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MINI-REVIEW

DDR Inc., one business, two associates

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Abstract
Eukaryotic cells activate cell cycle checkpoints in response to DNA damage. In *Saccharomyces cerevisiae*, the DNA damage response is achieved by the activation of the sensor kinases Mec1 and Tel1 and transmitted to the effector kinase Rad53. Rad9 and Mrc1 are thought to differentially mediate the activation of Rad53 depending on the cell cycle phase. Rad9 can respond to DNA lesions throughout the cell cycle, whereas Mrc1 responds to replication impediments in S phase. It was not clear if Rad9 and Mrc1 were triggering the same response to DNA damage occurring in S phase. By carefully studying the kinetics of activation of Rad53 by different types of replication stresses, we recently showed that Rad9 and Mrc1 cooperate in time and space to trigger a unique response to DNA damage in S phase. This primarily includes the control of both DNA replication initiation and elongation. After showing that Rad9 plays a preponderant role during S phase, the data presented here provocatively suggest that Mrc1 could also mediate the activation of Rad53 outside of S phase.

Keywords Rad9 · Mrc1 · DNA replication · S-phase checkpoint · Mediators · Replication stress

Signaling pathways have evolved in eukaryotic cells in response to DNA damage to coordinate DNA repair with the progression of the cell cycle. These pathways activate checkpoints during any cell cycle phase to prevent the persistence, duplication or transmission of damaged DNA molecules. These actions allow the maintenance of genome integrity, thus safeguarding cellular functions. At the molecular level, these sophisticated signaling cascades involve protein kinases acting as sensors and effectors. These kinases phosphorylate various targets to achieve both the activation and transmission of the signal throughout the cell to trigger an adequate response to DNA damage (Ciccia and Elledge 2010). DNA damage is detected as discontinuities in the double-stranded DNA molecule, i.e., gaps or breaks. DNA gaps contain single-stranded DNA (ssDNA) and 5′ junctions between single-stranded and double-stranded DNA fragments. In the budding yeast *Saccharomyces cerevisiae*, these structures are, respectively, recognized by the heterotrimer RPA (Rfa1-Rfa2-Rfa3) and 9-1-1 (Ddc1-Rad17-Mec3) complexes (Majka and Burgers 2003; Rouse and Jackson 2002; Zou and Elledge 2003). On one hand, RPA allows the recruitment of the sensor kinase Mec1 through its binding partner Ddc2 and, on the other hand, 9-1-1 activates it (Majka et al. 2006; Navadgi-Patil and Burgers 2009; Paciotti et al. 2000). Mec1 can also be activated by the scaffold protein Dpb11 and the DNA helicase/nuclease Dna2 (Kumar and Burgers 2013; Navadgi-Patil and Burgers 2008). DNA double-strand breaks (DSBs) are recognized by the MRX complex (Mre11-Rad50-Xrs2) and the sensor kinase Tel1 (Grenon et al. 2001; Nakada et al. 2003). Repair of DSBs requires the processing of DSB ends, generating ssDNA tails that can recruit and activate Mec1. Mec1 then phosphorylates the effector kinase Rad53 (Ma et al. 2006; Sanchez et al. 1999, 1996). Yet, this step requires the involvement of mediator proteins that bring Rad53 in close proximity to Mec1 at the sites of DNA damage and promote its autophosphorylation.

Rad9 is one of such mediators (Weinert 1998). It is recruited to DNA damage sites through the interaction with modified histones H3, methylated on lysine 79 by Dot1, and H2A phosphorylated on serine 129 by Mec1/Tel1 (Downs et al. 2000; Giannattasio et al. 2005; Grenon et al. 2007; Lee et al. 2014; Toh et al. 2006; Wysocki et al. 2005). It can be stabilized there by interacting with Dpb11 and the 9-1-1 complex (di Cicco et al. 2017; Pfander and Diffley 2011).

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Rad9 becomes phosphorylated by Mec1 and is then able to recruit two Rad53 molecules to stimulate their autophosphorylation and activation (Emili 1998; Gilbert et al. 2001; Sweeney et al. 2005). Another mediator of Rad53 activation is Mrc1 (Alcasabas et al. 2001; Tanaka and Russell 2001). Mrc1 is a constitutive component of the replication fork, where it links the DNA polymerase ε with the replicative helicase component Mcm6 (Katou et al. 2003; Komata et al. 2009; Lou et al. 2008). It also forms a complex with Csm3 and Tof1 to promote normal fork progression (Bando et al. 2009; Calzada et al. 2005; Nedelcheva et al. 2005; Szyjka et al. 2005; Tourrière et al. 2005; Yeeles et al. 2017). As a consequence of its location, Mrc1-mediated activation of Rad53 is dependent on DNA replication during S phase. Any impediment to the progression of replication forks is thought to induce the uncoupling between the helicase and polymerase activities or between the leading and the lagging strand synthesis, which would lead to the accumulation of ssDNA and 5′ ssDNA/dsDNA junctions at unligated Okazaki fragments. Alternatively, fork stalling could induce the controlled degradation of nascent DNA, also leading to the accumulation of the same DNA structures. Both scenarios are suited to promote the recruitment and activation of Mec1, which phosphorylates Mrc1, in turn mediating the activation of Rad53 (Alcasabas et al. 2001; Chen and Zhou 2009; Osborn and Elledge 2003; Smolka et al. 2006; Tanaka and Russell 2004; Xu et al. 2006).

Opposite to this cell cycle phase-specific activation of Rad53 via Mrc1, Rad9 can virtually mediate Rad53 activation at any time (Siede et al. 1993; Weinert 1998). Nevertheless, the slowdown of S phase in response to damage in the template DNA or any other impediment to replication fork progression was described to be dependent on Mrc1 and not on Rad9 (Alcasabas et al. 2001; Katou et al. 2003). This has led to a conceptual separation of activating pathways of the DNA damage response (DDR) during S phase, Mrc1 triggering the DNA replication checkpoint (DRC), and Rad9 the DNA damage checkpoint (DDC) (Branzei and Foiani 2010; Crabbé et al. 2010; Pardo et al. 2017). Yet, irrespective of the way by which Mrc1 or Rad9 are invoked, each of them alone can mediate almost identical phosphorylation patterns of Rad53 (Chen et al. 2014). This fact questions the pertinence of defining two separate checkpoint pathways and prompted us to investigate the interplay between Rad9 and Mrc1 in response to DNA damage in S phase.

In our recent work by Bacal, Moriel-Carretero et al. (2018), we aimed at looking if the DDC could repress the activation of late-firing replication origins, a well-described function of Rad53 when activated by the DRC. As a matter of fact, replication fork stalling caused by the lack of dNTPs in cells treated with hydroxyurea (HU) induces the repression of late origins through Mrc1, not Rad9 (Crabbé et al. 2010). Unlike HU, MMS induces a Rad9-dependent activation of Rad53 (Emili 1998; Schwartz et al. 2002). However, Rad53 has been shown to repress late origins when cells replicate DNA in the presence of MMS (Tercero and Diffley 2001; Tercero et al. 2003), suggesting that both Rad9 and Mrc1 are required to orchestrate the DDR to MMS. Indeed, we observed that the repression of late origins in MMS-treated cells depends primarily on Mrc1, but we could see that Rad9 is also involved in this process if the amount of MMS-induced DNA lesions does not impede replication fork progression. In this situation, DNA lesions can be bypassed and left behind forks, where Rad9 can be recruited (Bacal et al. 2018). This is in complete agreement with a recent work by García-Rodríguez et al. (2018), which shows that Rad9-mediated activation of Rad53 (DDC) in response to MMS is triggered by ssDNA gaps that form on nascent DNA after re-priming of DNA synthesis downstream of the DNA lesions.

Nevertheless, the role of Rad9 in repressing late origins was minor in these conditions. We could propose an explanation for this minor contribution of Rad9 considering the kinetics of Rad53 activation mediated by Mrc1 and Rad9. Mrc1 is required for the fast but transient activation of Rad53, whereas Rad9 is necessary for a slower but sustained activation (Fig. 1). We think that this transient DRC signaling is linked to the physical association

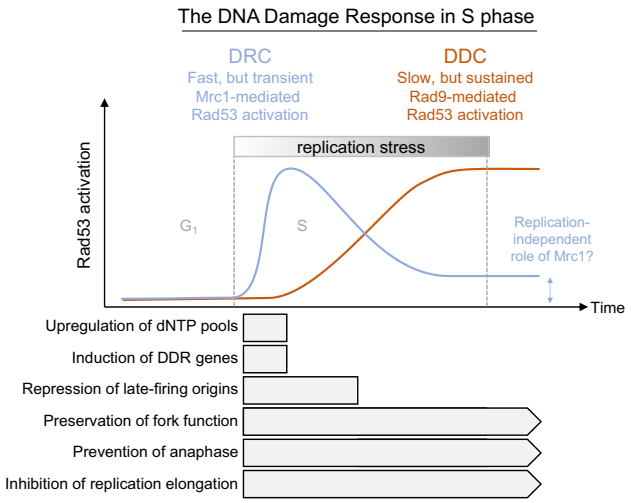

Fig. 1 Schematic representation of the kinetics of Rad53 activation by the DRC and the DDC and the S phase checkpoint functions during replication stress. We propose that the transcriptional response to up-regulate the dNTP pools and to express the genes required for DNA repair (Chabes and Thelander 2003; de Bruin and Wittenberg 2009; Dmowski and Fijalkowska 2017; Mikolaskova et al. 2018) is triggered by the DRC as an early event. The repression of late origins (Yoshida et al. 2013) remains possible until the last origins have been fired, and is mainly a DRC function. Finally, the preservation of the fork ability to restart (Labib and De Piccoli 2011), the slow-down of fork progression and the cell cycle arrest before the end of mitosis (Palou et al. 2017) are functions that are shared by the DRC and DDC and that remain activated until the resolution of the replication stress

of Mrc1 to replication forks and to the observation that a threshold number of stalled forks needs to be reached to activate this pathway (Shimada et al. 2002; Tercero et al. 2003). As cells progress through S phase, adjacent replicons gradually merge and the number of active forks irremediably decreases, thus reducing the number of fork-associated Mrc1 molecules below the threshold required to activate the DRC. Consequently, replicated DNA regions accumulate and provide an increasing number of sites for Rad9 recruitment. The Rad9-mediated activation of Rad53 is slow because histones must be modified prior to Rad9 recruitment. Moreover, the ssDNA gaps left behind replication forks need to be processed for an efficient DDC activation (Galanti and Pfander 2018; García-Rodríguez et al. 2018). Finally, Rad53 activation by Rad9 is dampened by the Slx4–Rtt107 complex, which competes with Rad9 for the binding to DNA damage sites (Balint et al. 2015; Cussiol et al. 2015; Ohouo et al. 2013). In contrast, Mrc1 is an integral component of the replisome and is ideally positioned to mediate a fast activation of Rad53 in response to any replication impediment (Alcasabas et al. 2001). We reasoned that the DDC should therefore be able to efficiently repress the activation of late-firing origins if activated early enough. We have validated this hypothesis by activating the DDC with a limited amount of DNA double-strand breaks in cells blocked in early S phase and showed that Rad9 can efficiently mediate the repression of late origins when cells are allowed to resume replication (Bacal et al. 2018).

Additionally, our study unambiguously clarified that the DDR can downregulate the progression of replication forks when the template DNA is damaged. We first observed that *rad9*Δ mutants progressed faster through S phase than wild-type cells when exposed to MMS, and this could not be entirely explained by the modest derepression of a subset of late origins (Bacal et al. 2018). Nevertheless, this was not enough to claim that replication forks in wild type cells were being actively slowed down by the DDC signaling and not by the presence of an alkylated DNA template, even if we used a low dose of MMS. We then set up conditions to induce a limited number of DSBs with Zeocin (around 10 breaks per genome when cells enter into S phase) to induce the DDC without impeding replication initiation. This allowed us to follow the progression of individual replication forks under high DDC and low DRC activation. Our results showed that Rad9-mediated Rad53 activation at DSBs was slowing down replication progression in *trans* (Bacal et al. 2018) (Fig. 1). These results raised the question of whether the down-regulation of replication fork progression was specific to the DDC. We do not think this is the case because replication fork stalling is the trigger for DRC activation and this precludes to appreciate a fork slowing down mediated by Rad53. Conversely, we have reported earlier that the DRC and not the DDC could prevent homologous recombination (HR) events in S phase by inhibiting the end processing of DSBs (Alabert et al. 2009). Yet, the Rad9-dependent DDR to DSBs is a well-described negative regulator of DSB end resection (Bonetti et al. 2015; Ferrari et al. 2015). In light of our new results describing the kinetics of Rad53 activation by Mrc1 and Rad9, we can now propose that Rad9 could mediate the inhibition of HR by the DDR during S phase if the DDC were activated early enough. Altogether, we conclude that the DDC involving Rad9-mediated activation of Rad53 by Mec1 is the main response to DNA damage during all the phases of the cell cycle. As the regulation of S phase progression in response to DNA damage requires the timely activation of Rad53 to delay the activation of late-firing replication origins, cells have evolved a complementary pathway to activate Rad53 in early S phase (DRC) involving a specific component of the replisome, Mrc1. Rad9 and Mrc1 thus mediate the continuous activation of Rad53, cooperating to ensure the early and late functions of a unique response to DNA replication stress (Fig. 1).

Intriguingly, we noticed that Rad53 phosphorylation was not completely abolished in the *rad9*Δ mutant when DSBs were induced in G_1 by Zeocin and cells released into S phase (Bacal et al. 2018) (Fig. 2). Indeed, we reproducibly observed a subtle phospho-shift of Rad53 from 90 min after release into S phase when cells had reached the G_2 phase, as observed by flow cytometry (Fig. 2b, c). This was confirmed by the accumulation of cyclin B2 (Clb2), an indicator of cell entry into G_2 phase (Fig. 2c). This was surprising, since the alternative pathway to activate Rad53 in the absence of Rad9 is only active in early S phase. Nevertheless, we have investigated whether this G_2-specific phosphorylation of Rad53 depends on Mrc1 by repeating the experiment in the *mrc1*Δ *rad9*Δ double mutant. We show here that the absence of Mrc1 mildly but consistently decreased the phosphorylation of Rad53 (Fig. 2c, d), raising the possibility that Mrc1 could mediate the phosphorylation of Rad53 in late S or in G_2 phases. As described by others (Doksani et al. 2009), replication forks encountering a DSB do not pause at the break site but are rapidly resolved into linear ends. This rules out the possibility that stalled forks accumulate at DSBs and reach the threshold required for DRC activation. Alternatively, this Mrc1-dependent signaling at persistent DSBs could relate to a DSB repair mechanism involving DNA synthesis such as Break-Induced Replication (BIR). BIR is described to occur during G_2, in agreement with the kinetics we report. Yet, the DNA synthesis machinery involved in BIR differs from normal replication forks and does not contain MCM helicases nor DNA polymerase ε (Lydeard et al. 2007; Wilson et al. 2013). It is therefore unclear whether Mrc1 plays an active role in BIR and could mediate Rad53 activation during this process. Alternatively,

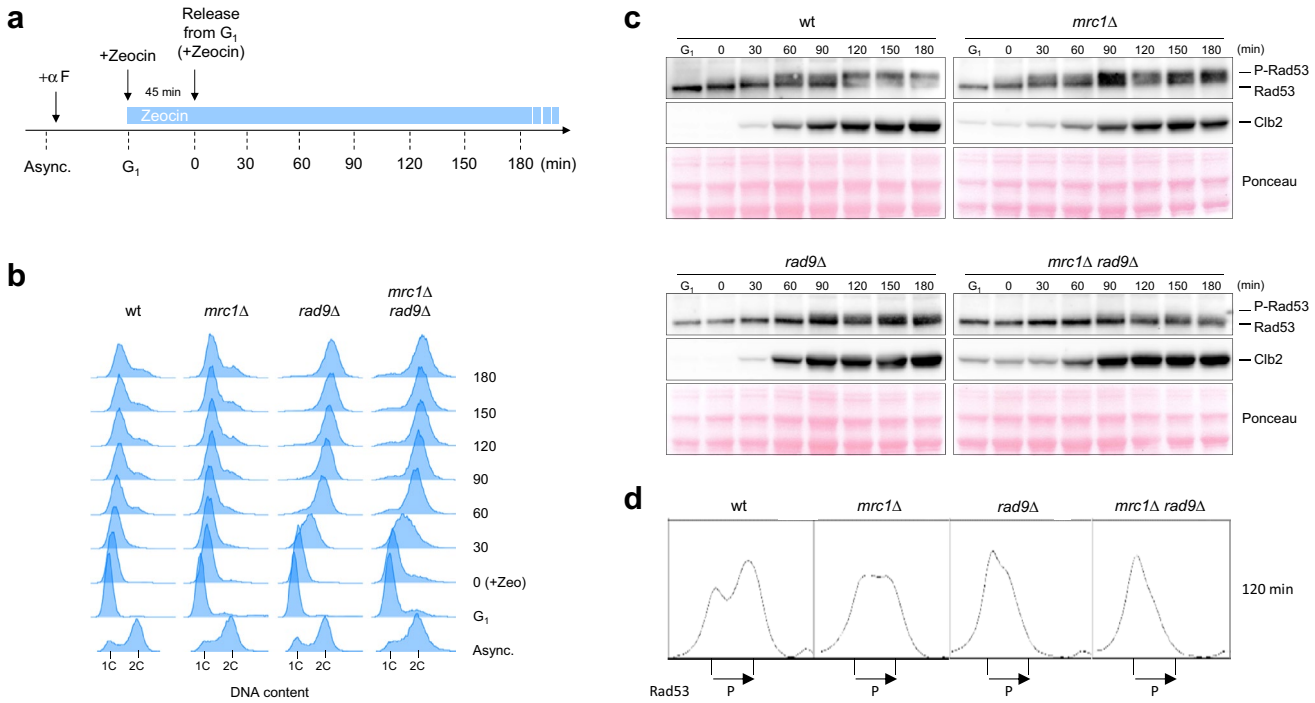

Fig. 2 Mrc1 can mediate Rad53 phosphorylation in late S/G_2 in the absence of Rad9 in response to DSBs in S phase. **a** Wild-type (PP3372), *mrc1Δ* (PP1196), *rad9Δ* (PP1197) and *mrc1Δ rad9Δ* (PP1195) cells containing a 2 µm plasmid overexpressing *RNR1* (Desany et al. 1998) were synchronized in G_1 with α-factor. 100 µg/ml Zeocin was added to the medium and cells were released from the G_1 arrest by the addition of pronase (75 µg/ml) 45 min later. Cells were collected at indicated time points. Protein extraction and Western blots were performed as described previously (Bacal et al. 2018), using anti-Rad53 (gift from C. Santocanale) and anti-Clb2 (y-180; SCBT) antibodies. **b** Analysis of DNA content by flow cytometry. **c** Analysis of Rad53 phosphorylation in G_1 prior to Zeocin addition (G_1) and at indicated times. **d** Densitometry profiles of Rad53 mobility shifts from **c** at 120 min. The increase in Rad53 phospho-shift is indicated by "P" and the arrow, from left to right

Mrc1 could contribute to Rad53 activation independent of DNA synthesis, as reported earlier for its orthologue Claspin in *Xenopus* (Yoo et al. 2006).

Still, low levels of Rad53 phosphorylation remained detectable in the *mrc1Δ rad9Δ* double mutant (Fig. 2c, d), suggesting that other factors mediate Rad53 activation in the absence of Mrc1 and Rad9. RFC^{Ctf18} and Sgs1 have been shown to facilitate Mec1-dependent phosphorylation of Rad53 at replication forks stalled by dNTP depletion in HU-treated cells (Bjergbaek et al. 2005; Crabbé et al. 2010; Kubota et al. 2011). RFC^{Ctf18} is an alternative loader/unloader for PCNA, a processivity factor for DNA polymerases (Bylund and Burgers 2005). It interacts with the DNA polymerase ε and has a function in sister-chromatid cohesion establishment during DNA replication (García-Rodríguez et al. 2015; Lengronne et al. 2006). Additionally, RFC^{Ctf18} is absolutely required for the DRC activation, for which it is epistatic with Mrc1 (Crabbé et al. 2010). As RFC^{Ctf18} has neither been described to be phosphorylated by Mec1/Tel1 in response to replication stress (Bastos de Oliveira et al. 2015; Chen et al. 2010; Smolka et al. 2007), nor to interact physically with Rad53, it cannot be considered as a canonical mediator of Rad53 activation. Instead, RFC^{Ctf18} could be required to maintain the replisome in a conformation that allows Mec1 and Mrc1 to activate Rad53 in response to replication impediments. In response to DSBs, RFC^{Ctf18} is also recruited to establish sister-chromatid cohesion but neither this process nor RFC^{Ctf18} itself are required for Rad53 activation in response to DSBs (Crabbé et al. 2010; Ogiwara et al. 2007). All these data make RFC^{Ctf18} a poor candidate for stimulating Rad53 phosphorylation in *mrc1Δ rad9Δ* cells in the presence of DSBs.

Unlike RFC^{Ctf18}, Sgs1 can interact directly with Rad53 after being phosphorylated by Mec1 (Hegnauer et al. 2012). Sgs1 is a DNA helicase that bears several functions in DSB processing, resolution of recombination intermediates and Rad53 activation (Bjergbaek et al. 2005; Cejka et al. 2010; Gravel et al. 2008; Mimitou and Symington 2008; Zhu et al. 2008). The absence of Sgs1 modestly affects the activation of Rad53 in cells exposed to HU or MMS and Sgs1 appears to act in the same pathway as Mrc1 for Rad53 activation (Bjergbaek et al. 2005; Hegnauer et al. 2012; Nielsen et al. 2013). The role of Sgs1 as a mediator of Rad53 activation is more evident in the absence of Rad9 or RFC^{Rad24},

another RFC-like complex responsible for the loading of the 9-1-1 complex (Bjergbaek et al. 2005; Hegnauer et al. 2012; Nielsen et al. 2013). For example, Rad53 activation in *rad9*Δ cells exposed to MMS is completely abolished in the absence of Sgs1 (Nielsen et al. 2013). Of importance, Rad53 is activated very late after S phase completion in *rad9*Δ cells treated with MMS (Nielsen et al. 2013), likely in G_2/M, similarly to our observations in *rad9*Δ cells treated with Zeocin. MMS has been shown to induce the formation of chromatin bridges, which are structures connecting the sister chromatids during late mitosis (Germann et al. 2014). Such structures recruit Mec1 and Sgs1 and activate Rad53 (Germann et al. 2014). Thus, Sgs1 appears as a potential candidate for activating Rad53 in G_2/M in the absence of Mrc1 and Rad9 in response to DNA damage. Further experiments will be needed to explore this possibility.

Finally, we do not discard the possibility that the sensor kinase Tel1 could be responsible for the residual Rad53 phosphorylation observed in *mrc1*Δ *rad9*Δ cells. In response to DSBs, Tel1 is able to phosphorylate Rad53 in the absence of Mec1, although less efficiently (Nakada et al. 2003). Supporting this hypothesis, Tel1 is involved in the resolution of replication termination at DSBs (Doksani et al. 2009).

Collectively, our recent work replaces Rad9 as a main actor of the DDR during S phase. The activation of the DDC by Rad9 allows Rad53 signaling to be maintained in time to ensure the integrity of challenged replication forks, slows down the progression of otherwise unchallenged forks and prevents the completion of mitosis until problems are solved. We also suggest that Mrc1 can unexpectedly signal DNA damage in late S or G_2 (Fig. 2). Altogether, these data support a model in which the checkpoint response to DNA damage is a single pathway with the sufficient versatility as to integrate temporal and spatial cues to safeguard the genome integrity during the cell cycle.

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