

7-3-2006

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### Recommended Citation

Weiss, Robert S.; Wang, Xiang; Hu, Baocheng; and Wang, Ya, "The effect of Hus1 on ionizing radiation sensitivity is associated with homologous recombination repair but is independent of non-homologous end-joining" (2006). *Department of Radiation Oncology Faculty Papers*. Paper 1.  
<http://jdc.jefferson.edu/radoncfp/1>

**The effect of Hus1 on ionizing radiation sensitivity is associated with homologous recombination repair but is independent of non-homologous end-joining**

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Running Title: A link between Hus1 and HRR

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**Abstract**

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Mammalian Hus1 plays an important role in maintaining genomic integrity. Cells lacking mouse Hus1 are hypersensitive to DNA damage inducers including UV and camptothecin (CPT). By using clonogenic assay, we show here that Hus1 deficient mouse cells are hypersensitive to ionizing radiation (IR) compared with their Hus1-positive counterparts. However, these cells show similar induction levels and similar rejoining rates of DNA double strand breaks (DSBs) following IR, indicating that the effect of Hus1 on cell radiosensitivity is independent of non-homologous end-joining (NHEJ). By combining an I-SceI-induced-DNA DSBs system and a siRNA approach, we also show that knocking down *Hus1* decreases the efficiency of homologous recombination repair (HRR), which is associated with the cellular sensitivity to IR-induced killing. Together, these results indicate that the role of Hus1 affecting the sensitivity of cells to IR-induced killing is independent of NHEJ but might be linked to HRR.

### **Introduction**

Hus1 is an important checkpoint protein that contributes to the resistance of mammalian cells against multi-DNA damage inducers, including UV radiation and camptothecin (CPT) treatment (Wang et al., 2004b; Weiss et al., 2003; Weiss et al., 2002). Compared with their Hus1-positive counterparts, Hus1 deficient cells show normal sensitivity to ionizing radiation (IR) induced killing when measured in short-term viability assays (Weiss et al., 2000). However, as shown here, Hus1 deficient cells show increased sensitivity to IR-induced killing when using the clonogenic assay that measures reproductive ability. These results support the model that Hus1 associates with Rad1 and Rad9 as a 9-1-1 complex to respond to IR-induced DNA damage because Rad9 deficient cells are hypersensitive to IR-induced killing (Hopkins et al., 2004; Roos-Mattjus et al., 2003). IR induced DNA double strands breaks (DSBs) are the most severe threat for cell survival. Two major pathways: non-homologous end-joining (NHEJ) and

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homologous recombination repair (HRR) play important roles in repairing DNA DSBs of irradiated mammalian cells (Couedel et al., 2004; Mills et al., 2004). Thus, to elucidate which repair pathway, NHEJ or HRR or both, is affected by Hus1, is an essential step in understanding how Hus1 is involved in protecting mammalian cells from IR-induced killing, and how Hus1 is involved in maintaining genomic integrity following DNA damage.

## **Results**

### **Hus1 deficient cells are hypersensitive to IR-induced killing**

Hus1 is believed to associate with Rad1 and Rad9 as a 9-1-1 complex in cells to respond to DNA damage (Hang & Lieberman, 2000; Lindsey-Boltz et al., 2001; Rauen et al., 2000). Rad9 is involved in IR-induced DNA damage response and Rad9 deficient cells are hypersensitive to IR-induced killing (Hopkins et al., 2004; Roos-Mattjus et al., 2003). It is reasoned, then, that Hus1 should also affect the sensitivity of mammalian cells to IR-induced killing. Although a previous study showed that Hus1 deficient cells show normal sensitivity to IR-induced killing by trypan blue staining (Weiss et al., 2000), such short-term viability assays are limited to measuring the immediate death of cells and might hide the effect of Hus1 on protecting cells from IR-induced death due to loss of reproductive ability. To test this hypothesis, we examined the radiosensitivity in Hus1 deficient cells using clonogenic survival assay. The results showed that Hus1 deficient cells were more sensitive to IR induced killing than were their Hus1-positive counterparts (Figure 1). When Hus1 was reintroduced into Hus1 deficient cells, normal sensitivity to IR was restored (Figure 1), indicating that the sensitivity to IR-induced killing shown in Hus1 deficient cells is because of the absence of Hus1. These results show for the first time that Hus1 plays an important role in protecting mammalian cells from IR-induced killing.

**Hus1 deficient cells and their wild type counterparts show similar DSB induction levels and similar DSB rejoining rates following IR**

IR-induced DNA DSBs are the most damaging events affecting cell survival. NHEJ and HRR are the two major pathways for repairing DNA DSBs in mammalian cells. Compared with their Hus1-positive counterparts, Hus1 deficient cells are sensitive to IR-induced killing (Figure 1), suggesting that Hus1 might be involved in DNA repair either directly or indirectly. Hus1 could affect cellular radiosensitivity through NHEJ or HRR, or both. Rad9, the partner of Hus1 in the 9-1-1 complex, is phosphorylated by ATM following IR, which is essential for the role of Rad9 in protecting human cells from IR-induced killing (Chen et al., 2001). The ATM pathway that affects cell radiosensitivity is linked to HRR but is independent of NHEJ (Golding et al., 2004). Therefore, the effect of Hus1 on radiosensitivity also might be independent of NHEJ but linked to HRR. To test this hypothesis, we compared the efficiency of NHEJ between Hus1 deficient cells and their wild type counterparts by using asymmetric field inversion gel electrophoresis (AFIGE) assay. The results show that cells with or without Hus1 have similar induction levels and similar rejoining rates of DNA DSBs following IR (Figure 2) although they do have different sensitivities to IR-induced killing (Figure 1). These results indicate that the effect of Hus1 on cell radiosensitivity is not by affecting NHEJ repair but might be linked with HRR.

**Hus1 siRNAs inhibits HRR efficiency**

To study the effects of Hus1 on HRR efficiency following DNA DSBs, we combined the pDR-GFP-I-SceI system (Pierce et al., 1999) and siRNA approaches. The I-SceI system can be used to examine the types of recombination events induced by DSBs at a defined chromosomal locus in mammalian cells by the nuclease I-SceI, which is known to stimulate recombination in mammalian cells. The advantage of pDR-GFP-I-SceI system is using a modified gene for green

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fluorescent protein (GFP) as a recombination reporter (Pierce et al., 1999). The 18-bp *I-SceI* site is inserted within *GFP* gene and inactivates it. When the DSBs are induced by *I-SceI* endonuclease, a homologous repair event with a linked donor *GFP* gene fragment restores functional GFP expression. These gene conversion events can be readily detected by flow cytometry in 2-4 days (Pierce et al., 1999).

To evaluate HRR of DNA DSBs, we first established the stable cell lines (F-DRGFP cells) that have incorporated the substrate of *I-SceI* endonuclease by transfecting the pDR-GFP plasmid (provided by Dr. Jasin) (Pierce et al., 1999) to transformed mouse kidney fibroblast cells (Hu et al., 2005). Then we transfected F-DRGFP cells with the plasmid that encoded *I-SceI* endonuclease to observe the intensity of GFP signals measured by flow cytometry. For this purpose, F-DRGFP cells were either transfected with pGFP (containing full-length cDNA of GFP and as a control of transfection efficiency) or transfected with pCMV3xnl*I-SceI* plasmid (encoding full-length *I-SceI* expression sequences, provided by Dr. Nickoloff) (Nickoloff & Brenneman, 2004). To observe the effect of Hus1 on HRR of DNA DSBs, we combined the pDR-GFP-*I-SceI* system and *Hus1* siRNA approaches. The sequence of 21 nucleotides of mouse *Hus1*, which we chose to target with siRNA, has a complete homologue in human *Hus1*. We treated these cells with either *Hus1* siRNA or control RNA twice (at 0 and 24 h after pCMV3xnl*I-SceI* plasmid transfection) and collected the cells for measuring the GFP signal at 48 h after the siRNA for the second time treatment because the GFP signals were most intense at 3 days following pCMV3xnl*I-SceI* plasmid transfection and Hus1 was efficiently inhibited at 24-48 h following *Hus1* siRNA treatment in these cells (Figure 3a). The results show that when Hus1 expression was inhibited in F-3 (F-DRGFP-clone3) or F-6 (F-DRGFP-clone6) cells (Figure 3a), the efficiency of HRR in these cells was much lower than that in the control RNA treated cells (Figure 3b and 3c), thus demonstrating that Hus1 is involved in HRR. To further study

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whether the effects of *Hus1* siRNAs on HRR are linked to cell sensitivity to IR-induced killing, we examined the clonogenic survival capacity in F-DRGFP cells treated with *Hus1* siRNA following IR. The results show that when the level of Hus1 protein is reduced by *Hus1* siRNA treatment (Figure 3a), both F-3 and F-6 cells show increased sensitivity to IR-induced killing (Figure 3d). This suggests a functional connection between radiosensitivity and HRR, with lower HRR efficiency of HRR correlating with increased radiosensitivity. Taken together, these results suggest an explanation why Hus1 deficient cells are sensitive to IR-induced killing (Figure 1). The mechanism by which Hus1 affects HRR following DNA DSBs needs to be elucidated in the near future.

In summary, we show here that Hus1 plays an important role in protecting mammalian cells from IR-induced killing. The effect of Hus1 on radiosensitivity of mammalian cells is independent of NHEJ but might be linked to HRR.

### **Acknowledgements**

We thank Drs. M. Jasin and J.A. Nickoloff for reagents and Nancy Mott for help in the preparation of the manuscript. This work is supported by a grant from NASA NAG2-1628 (to Y.W.).

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### Figure legends

**Figure 1** Hus1 deficient cells are much more sensitive to IR-induced killing than their Hus1-positive counterparts. *Hus1<sup>+/+</sup>p21<sup>-/-</sup>* and *Hus1<sup>-/-</sup>p21<sup>-/-</sup>* mouse embryonic fibroblasts (MEFs) (Weiss et al., 2000), as well as the corresponding complemented cell pools: *Hus1<sup>+/+</sup>p21<sup>-/-</sup>* + vector, *Hus1<sup>-/-</sup>p21<sup>-/-</sup>* + vector and *Hus1<sup>-/-</sup>p21<sup>-/-</sup>* + Hus1 (Weiss et al., 2002), were exposed to the indicated doses of X-rays (310 kV, 10 mA, 2-mm Al filter), and were then collected and plated aiming at 20-200 colonies per 100 mm dish. Two replicates were prepared for each datum point and incubated for one week in the absence of drugs to allow colonies to develop. Colonies were stained with crystal violet (100% methanol solution) before counting. Data shown are the average from three independent experiments.

**Figure 2** Hus1 deficient cells and their Hus1-positive counterparts show similar induction levels and similar rejoining rates of DNA DSB. The induction and rejoining of DNA DSBs were performed by using asymmetric field inversion gel electrophoresis (AFIGE) assay as described before (Wang et al., 2004a). Briefly, cells in cold medium were irradiated and returned to the incubator at 37°C. At various times thereafter cells were collected and mixed with an equal volume of 1% agarose (InCert agarose, FMC). A similar protocol was also employed to determine DSB induction except that, in this instance, cells were embedded in agarose blocks prior to irradiation, and were placed in lysis buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 0.5 M EDTA, 2% N-lauryl sarcosyl, 0.1 mg/ml proteinase E) immediately after irradiation. DNA DSBs were quantitated by calculating the FAR (fraction of activity released from the well into the lane)

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in irradiated and non-irradiated samples by means of a fluorescence image measured with a PhosphoImager (Typhoon 8600, Molecular Dynamics). (a) and (b) show the induction of DNA DSBs. (c) and (d) show the kinetic rejoining of DNA DSBs after exposure to 40 Gy X-ray as described before (Hu et al., 2001). (b) and (d) are the quantification of the gel results shown in (a) and (c). Data shown are the average and standard error from three independent experiments.

**Figure 3** Reduced Hus1 expression impairs HRR. (a) The levels of Hus1 expression were measured with the whole cell lysates from either *Hus1* siRNA or control RNA treated F-DRGFP cells. Hus1 antibody was prepared as follows. A Hus1 fragment (Hus1f, 606-846bp) from mRNA of NIH3T3 cells was amplified by using RT-PCR. The PCR products were inserted into pET-28a(+) vector; the plasmid was transformed into host cell BL21(DE3) for the Hus1f expression. The expressed protein was purified by chelating column (Ni-NTA, Qiagen). Polyclonal antibody was made by immunizing mice using the purified protein and was confirmed by Western blot to recognize the Hus1 fragment protein specifically. *Hus1* siRNA that specifically targets the sequences of the mouse *Hus1* mRNA (5'-CCUGCACCCUCCGCAUCAGUU-3') was designed. The designed siRNA was synthesized by Dharmacon Company. The Scrambled RNA (Dharmacon Company) was used as the transfection control. The transfection of siRNAs was performed with the Oligofectimine (Invitrogen) following the manufacturer's instructions. The cells were prepared for further examination (HRR, western blot and IR) at 48 h after transfection. Western blotting was performed with whole cell lysates using antibodies against Hus1 and CHK1 (sc-8404, Santa Cruz Biotechnology, Inc.) as described (Hu et al., 2005). CHK1 was used as the internal loading control. (b) Measurement of HRR was as described (Hu et al., 2005). Representative flow cytometric analyses of GFP signal in F-3 cells following DSBs. The cells were either transfected with pGFP

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(alone or co-transfected with siRNA) or pCMV3xnlsl SceI (pI-SceI) (alone or co-transfected with siRNA) with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cells were then transfected with either *Hus1* siRNA or control RNA by using the oligofectimine (Invitrogen) following the manufacturer's instructions at 24 h after the first transfection. The cells were collected at 48 h following the siRNA transfection. **(c)** HRR efficiency was calculated by determining the frequency of GFP signal in I-Sce I transfected F-DRGFP cells subtracting the background from non-transfected controls and dividing by the frequency of GFP signal from pGFP transfected F-DRGFP cells. Data shown are the average from three independent experiments, with error bars representing the standard error. **(d)** Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described in Figure 1. The cells were exposed to X-rays (4 Gy) at 48 h after siRNA transfection and were then collected for clonogenic assay. Data shown are the average from three independent experiments.