



DNA Repair Processes and Checkpoint Pathways in Human Cells Exposed to Heavy Ion Beams

Hirohiko Yajima, PhD¹; and Lian Xue, PhD²

¹Research Center for Charged Particle Therapy, National Institute of Radiological Sciences, Chiba, Japan

²School of Public Health, Medical College of Soochow University, Suzhou, China

Abstract

The DNA double-strand break (DSB) is the most deleterious of the ionizing radiation-induced DNA damages. Two major repair pathways for DSBs have been well studied, nonhomologous end-joining and homologous recombination. It is known that high linear energy transfer radiation, such as heavy ion beams, induces complex DSBs with clustered damages at the end and that, as a result, the efficiency of nonhomologous end-joining in repairing these DSBs is diminished. We have shown that more than 80% of complex DSBs in S/G2 human cells are subjected to DNA end resection, an early step in homologous recombination to generate single-strand DNA. Furthermore, recent work, including ours, revealed that a subpopulation of human G1 cells exhibit resection activity following ionizing radiation, which is dependent on CtIP, as in other cell cycle phases, and also dependent on the complexity of the DSB. Collectively, this recent progress indicates that the complexity of the DSB structure drastically enhances end resection, with CtIP being a significant factor required for complex DSB repair throughout the cell cycle. We further revealed that the ATR pathway, which is activated by end resection, plays a pivotal role in regulating early G2/M arrest in ATM-deficient cells exposed to high linear energy transfer ion beams. This suggests that the complexity of the DSB also influences the choice of the signaling pathway via the enhanced resection. Additionally, we discuss a possibility that CtIP has an additional function (or functions) after the initiation of resection. In conclusion, new findings and insight are pivotal to allow innovative progress in heavy ion-particle therapy by shedding light on the whole response at the molecular level in cells exposed to heavy ion beams.

Keywords: DNA repair; checkpoint; resection; nonhomologous end-joining, homologous recombination

Introduction

Exposure to ionizing radiation (IR) induces various types of DNA damage in human cells. The DNA double-strand break (DSB) is the most deleterious of the IR-induced damage types, and its inappropriate repair leads to cell death or a wide variety of genetic alterations, including large- or small-scale deletions, loss of heterozygosity, translocations, and chromosome loss [1]. Therefore, cells have developed a sophisticated system, called the DNA damage response (DDR), to cope with the damage. As a crucial part of the DDR, 2 major DSB repair pathways function in human cells, nonhomologous end-joining (NHEJ) and homologous recombination (HR). Defects in these pathways cause genomic instability

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Corresponding author:
Hirohiko Yajima
Medical Physics Research
Program
Research Center for Charged
Particle Therapy
National Institute of
Radiological Sciences
4-9-1 Anagawa, Inage-ku
Chiba 263-8555 Japan
Phone: +81 (43) 206-3233
Fax: +81 (43) 206-4149
hyajima@nirs.go.jp

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and lead to the promotion of tumorigenesis. The NHEJ repair pathway directly rejoins the 2 broken ends through the action of DNA-PK and can function in all cell cycle phases, while HR functions in S/G2. The HR requires an early process, called DNA end resection, to generate the single-strand DNA (ssDNA). Multiple studies, predominantly involving lower organisms, have strongly suggested that resection is initiated by the MRE11 nuclease together with CtIP (also known as RBBP8), which was reported to be a BRCA1 binding partner with a function in transcription [2–5]. The CtIP is phosphorylated by ATM following DNA damage [6]. The activity of MRE11 and CtIP for resection is thought to be restricted to the vicinity of the DNA ends. Subsequently, a large-scale expansion of resection is executed by other nucleases and RecQ family helicases, such as EXO1 and BLM [2–4, 7, 8]. After the exposure of ssDNA, RPA (an ssDNA binding protein complex consisting of RPA1, RPA2, and RPA3) accumulates and is phosphorylated by PIKK family kinases [9, 10]. The NHEJ pathway is initiated by the binding of the Ku70/Ku80 heterodimer to DSB termini, which in turn recruits and activates a large protein kinase, DNA-PKcs, to mediate the regulation of other NHEJ factors by phosphorylation. The process culminates with the ligation of the 2 DNA ends by the Ligase4 complex recruited by the Ku heterodimer [11, 12]. High linear energy transfer (LET) radiation, such as heavy ion beams, induces complex DSBs with clustered damages, which will be discussed in detail in the next section. In this review, we describe how critical a factor the complexity of the DSB structure is for the choice of repair pathway and the consequent checkpoint signaling. We will show that the ATR checkpoint pathway is efficiently activated following exposure to high LET radiation. In addition, we describe how CtIP is required for the repair of complex DSBs throughout the cell cycle as well as the possibility that CtIP has an additional function that has not hitherto been described.

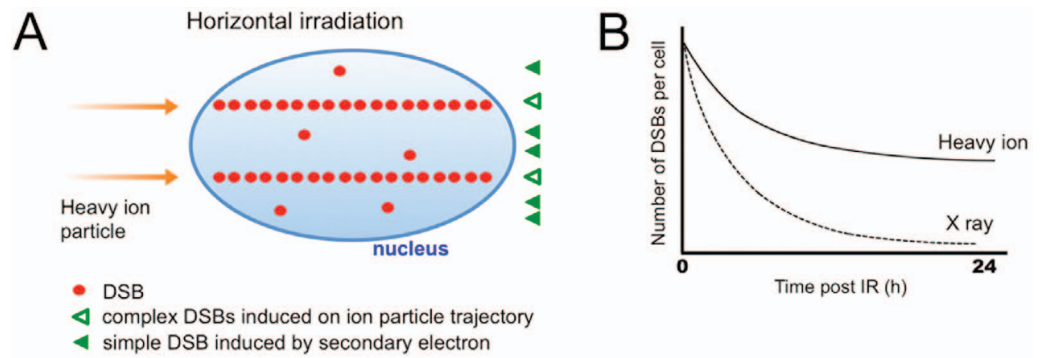
The Complexity of DNA Damage and Repair Pathway Choice

The loss of either one of the DSB repair pathways can be compensated for in most cells by another DSB repair mechanism, indicating that they compete to some extent for repair of a defined DSB. In this way, the choice between the NHEJ and HR-related pathways to repair a defined DSB is a critical aspect of DSB repair. However, how this choice is performed is far from understood. Mechanisms that have been proposed to participate in this decision include cell type, age of the cell, cell cycle phase, and heterochromatic status of the DNA, as well as the complexity of the damage. Of these, the latter 2 factors are regarded as the most significant factors influencing the switch from NHEJ to HR [13]. Here we mainly focus on the complexity of DSBs and how that complexity influences repair pathway choice.

A classification of DSBs on the basis of increasing complexity has been made previously [14]. Isolated DNA lesions (mainly induced by low LET radiation), including DSBs, single-strand breaks (SSBs), and damaged bases located distant from each other are generally repaired efficiently. Substantial evidence indicates that high-LET radiation induces complex DNA damage, a unique class of DNA lesions that includes 2 or more individual lesions within 1 or 2 helical turns of the DNA. These lesions can be apurinic/apyrimidinic (AP) sites, damaged bases, SSBs, or DSBs. A recent study used immunofluorescence (IF) staining of repair proteins as surrogate markers to examine DSBs, SSBs, and base damages in human cells to directly visualize the induction and repair of clustered DNA lesions at the single-cell level [15]. Results showed that a large fraction of 53BP1, XRCC1, and hOGG1 foci colocalized with another marker in cells irradiated with iron and silicon ions, suggesting that the majority of lesions were complex in nature. The complexity and yield of radiation-induced clustered DNA damage increases with the increasing LET value of the radiation and convincing evidence indicates that complex DNA lesions are more difficult to repair than isolated lesions and in some instances are irreparable.

In NHEJ deficient mammalian cells, the relative biological effectiveness for the cell survival does not increase with LET in contrast to repair proficient cells, indicating that high-LET radiation induces a higher yield of DNA lesions that cannot be repaired by the NHEJ pathway [16]. Therefore, we determined if heavy ion beam-induced complex DSBs could enhance the level of end resection. Given that the DDR network is critically regulated via phosphorylation, we focused on the analysis of the signal. A key kinase working in IR-exposed human cells is ATM, and it phosphorylates a number of DDR-related proteins, including CtIP and RPA. When the DNA broken end is resected, the exposed ssDNA rapidly becomes coated with RPA, with each subunit of RPA (e.g., RPA2) undergoing phosphorylation at the DSB site. We showed that heavy ion beam irradiation induces more CtIP and RPA2 phosphorylation compared with X rays. In particular, exposure to 10 Gy of iron ion resulted in nearly all CtIP molecules being converted to a phosphorylated form by 30 minutes [17]. After horizontal irradiation of adherent cells, clear trajectories of ion particles were observed as tracks of γ H2AX, a DSB marker, when examined by IF (see **Figure 1A** for a schematic representation). Using this method, we observed that the majority of γ H2AX foci colocalized with pRPA foci in U2OS cells. Thus, we conclude that more than 80% of the DSBs along the high LET heavy ion particle trajectory are

Figure 1. High LET heavy ion particle-induced DSB and repair kinetics. (A) Horizontal irradiation of heavy ion beams produces complex DSBs along particle trajectories as well as simple DSBs by secondary electrons [20]. (B) Repair kinetics of DSBs induced by high LET heavy ion beams are much slower than those by low LET radiation in human cells. Abbreviations: DSB, double-strand break; LET, linear energy transfer.



subjected to resection in S/G2 human cells [17]. Collectively, our observations indicate that the complexity of the DSB damage is a critical factor enhancing end resection.

Are the complex DSBs efficiently repaired by HR following end resection? Slower repair kinetics of complex DSBs is observed compared with simple DSBs [13, 18–20], which is shown in a schematic image in **Figure 1B**. This has been attributed to the difficulty of complex DSB repair by NHEJ; accordingly, it was suggested that the complex DSB repair in G2 cells is dependent on BRCA2/RAD54-mediated HR [13]. On the other hand, a much smaller difference of repair kinetics between low and high LET radiation-induced DSBs was exhibited in Chinese hamster cells, and NHEJ was suggested to be a major repair pathway even for carbon ion-induced DSBs in these cells [21]. These results suggest that there may be a difference of complex DSB repair pathway choice between species. Therefore, further analysis will be required to clarify the actual repair pathway usage for complex DSBs in human cells.

Resection Activity in G1 Phase Human Cells

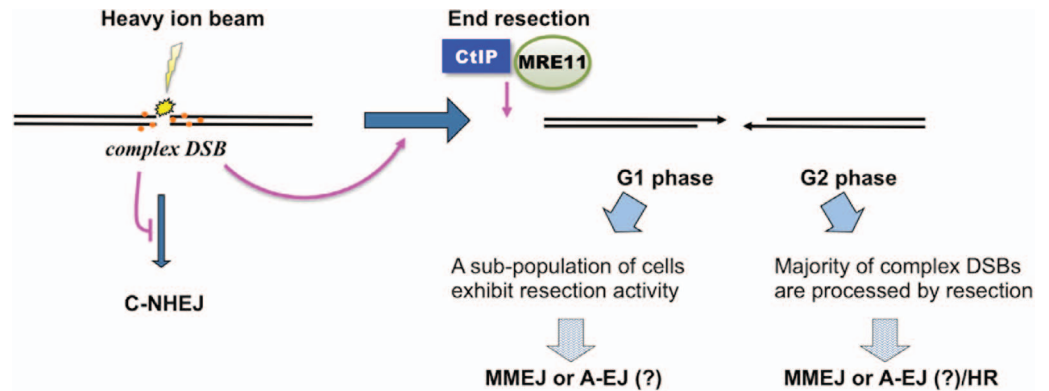
DNA end resection is an early step of HR, and HR is utilized only in late S and G2 phase of the cell cycle because HR requires sister chromatids as the recombination partner. However, it was suggested that CtIP function contributes to the repair of DSBs throughout the cell cycle, including the G1 phase, in chicken DT40 cells [22]. To determine if CtIP-dependent resection activity is observed in human G1 cells, we used a cell cycle marker to identify the cell cycle phase of the cells undergoing resection after heavy ion beam exposure. Our results obtained in this way indicated that around 30% of the G1 U2OS or 1BR-hTERT (human immortalized fibroblast) cells were resection signal-positive [17].

Although our results imply that a subset of cells in the G1 phase possess the capacity for resection, which is dependent on CtIP as with other cell cycle phases [17], HR is not available in G1 cells because of the absence of a sister chromatid.

What is the function of CtIP-dependent resection in the G1 phase other than HR? It has been reported that CtIP expression is decreased in the G1 phase and that it is upregulated by cyclin-dependent kinases (CDKs) in the S and G2 phases [23, 24]. However, previous analysis using chicken DT40 cells proposed that a low level of CtIP activity can promote small-scale end resection to enable microhomology-mediated end joining (MMEJ) in G1 cells [22]. Therefore, our results raise the possibility that in human cells also, resection in the G1 phase may promote MMEJ to repair heavy ion-induced complex DSBs that cannot be efficiently repaired by canonical NHEJ (C-NHEJ) [17]. Following our 2013 publication, resection in human G1 cells was further confirmed by other groups in 2014 [25, 26]. One of these studies revealed further that, although CtIP is phosphorylated at several sites and activated by CDK kinases in S and G2 cells, PLK3 phosphorylates the sites instead of CDK in G1 cells, and PLK3-dependent phosphorylation is required for CtIP function and DSB repair in G1 cells [25]. The resection signal in G1 cells is detectable only following high LET radiation [25, 26], and the percentage of resection-positive G1 cells correlates with the LET value [26]. Taken together, these results suggest that complex DSBs produced in the G1 phase are processed by end resection in a complexity-dependent manner and that the DNA ends are end-joined in some way.

Recently an alternative end-joining mode has received attention as a third player for DSB repair, which is generally defined as being independent of C-NHEJ factors, such as Ku or LIG4 [27]. Meanwhile, NHEJ often uses microhomology, and it may be inadequate to completely separate C-NHEJ and the alternative end-joining process [28]. In view of this, the involvement of C-NHEJ factors in MMEJ in G1 cells should not be excluded. Although actual mechanisms of resection-dependent end-joining

Figure 2. The complexity of DSB structure is a critical factor enhancing end resection throughout the cell cycle. Abbreviations: A-EJ, alternative end-joining; DSB, double-strand break; MMEJ, microhomology-mediated end-joining.



mode in the G1 phase is still largely unknown, the mode appears to be crucial for complex DSB repair, regardless of the cell cycle, and further understanding of it is required to elucidate the DDR of cells exposed to heavy ion particles (**Figure 2**). The slow repair kinetics of complex DSBs shown in **Figure 1B** occurs in G1 cells [13, 20, 25, 26], and CtIP is at least partially required for the repair [25, 26]. Whether the majority of complex DSBs will be repaired in the long term remains unclear but is a critical issue. In cycling cells unrepaired DSBs induced in the G1 phase might lead to cell death or might be carried over to the S phase. How such the broken ends are processed in the S phase is of interest.

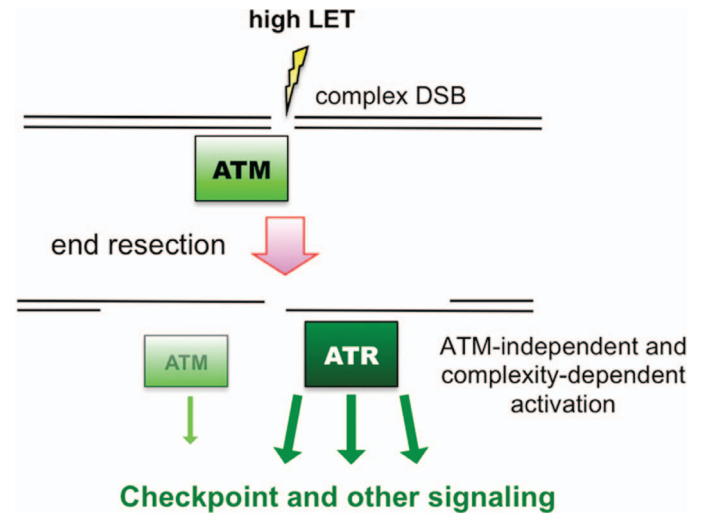
Another issue is whether cells have a G1/S checkpoint, which may prevent progression into the S phase. This is particularly relevant as many cancer cells lose p53 function, which regulates G1/S checkpoint arrest. Furthermore, considering cancer recurrence after a long period following heavy ion therapy, it is crucial to know the consequence of DSBs produced in cancer stem cells resting in G0/G1 phase. Additionally, it is an important challenge to know the response of normal tissue exposed to heavy ion beams during the carbon therapy. Since the majority of cells in the adult normal tissue are in the G0/G1 phase, it is vital to elucidate their long-term response in the G0/G1 phase for the protection.

Conversion of the Primary Kinase in Heavy Ion-Irradiated Cells

Two primary kinases activated by DSBs are ATM and ATR. It is generally regarded that ATM plays a primary role in the response to DSBs, which is critical for the initial response and subsequent ATR activation [29, 30]. However, it is not clear how ATM and ATR are coordinated at DSBs. It was shown by an in vitro ATM/ATR activation assay that ATM and ATR are activated by similar yet distinct DNA structures at resected DSBs [31]. Although both ATM and ATR depend on the junction of ssDNA and double-strand DNA for activation, they are oppositely regulated by the lengthening of single-strand overhangs. The single-strand overhangs simultaneously attenuate ATM activation and potentiate ATR activation, thereby promoting an ATM-to-ATR switch during the process of DSB resection [31]. These findings provide mechanistic insights into how ATM and ATR function in concert to bring about the biphasic DSB response, such as G2/M checkpoint regulation. Cells in the G2 phase at the time of irradiation can rapidly arrest to avoid division with DNA damage. This early G2 arrest is ATM dependent [32], while a late and dose-dependent arrest in G2 of the cells that were irradiated in the S or G1 phase is ATM independent but relies on ATR [33]. However, the sequential activation of ATM and ATR does not tell the whole story; the relationship between ATM and ATR is more complex following ionizing radiation.

We elucidated that the complexity of DSBs is a critical factor enhancing DNA end resection, which could more efficiently trigger HR repair following particle radiation [17]. As the recruitment of ATR to DSBs requires RPA-coated ssDNA, a structure generated by nuclease-mediated resection of DSBs, it is reasonable to speculate that the ATR signaling pathway may be more activated in cells exposed to heavy ion beams than x-rays. In fact, our observation that the ATR pathway functions in the early G2/M checkpoint arrest upon IR in ATM-deficient cells revealed that the ATR pathway can be rapidly activated in an ATM-independent manner and that DNA end resection is a crucial factor for the response (**Figure 3**) [34]. Meanwhile, the LET-dependent early G2/M checkpoint arrest activated by different kinds of radiation indicated that ATR plays a more important role in cells irradiated with ion particles compared with x-rays. Furthermore, we observed that an ATR inhibitor had a more effective radiosensitizing effect on survival after carbon ion beams compared with the response following x-ray exposure, while conversely, ATM inhibitor had the opposite effect [34]. Wang et al [35, 36] have also reported that the effects of ATR and CHK1 on radiosensitivity are independent of NHEJ. By examining the survival fraction by low and high LET radiation, they also reported that the checkpoint response plays a more protective role in HZE particle-irradiated cells than in x-ray-irradiated cells

Figure 3. Complex DSBs activate the ATR pathway for G2/M checkpoint arrest regardless of ATM function via enhanced end resection. Abbreviation: DSB, double-strand break.



[37]. The ATM-to-ATR switch after low and high LET radiation was investigated in a recent article, and a prolonged signal of phospho-ATM (Ser1981) and phospho-ATR (Ser428) was observed in high LET radiation-exposed cells, in accordance with our results [38]. However, it has been reported that the phosphorylation of ATR at Ser428 does not correlate with ATR activity [39, 40], and the regulation and significance of the phosphorylation at the site has not yet been well elucidated, although antibodies specific to pS428-ATR are commercially available. Collectively, since these data may indicate that the role of the ATR pathway can be considered as a target for radiosensitization after heavy ion particles in cancer therapy, further analysis of the activation and regulation of the ATR pathway is necessary to assess this possibility (**Figure 3**).

Additional Function of CtIP

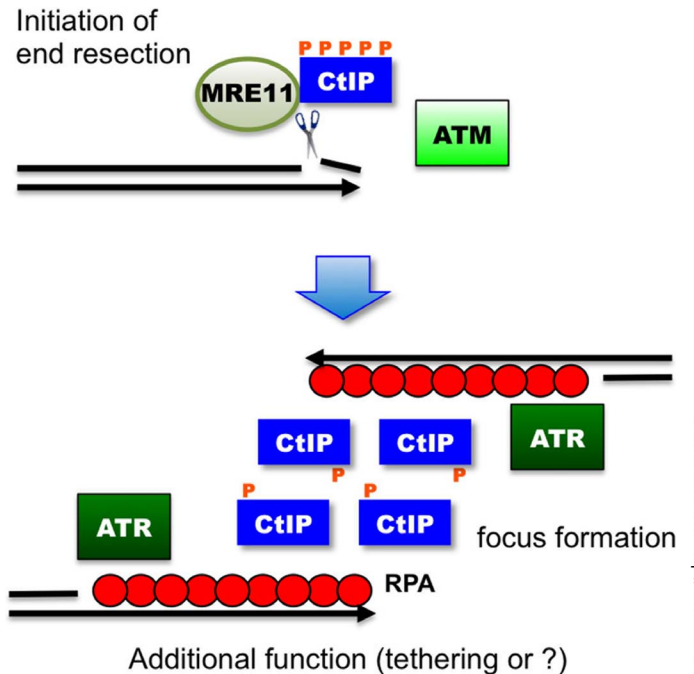
Recent work, including our report, has emphasized the importance of CtIP for the repair of heavy ion-induced DSBs throughout the cell cycle. Since ATM-dependent hyperphosphorylation of CtIP arises within 20 minutes after heavy ion exposure, we propose that the hyperphosphorylation of CtIP correlates with the initiation of resection together with MRE11 [17]. However, our IF experiments revealed that the number of CtIP foci increased up to 4 hours after irradiation, and it was maintained for at least 15 hours with only a slight decline, even though hyperphosphorylation of CtIP has been lost [41].

The kinetics of CtIP focus formation is similar to that of RPA. Our results, therefore, imply that CtIP accumulates and forms foci at DSB ends that have undergone resection, and that the CtIP foci are maintained until the completion of DSB repair. Our study further revealed that CtIP molecules in the foci at DSBs are rapidly degraded, and newly synthesized CtIP molecules are required for the maintenance of CtIP foci at the DSBs. Additionally, at late times after heavy ion beam irradiation, the hypophosphorylation state of CtIP is regulated by ATM and ATR [41]. Collectively, these novel characteristics of CtIP strongly suggest that CtIP has an additional function (or functions) after the initiation of resection (**Figure 4**). For example, CtIP may regulate the progression of resection or the replacement of RPA with Rad51, which is required for recombination with the sister chromatid. Phosphorylation of CtIP by ATR was previously reported using the *Xenopus* egg extract system [42]. The phosphorylation of CtIP at a conserved threonine residue (Thr818 in *Xenopus*) is required for the chromatin binding of CtIP and an extensive resection. The authors proposed that CtIP might have a direct role in the extension of resection, together with DNA2 nuclease. Although phosphorylation of human CtIP at the conserved site (Thr859) was confirmed using human 293 cells [42], detailed investigation of the regulation and significance of damage-induced phosphorylation of CtIP in human cells is required to clarify its functions. A very recent study reported that the CtIP N-terminal region forms a tetramer by head-to-head association and that this tetramerization is required for resection/HR [43]. The authors suggested that a CtIP tetramer might function in tethering 2 DNA ends to promote repair [43] (**Figure 4**). Further analysis of CtIP regulation and functions is critical to understand how heavy ion-induced complex DSBs are processed in human cells.

Conclusion

It has been well accepted that high LET heavy ion beams induce complex DSBs with clustered damages, and now it is obvious that the complexity of DSB is one of the critical factors for enhancing DNA end resection. Furthermore, the complexity

Figure 4. Novel characteristics of CtIP suggest that CtIP has functions in addition to promoting the initiation of resection during HR. Abbreviation: HR, homologous recombination.



consequently leads to activation of the ATR signaling pathway for G2/M checkpoint regulation via enhanced resection. This means that the DSB repair pathway choice naturally influences the signal pathway choice (**Figure 3**). Therefore, it is challenging to determine a sequence of response connecting the repair pathway choice and cell fate decision. Furthermore, CtIP is required throughout the cell cycle for the repair of DSBs, especially the complex DSBs induced by high LET radiation. For this reason, the analysis of CtIP functions should provide further insight into the DDR in human cells induced by exposure to the heavy ion beams, as well as general mechanisms of resection/HR. In addition, a recent study suggested a strong relationship between no or low expression of CtIP and poor breast cancer prognosis as well as a correlation between CtIP and RB1 expression level in breast cancer biopsies [44]. Although the mechanism by which the expression level of CtIP in cancer cells modulates the response of the cells to radiotherapy has not been elucidated, the observation raises expectations that a better understanding of CtIP regulation will lead to improvement in carbon ion radiotherapy. In conclusion, for innovative progress in heavy ion-particle therapy, an improvement in the comprehension of the DDR at the molecular level in human cells is clearly required.

ADDITIONAL INFORMATION AND DECLARATIONS

Conflicts of interest: The authors have no conflicts of interest to disclose.

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