formation.

Claspin has also been linked to oncovirus-associated cancer formation (Benevolo et al., 2012; Koganti et al., 2014, 2020; Spardy et al., 2009). It has been shown that several oncogenic viruses interfere with the ATR-Claspin-Chk1 pathway. Oncogenic Epstein–Barr virus (EBV) activates STAT3 in B lymphocytes, a transcriptional factor that induces a caspase cascade involving caspase 9 and caspase 7. Caspase 7 then targets Claspin for proteasomal degradation and inhibits Chk1 phosphorylation at Ser-345 (Koganti et al., 2020). Disrupted Claspin–Chk1 pathway permits EBV-infected cells to continuously proliferate regardless of oncovirus-induced replication stress and DNA lesions, leading to active viral replication and tumorigenesis.

To conclude, acquired mutations and anomalous expression in any component of the ATR-Claspin-Chk1 axis or disruption of its operation can perturb replication stress signaling pathway. The level of Claspin can be high or low in different cancer cell lines, and thus, the high level of Claspin expression could trigger oncogenesis in some cases and in others suppress oncogenesis (Bianco et al., 2019; Cai et al., 2021; Kobayashi et al., 2019, 2020; Wang et al., 2017; Yuan et al., 2014). This suggests the potential of the ATR-Claspin-Chk1 axis as an attractive therapeutic target for cancer treatment, but the strategy could be different depending on the nature of the cancer cells.

Targeting the ATR-Claspin-Chk1 axis and its utilization as biomarkers for cancer therapeutics

Manipulation of the ATR-Claspin-Chk1 replication checkpoint pathway could be a potential target of novel cancer treatment strategy (Azenha et al., 2017). Cancer cells possess accumulating genetic alterations due to the defective DDR responses (Dieltein et al., 2014; Burgess et al., 2020). The aberrations of replication checkpoint and the subsequent impairment of the DDR signaling pathways would permit cancer cells to continue to progress through the cell cycle in the presence of replication stress. Frequent upregulation of components for the ATR-Claspin-Chk1 axis in clinical cancer samples leads to the proposal that cancer cells are more heavily dependent on the ATR-Claspin-Chk1 pathway for survival through replication stress compared to normal cells. Thus, targeting this pathway and the associated DDR in cancer cells may render them more susceptible to replication impediments, thereby inducing cancer cell death (Bianco et al., 2019; Cai et al., 2021; Choi et al., 2014; Gilad et al., 2010; Kobayashi et al., 2019, 2020; Tsimaratou et al., 2007). Accordingly, inhibition of the ATR-Claspin-Chk1 axis in combination with added replication stress has been exploited to suppress cancer cell growth and now is under clinical trials for several cancer treatment regimen (Boudny & Trbusek, 2020; Gralewska et al., 2020; Sanjiv et al., 2016; Barnieh et al., 2021; Dent, 2019) (Tables 1, 2, 3, 4).

For instance, VE-821, an efficacious ATR inhibitor, strongly suppresses ATR signaling exemplified by significantly downregulated Chk1 phosphorylation at Ser345, a marker for checkpoint activation and a critical residue for subsequent replication checkpoint activation (Huang & Zhou, 2020). The drug increases γH2AX signals and decreases Rad51 foci in primary and cultured pancreatic cancer cells (Prevo et al., 2012), suggesting reduced DNA repair in the presence of the ATR inhibitor. Moreover, AZD7762, a Chk1/2 inhibitor, greatly sensitizes cancer cells to gemcitabine, an inhibitor of DNA synthesis, in urothelial cancer cell lines (UCCs). In MCF-10A (human normal breast epithelial cell cells) and B16-F10 (melanoma cells), AZD7762 in combination with ionizing radiation results in abscopal tumor response through increased micronuclei formation and immune activation signaling (Chao et al., 2020; Prevo et al., 2012). AZD7762 also sensitizes urothelial carcinoma cells to gemcitabine (ionizing radiation mimetics) by inhibiting DNA repair and disturbing checkpoints, supporting the combination of gemcitabine with Chk1 inhibition as

Table 1 ATR inhibitors under clinical trials

Agent	Effects	Target cells	References
VX-970 (VE-822)	Inhibition of Chk1 phosphorylation, retarded tumor growth	PDAC, Non-small cell lung cancer (NSCLC) cell lines	Barnieh et al. (2021) and Hall et al. (2014)
AZD6738, an improved form of AZ20	Accumulation of increased unrepaired DNA damage, mitotic catastrophe in ATM-deficient cells, inhibition of cancer cell growth, production of micronuclei	NSCLC, HT29, A549, Cal27, FaDu, HCT116, H460, PDAC	Foote et al. (2015) and Dillon et al. (2017)
BAY1895344	Increased unrepaired DNA damage, anti-proliferation of cancer cells in combination with chemotherapies	M059J, HT29, LoVo, MCF-7, LAPC-4, MDA-MB-436, PC-3, MDA-MB-468, Caco2, U-87MG HCC70, HCT116, HeLa	Foote et al. (2015) and Wengner et al. (2020)
M4344 (VX-803)	Inhibition of 308-kinase activities and tumor regression	A panel of 92 cancer cell lines	Foote et al. (2015) and Zenke et al. (2019)

a promising urothelial cancer therapy (Isono et al., 2017). AZD7762 also induces synthetic lethality in combination with ATR inhibition (VE-821) in U2OS osteosarcoma cancer cell line, through replication fork arrest, ssDNA accumulation and replication collapse (Sanjiv et al., 2016).

As with other factors for replication checkpoint, Claspin is more frequently upregulated in cancer tissues. Although no Claspin inhibitors have been developed for clinical trials so far, Claspin has been utilized as a proliferation biomarker since its anomalous expression is associated with oncogenic progression, resistance to cancer chemotherapies, and metastases (Choi et al., 2014; Tsimaratou et al., 2007). Upregulation of Claspin expression either at the mRNA or at the protein level has been clinicopathologically shown in certain types of cancer, including, GC, renal cell carcinoma, colorectal carcinoma, prostate cancer, lung cancer, brain cancer, and so on (Bianco et al., 2019; Cai et al., 2021; Choi et al., 2014; Kobayashi et al., 2019, 2020; Tsimaratou et al., 2007). For instance, in prostate cancer, a recent report shows that Claspin overexpression is associated with tumor progression, more aggressive and metastatic nature of the tumor, and relatively poor survival rate in patients, indicating an oncogenic role of Claspin (Kobayashi et al., 2019). Elevated levels of Claspin mRNA and protein in clinical samples may suggest the therapeutic as well as diagnostic and prognostic potential of Claspin in these types of cancers.

Conclusion

In past decades, a wide range of studies on the ATR-Claspin-Chk1 replication checkpoint pathway has revolutionized our perspective regarding the molecular mechanisms of replication checkpoint in oncogenic signaling and its utilization for novel cancer therapies. However, Claspin is differentially involved in the regulation of replication and checkpoint depending on the cell types, tissue, and cancer types in clinical settings. For example, Cdc7-mediated phosphorylation of Claspin at CKBD may be more specific to cancer cells, and in normal cells, CK1γ1 would play a more significant role. In fission yeast, Mrc1 exerts brake for initiation, and Cdc7(Hsk1)-mediated phosphorylation releases this break for initiation. Tumor suppressive role of Claspin in some cancer cells may reflect a potential negative role of Claspin for initiation. Thus, development of anti-Claspin agents for cancer therapy would require further careful investigation.

Future perspectives

Recent studies on Claspin described above have deepened our understanding of how Claspin facilitates initiation and replication fork progression, and maintains genome integrity in the presence of replication stress and how its dysfunction potentially gives rise to oncogenesis. Claspin/Mrc1 plays positive roles in replication initiation and fork progression during normal course of DNA replication, and it would negatively regulate replication upon replication stress and also potentially the initiation stage. Studies on budding yeast Mrc1 indicate that it may act as a brake for replication initiation for fine tuning of initiation timing (Matsumoto et al., 2017). The mechanisms of this negative regulation by Clapsin/Mrc1 of both DNA chain elongation and initiation need to be clarified. Indeed, a recent report indicates Rad53 (effector kinase)-mediated phosphorylation of Mrc1 in response to replication stress inhibits its fork progression activity (McClure & Diffley, 2021).

Besides, roles of Claspin in various cell types and during development have not been investigated. Knockout of Claspin is embryonic lethal at E12.5, suggesting its crucial roles for early development (Yang et al., 2016). However, tissue-specific KO of Claspin may lead to different phenotypes. Claspin's roles as negative or positive regulator of

Table 2 ATR inhibitors under preclinical development

Agent	Effects	Target cells	References
AZ20	Downregulation of Chk1 phosphorylation, accumulation of unrepaired DNA damage	GRANTA-519, JVM2, LoVo	Barnieh et al. (2021)
Schisandrin B	Inhibition of UV-activated G2/M and S-phase checkpoints, suppression of ATM kinase, block of epithelial-mesenchymal transition (EMT)	Primary breast cancer cells, 4T1, MDA-MB-231, S180	Barnieh et al. (2021), Liu et al., (2012a, 2012b) and Xu et al. (2011)
NU6027	Inhibition of homologous recombination, synthetic lethality with PARP inhibition	MCF7, L1210	Barnieh et al. (2021) and Peasland et al. (2011)
Dactolisin (NVP-BEZ235)	Inhibition of PI3K/mTOR activities, other ATR homologs, ATM, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), suppression of DNA damage responses	U2OS	Barnieh et al. (2021) and Toledo et al. (2011)
EPT-46464	Inhibition of PI3K/mTOR activities, other ATR homologs, ATM, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs)	U2OS	Barnieh et al. (2021) and Toledo et al. (2011)
Torin-2	Inhibition of PI3K/mTOR activities, induction of replication and mitotic catastrophe, cancer cell death	NIH3T3, MCF-10A, MCF-10F, MCF-7, MDA-MB-23	Barnieh et al. (2021), Gilad et al. (2010) and Chopra et al. (2020)
VE-821	Inhibition of Chk1 phosphorylation at Ser-345 after treatment with gemcitabine and radiation, disruption of cell cycle checkpoints, induction of replication and mitotic catastrophe, retarded cancer cell growth	PSN-1, MiaPaCa-2, PANC-1, HCT116	Barnieh et al. (2021), Foote et al. (2015) and Prevo et al. (2012)

Table 3 Chk1 inhibitors under clinical trials

Agent	Effects	Target cells	References
UCN-01	Inhibition of Chk1/2, CDK1/2, MK2, protein kinase C (PKC) and p53, enhanced sensitivity to histone deacetylase inhibitors (HDACi) in normal cells, sensitization of tumor cells to chemotherapies and ionizing radiation (IR)	HCT116, A549	Qiu et al. (2018), Yu et al. (2002) and Lara et al. (2005)
AZD7762	Sensitization of tumor cells to chemotherapies and IR, enhanced sensitivity to histone deacetylase inhibitors (HDACi) in normal cells, synthetic lethality when in combination with ATRi	4T1.2, MDA-MB-231, MCF-7 RAW264.7, MC3T3, MLO-A5, UCCs MCF-10A, B16-F10	Qiu et al. (2018), Prevo et al. (2012), Chao et al. (2020), Isono et al. (2017) and Wang et al. (2018)
LY2603618	Selective inhibition of ATP-binding sites in Chk1, inactivation of G2/M DNA damage checkpoint, suppression of tumor growth in combination with gemcitabine	HeLa, Calu-6, HT29, HCT116	Qiu et al. (2018) and King et al. (2014)
MK-8776	Radio-sensitization of tumor cells by aggravating IR-induced mitosis and inhibiting autophagy	MDA- MB-231, BT-549, CAL-51, EMT6, HeLa	Qiu et al. (2018), Zhou et al. (2017) and Suzuki et al. (2017)
PF-00477736	Inhibition of cytosolic translocation of phosphorylated Cdc25C, induction of anti-proliferative activities in combination with docetaxel, gemcitabine and carboplatin by interfering G1/S and mitotic checkpoint, potentiation of apoptosis	COLO205, MDA- MB-231,HT29, human umbilical vein endothelial cells	Qiu et al. (2018), Zhang et al. (2009) and Blasina et al. (2008)
LY2606368	Induction of DNA damage, loss of DNA damage checkpoint, replication and mitotic catastrophe, growth retardation of tumor cells	NCI-H460, HeLa,HT29, HCT 116,Calu-6, U-2OS	Qiu et al. (2018) and King et al. (2015)
XL-844 (EXEL-9844)	Block Chk1-induced Cdc25A degradation, higher level of gH2AX phosphorylation in association with gemcitabine, premature mitotic onset, enhanced radiosensitivity of cancer cells	PANC-1, HY29	Qiu et al. (2018), Matthews et al. (2007) and Riesterer et al. (2011)
CBP501	Induction of higher level of cytotoxic T cells in combination with cisplatin, enhanced tumor cell death in combination with immune checkpoint inhibitors and cisplatin	CT26WT, HCT15, COR-L23, NCI-H226, MIAPaCa2, HCT116, HT29, human umbilical vein endothelial cells	Qiu et al. (2018), Sakakibara et al. (2017) and Mine et al. (2011)

Table 4 Chk1 inhibitors under preclinical development

Agent	Effects	Target cells	References
SAR-020106	Significant accumulation of cells in S phase, decrease of cells in G2/M arrest, sensitization of tumor cells and induction of tumor cell death to chemotherapies and IR	LN405, T98G, A172, DBTRG, primary glioblastoma cells	Qiu et al. (2018) and Patties et al. (2019)
CHIR-124	Abrogation of Chk1-mediated cell cycle checkpoint, enhanced sensitivity to HDACi in normal cells, induction of cancer cell death in combination with topoisomerase I inhibition	MDA-MB-435, HCT116, MDA-MB-231, SW620, COLO 205, HFS, A549, LNCaP	Qiu et al. (2018), Archie et al. (2007) and Lee et al. (2011)
GNE-783	Enhanced efficacy and reduction of tumor cell growth in xenograft models in combination with gemcitabine, CPT-11, and temozolomide	HT29, HCT116	Qiu et al. (2018) and Xiao et al. (2013)
GNE-900	Enhanced efficacy and reduction of tumor cell growth in vitro and in vivo in combination with gemcitabine	HT29, HCT116	Qiu et al. (2018) and Xiao et al. (2013)
CCT244747	Inhibition of Chk1 phosphorylation, abrogation of cell cycle checkpoints, increased sensitivity of tumor cells to radiation and gemcitabine	SW620, HT29, MiaPaCa-2, Calu6, HN4, HN5, SCC090	Qiu et al. (2018), Walton et al. (2012) and Barker et al. (2016)
AR323	Reduction of proliferation and viability in melanoma cells through inducing apoptosis, premature S-phase exit and mitotic onset	A panel of 17 melanoma cell lines, HeLa	Qiu et al. (2018) and Brooks et al. (2013)
AR678	Reduction of proliferation and viability in melanoma cells through inducing apoptosis, premature S-phase exit and mitotic onset	A panel of 17 melanoma cell lines, HeLa	Qiu et al. (2018) and Brooks et al. (2013)
CH-01	Inhibition of aurora A, accumulation of DNA damage and lower cell viability in hypoxic cancer cells	RKO, A549, H1299, H1975	Qiu et al. (2018) and Cazares-Körner et al. (2013)

DNA replication in various organs or tissues need to be carefully evaluated.

Moreover, there are some reports that Claspin–Chk1 is activated by other cellular stresses. In budding yeast, osmotic shock activates Mrc1 through phosphorylation by Hog1, resulting in the inhibition of DNA replication (Duch et al., 2018). Effects of various biological stresses, such as heat, nutrition deprivation, hypoxia and so forth, on the Claspin–Chk1 axis need to be evaluated to clarify how these stress pathways may crosstalk with replication checkpoint pathway.

Furthermore, the structural basis of Claspin is still lacking. It has strongly been indicated that Claspin undergoes intramolecular interaction, which may be regulated by Cdc7-mediated phosphorylation (Masai et al., 2017; Yang et al., 2016). Claspin, predicted to harbor large segments of intrinsically disordered polypeptide (IDP), may adopt variable structures depending on its bound partners and covalent modifications. Clarification on structures of Claspin under various conditions will provide important information on its modes of action at the initiation, replication fork progression and replication checkpoint.

Finally, it is also important to clarify the roles of Claspin during oncogenesis of various cancer types. Taken together, these future endeavors will lead to identification of novel diagnostic and prognostic cancer biomarkers as well as effective therapeutic strategies involving Claspin as a target.

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