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The Maintenance of ATM Dependent G2/M Checkpoint Arrest Following Exposure to Ionizing Radiation

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The G2/M checkpoint is important in preventing cells with unrepaired DNA double strand breaks (DSBs) entering mitosis, an event which is likely to result in genomic instability. We recently reported that checkpoint arrest is maintained until close to completion of DSB repair and that the duration of checkpoint arrest depends on the dose and DSB repair capacity rather than lasting for a fixed period of time. ATM leads to phosphorylation of Chk1/2 in G2 phase following exposure to ionizing radiation. These transducer kinases can phosphorylate and inhibit Cdc25 activity, which is the phosphatase regulating mitotic entry. In this study we dissect three processes that contribute to the maintenance of checkpoint arrest in irradiated G2 phase cells. First, the ATR-Chk1 pathway contributes to maintaining checkpoint arrest, although it is dispensable for the initial activation of checkpoint arrest. Second, ongoing ATM to Chk2 signalling from unrepaired DSBs contributes to checkpoint arrest. This process plays a greater role in a repair defective background. Finally, slow decay of the initially activated Chk2 also contributes to the maintenance of checkpoint arrest. 53BP1 and MDC1 defective cells show an initial checkpoint defect after low doses but are proficient in initial activation of arrest after high doses. After higher radiation doses, however, *53BP1*$^{-/-}$ and *MDC1*$^{-/-}$ MEFs fail to maintain checkpoint arrest. Furthermore *53BP1*$^{-/-}$ and *MDC1*$^{-/-}$ MEFs display elevated mitotic breakage even after high doses. We show that the defect in the maintenance of checkpoint arrest conferred by 53BP1 and MDC1 deficiency substantially enhances chromosome breakage.

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Introduction

A DNA double strand break (DSB) is a significant lesion and activates a DNA damage response (DDR), which encompasses a complex network of proteins that collectively function to enhance survival and maintain genomic stability. The DDR encompasses pathways of DSB repair and a signal transduction response that effect cell cycle checkpoint arrest and apoptosis.[1] After induction of DSBs, ataxia telangiestasia mutated (ATM) phosphorylates H2AX, which occurs over megabase-pair regions surrounding the DSB. ATM dependent signalling functions to change the chromatin structure and helps the recruitment and/or maintenance of mediator proteins, such as MDC1, 53BP1 and BRCA1.[2-5] The G2/M checkpoint is important for the maintenance of genomic stability by preventing the entry of cells with unrepaired DSBs into mitosis. ATM establishes the G2/M checkpoint machinery within 1-2 hr post ionizing radiation (IR). Activated ATM leads to phosphorylation of Chk1/2 in G2 phase, followed by the inhibition of Cdc25 activity, which is the phosphatase that regulates mitotic entry.[6] Chk2 is a direct target of ATM whilst Chk1, a substrate of ATR, is indirectly activated following ATM-dependent DSB resection.[7] Therefore G2/M arrest of irradiated G2 phase cells via either Chk1 or Chk2 is ATM-dependent. Although much is known about the mechanism leading to activation of G2/M checkpoint arrest, few studies have addressed how arrest is maintained. We recently showed that the duration of arrest is dependent upon the dose and repair capacity post IR.[8] Further we observed that checkpoint arrest is maintained until the defined threshold of DSBs is reached. Therefore it is likely that the checkpoint signaling machinery from unrepaired DSBs, i.e. ongoing signaling, monitors checkpoint maintenance. We have previously found that non-homologous end joining (NHEJ) represents the major DSB repair mechanism in G1 and G2.[9,10] We have recently found that only 15~20 % of IR induced DSBs undergo resection in G2 and processing by homologous recombination (HR), and that these represent DSBs repaired with slow

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kinetics (Beucher et al, submitted). Here, we investigate the contribution of ongoing signalling and Chk1/Chk2 on the maintenance of checkpoint arrest and discuss the potential role of mediator proteins in this process.

Results and Discussion

ATR is required for the maintenance of checkpoint arrest but is dispensable for the initiation of checkpoint arrest

ATM regulates G2/M checkpoint arrest via both Chk2 and Chk1 activation. ATM promotes DNA end resection in G2 phase after IR and ATR, which is activated by single stranded regions of DNA, phosphorylates Chk1 at such regions generated by resection. We, therefore, examined whether the ATR-Chk1 pathway contributes to the maintenance of checkpoint arrest in irradiated G2 phase cells.

To investigate the contribution of the ATR-Chk1 pathway to the maintenance of checkpoint arrest, we examined the timing of checkpoint release in ATR-Seckel (ATR-SS) cells, which have impaired ATR function. To examine the maintenance of arrest specifically in irradiated G2 cell, we added aphidicolin, which is a replication polymerase inhibitor, to prevent progression of G1 and S cells into G2 phase during analysis. Interestingly, although G2/M arrest is normal in ATR-SS hTERT cells following 3 Gy IR, they are released slightly earlier (4-6 h) than control cells (6-8 h). We recently observed that resected DSBs are repaired by HR with slow kinetics in G2 phase mammalian cells. Consistent with this observation, the ATR-Chk1 pathway contributes to the maintenance of checkpoint arrest but is dispensable for initial arrest.

It is likely that the relatively modest contribution of p-Chk1 is consistent with the recent evidence that only ~15 % of IR induced DSBs undergo resection. However, the contribution of Chk1 activation might have a greater role in situations where resection occurs but HR fails (eg BRCA2$^{-/-}$ cells).

Ongoing ATM signalling contributes to the maintenance of checkpoint arrest

However, ATR-SS hTERT cells initiate checkpoint arrest normally and maintain arrest for a limited period post irradiation. In mammalian G2 cells, 80~85 % of IR induced DSBs are repaired by NHEJ, suggesting that DSB ends, which do not undergo resection, activate Chk2 by ATM. Therefore, we considered that Chk2 might also contribute to the maintenance of checkpoint arrest. Furthermore, we considered that the initially activated Chk2 and ongoing ATM to Chk2 signalling from unrepaired DSBs might contribute to the maintenance of checkpoint arrest. To investigate whether ongoing signalling contributes to checkpoint maintenance, we added the ATM specific inhibitor, KU55933 (hereafter designated ATMi) at 30 min post IR, when ATM dependent G2/M checkpoint arrest has been established. Interestingly, ATMi-treated cells show premature checkpoint release (4-6 h) compared to control cells (6-8 h) after 3 Gy IR. Indeed, since the number of unrepaired DSBs at 6 h, the time of checkpoint release, is greater in ATMi treated versus control cells, the impact of ongoing ATM signalling is more marked than apparent from the comparison with control cells. Importantly ATMi-treated ATR-SS cells show very early checkpoint release (at 2-4 h), suggesting that both Chk1 and ongoing ATM to Chk2 signalling contribute to the checkpoint maintenance. In contrast, to examine the impact of loss of Chk1/2 activity, we added SB218078, a Chk1 and Chk2 inhibitor at 30 min post IR. In the presence of the inhibitor, cells are released earlier than ATMi-treated ATR-SS cells, suggesting that residual Chk1/Chk2 activity remains and/or that the initially activated Chk2 contributes the maintenance of checkpoint arrest (see below).

Ongoing ATM signalling plays a significant role in a repair defective background

NHEJ defective cells show a substantial DSB repair defect in G2 phase as well as in G1 and prolonged checkpoint arrest. We therefore considered that ongoing signalling from unrepaired DSBs might have a greater role in maintaining checkpoint arrest in a NHEJ defective background. To examine this possibility, we carried out the checkpoint maintenance assay in 2BN hTERT cells, which are defective in XLF/Cernunnos, a core NHEJ component. As expected, 2BN hTERT cells show substantially reduced DSB rejoining in G2 phase confirming that NHEJ is the major DSB repair process in G1 and G2. Then we tested the impact of loss of ongoing signalling in 2BN cells by adding ATMi at 30 min post IR. Interestingly we observed premature release at 4-6 h in ATMi-treated 2BN cells. In contrast, cells are arrested for >12 h in the absence of ATMi. Thus, ongoing ATM signalling plays a significantly greater role in maintaining checkpoint arrest in a repair defective background.

Ongoing ATM signalling is required for maintaining the levels of phosphorylated Chk2

To investigate the impact of ongoing ATM to Chk2 signalling to the maintenance of checkpoint arrest, we quantified the levels of phosphorylated Chk2 (p-Chk2) by immunofluorescence (IF) specifically in G2 phase. G2 phase cells were identified by CENPF staining. To confirm antibody specificity of p-Chk2, we measured the levels of p-Chk2 in Chk2 knockdown cells. Since Chk2 knockdown cells show significantly reduced p-Chk2 levels, we concluded that p-Chk2 antibody specifically recognizes phosphorylated Chk2 by IF.

Consistent with the DSB repair defect in 2BN cells, we observed that the p-Chk2 signal in 2BN cells at 30 min post IR is greater than in control cells and that it remains elevated for up to 8 hr. After addition of ATMi at 30 min post IR, p-Chk2 levels decrease dramatically in 2BN cells. These results suggest that ongoing ATM to Chk2 signalling maintains p-Chk2 levels in control cells, and more strikingly in a NHEJ defective background.

The initially activated p-Chk2 decays slowly

Since, in the presence of Chk1/Chk2 inhibitor, cells are released

earlier than ATMi-treated ATR-SS cells, we considered that the initially activated p-Chk2 might maintain checkpoint arrest for several hours. To examine this quantitatively, we measured the p-Chk2 levels by immunoblotting in irradiated G0/G1 cells (used to eliminate the impact of newly generated DSBs in S phase). As in the checkpoint experiment, we added ATMi at 30 min post IR. In the presence of ATMi, the level of p-Chk2 at 1 and 2 h post IR was lower compared to non-ATMi treated cells but was above background levels, suggesting that the initially activated p-Chk2 also can partially contribute to the maintenance of checkpoint arrest without ongoing ATM signalling.

The involvement of 53BP1 and MDC1 in the maintenance of checkpoint arrest

In this study, we dissected three processes that contribute to the checkpoint maintenance. The first is the ATR/Chk1 pathway. The second is ongoing ATM to Chk2 signalling and this is observed most strikingly in a NHEJ defective background. And finally, slow decay of the initially activated ATM signalling. It has been reported that the loss of mediator proteins such as 53BP1 and MDC1 show a checkpoint defect.[3,4] However this defect is only observed after low doses and not after high doses. Indeed, we observed an initial checkpoint defect in *53BP1*$^{-/-}$ and *MDC1*$^{-/-}$ MEFs at 1 hr after 1 Gy IR, with normal checkpoint arrest after 3 Gy IR. Interestingly we found that *53BP1*$^{-/-}$ and *MDC1*$^{-/-}$ MEFs show premature checkpoint release after 3 Gy IR. Mediator proteins function has been suggested to amplify ATM signalling by the retention of repair proteins at the DSB site. 53BP1 and MDC1 defective cells fail to form foci of p-ATM, p-Chk2 and BRCA1 at the site of DSB.[4,11,12] Therefore it is likely that mediator proteins are required for ongoing ATM to Chk2 signalling at the site of unrepaired DSBs. Furthermore, since *53BP1*$^{-/-}$ and *MDC1*$^{-/-}$ MEFs show a very early checkpoint release compared to ATMi-treated cells, the mediator proteins might be required for the activation of Chk1. We also observed increased chromosomal aberrations in the mitotic cells of *53BP1*$^{-/-}$ and *MDC1*$^{-/-}$ MEFs, following their release from G2/M checkpoint arrest even after high doses. Therefore, both the maintenance and initiation of checkpoint arrest is important in preventing chromosome breakage.

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