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ORIGINAL RESEARCH

Role of a DNA Damage Checkpoint Pathway in Ionizing Radiation-Induced Glioblastoma Cell Migration and Invasion

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Abstract Ionizing radiation (IR) induces a DNA damage response that includes activation of cell cycle checkpoints, leading to cell cycle arrest. In addition, IR enhances cell invasiveness of glioblastoma cells, among other tumor cell types. Using RNA interference, we found that the protein kinase MRK, previously implicated in the DNA damage response to IR, also inhibits IR-induced cell migration and invasion of glioblastoma cells. We showed that MRK activation by IR requires the checkpoint protein Nbs1 and that Nbs1 is also required for IR-stimulated migration. In addition, we show that MRK acts upstream of Chk2 and that Chk2 is also required for IR-stimulated migration and invasion. Thus, we have identified Nbs1, MRK, and Chk2 as elements of a novel signaling pathway that mediates IR-stimulated cell migration and invasion. Interestingly, we found that inhibition of cell cycle progression, either with the CDK1/2 inhibitor CGP74514A or by downregulation of the CDC25A protein phosphatase, restores IR-induced migration and invasion in cells depleted of MRK or Chk2. These data indicate that cell cycle progression, at least in the context of IR, exerts a negative control on the invasive properties of glioblastoma cells and that checkpoint proteins mediate IR-induced invasive behavior by controlling cell cycle arrest.

Keywords Glioblastoma · Cell cycle arrest · Checkpoint · Migration · Invasion

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Introduction

Ionizing radiation (IR) is used as a standard therapeutic modality in many types of cancers including glioblastoma multiforme (GBM), the most aggressive form of astrocytoma, where it prolongs median survival for 6–8 months (Walker et al. 1979; Walker et al. 1980). GBM is characterized by high invasiveness that renders complete surgical extirpation and local radiation ineffective.

DNA damage stimulates a variety of signaling events including cell cycle checkpoints that inhibit cell cycle progression while cells attempt DNA repair. A central component of the DNA damage checkpoint network is the Mre11–Rad50–Nbs1 (MRN) complex, which recognizes double strand break lesions (Petrini and Stracker 2003) and contributes to activation of the Chk2 protein kinase

(Bartek and Lukas 2003). Chk2 in turn phosphorylates the phosphatase CDC25A, leading to its degradation and subsequent cell cycle arrest (Bartek and Lukas 2003; Zhou and Elledge 2000). MDC1 is an adaptor/mediator protein that works upstream of Nbs1, with which it physically interacts (Stucki et al. 2005), and coordinates the assembly of other repair/checkpoint proteins onto the surrounding chromatin (Jungmichel and Stucki 2010).

Previous studies in our laboratory have identified MRK, also known as MLTK (Gotoh et al. 2001) or ZAK (Liu et al. 2000), as a MAP3K protein that is activated by IR and is necessary for the S and G2/M checkpoint responses following DNA damage (Gross et al. 2002). We have also demonstrated that MRK is required for full activation of Chk2 after IR and that siRNA-mediated depletion of MRK sensitizes cancer cells to IR (Tosti et al. 2004). The signaling mechanisms that regulate the activation of MRK by DNA damage remain to be identified, however.

Besides its therapeutic effect, irradiation has been shown to promote the invasive behavior of many types of cancer cells both in vitro and in vivo (Camphausen et al. 2001; Cheng et al. 2006; Jung et al. 2007; Ohuchida et al. 2004; Qian et al. 2002; Wild-Bode et al. 2001). The effect of sublethal irradiation on migration and invasiveness of glioma cells in vitro and in vivo led Wick and co-authors (2001) to propose that glioblastoma recurrence in close proximity of the primary tumor or resection site could result from a geographic "miss" as tumor cells migrate out of the fixed radiation portal during the course of radiotherapy.

Several studies have identified signaling components that are required for radiation-induced invasion of glioma cells. These include activation of the RhoA pathway (Ader et al. 2003; Zhai et al. 2006), activation of integrins (Monferran et al. 2008), and production of MMP-2 and MMP-9 matrix metalloproteinases (Badiga et al. 2011; Nirmala et al. 2000; Park et al. 2006). In this study, we have investigated the role of MRK in migration and invasion stimulated by IR in glioblastoma cells and found that MRK and members of the checkpoint pathway regulate IR-induced invasive behavior of glioblastoma cells. Analysis of the contribution of cell cycle arrest to IR-induced tumor cell invasion properties identified an inhibitory role for cell cycle progression on tumor cell migration and invasion induced by IR, providing direct evidence for an antagonistic role of the cell cycle on cell migration in the context of IR.

Materials and Methods

Cell Lines and Tissue Culture Conditions

Human glioblastoma cell lines, SNB19 and U87 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown as adherent monolayers and were cultured in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker, Walkersville, MD, USA) supplemented with 10 % Fetal Bovine Serum (Hyclone, South Logan, UT, USA) and 1 % penicillin–streptomycin, 1 % glutamine at 37 °C in a humidified 5 % CO_2 incubator. The M28 cell line was derived from U2-OS Tet-On cells (Clontech, Mountain View, CA, USA) that were engineered to express a transactivator responsive to doxycycline, a tetracycline derivative. These cells were transfected with a pTRE plasmid expressing the MRK gene under the control of a Tet-inducible promoter. Individual clones were selected for puromycin resistance and tested for MRK inducibility following doxycycline treatment. Clone M28 was identified for good inducibility and used in these studies.

Chemical Reagents

CGP74514A, *N*-(*cis*-2-aminocyclohexyl)-*N*-(3-chlorophenyl)-9-ethyl-9*H*-purine-2,6-diamine was obtained from Calbiochem (San Diego, CA, USA).

Small Interfering RNA and Transfection Treatment

siRNA transfections were performed using the DharmaFECT1 reagent (Dharmacon, Lafayette, CO) following the manufacturer's recommendations, with 2 µl/well of lipid reagent in a six-well plate. All of the siRNA duplexes used in this study were supplied by Dharmacon and used at a concentration of 10 nM. Specific oligonucleotide sequences for each target gene were as follows:

5-GAAUGUCUGAGGAGUCUUAdTdT targeting MRK; 5-UUUAUGGCUACUUUCUUAC (Chk2-1) and 5-UGACACUUGAGUCCUAUGC (Chk2-2) targeting Chk2; 5-GAAUACAUUCCCUACCUCAdTdT targeting CDC25A; 5-CGUACGCGGAAUACUUCGAdTdT (Luciferase) as control; smart pools were used to down regulate Nbs1 (M-009641-00).

Western Blot Analysis and Antibodies

Forty-four to seventy-two hours after transfection, cells were lysed in 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, protease inhibitors tablets (Roche Applied Science, Indianapolis, IN, USA), 10 % Nonidet P-40 substitute, and 0.4 U/ml Benzonase (Novagen, EMD Chemicals, Gibbstown, NJ, USA). The different proteins were detected by western blot analysis with the following specific antibodies: the 4-23 monoclonal anti-MRK, the 40-5 polyclonal anti-MRK and the phospho-specific MRK antibody, which recognizes autophosphorylated sites (Gross et al. 2002), the Nbs1 antibody (Novus Biologicals,

Littleton, CO, USA), the Chk2 and the βIII tubulin antibodies (Upstate Biotechnology, Lake Placid, NY, USA), the MDC1 antibody (Bethyl Laboratories, Montgomery, TX, USA), the Cdc25A antibody (Ab3; NeoMarkers, Fremont, CA, USA), and the phospho-Chk2 (Thr^{68}) antibody (Cell Signaling Technology, Beverly, MA, USA).

Invasion and Migration Assays

Fifty thousand cells were imbedded in 50 µl of Matrigel (10 mg/ml) (Trevigen, Gaithersburg, MD, USA) and added to the top well of a 24-well transwell plate (BD, Franklin Lakes, NJ, USA). The matrix was allowed to solidify at 37 °C for 30 min. Subsequently, serum-free DMEM media was added to both the top and the bottom of the transwell, followed by 2 h of incubation at 37 °C. Various doses of IR were applied to the transwells using irradiator Gammacell 1000 (^{137}Cs; Atomic Energy of Canada, Ltd., Mississauga, Ontario, Canada). The irradiated cells were further incubated for 24 h in serum-free DMEM media (37 °C, 5 % CO_2). Invaded cells were fixed and stained with crystal violet and counted under the microscope (Olympus IX70).

For migration assay, cells were transfected with the appropriate siRNAs and 60 h after transfection sixty thousand cells were seeded in migration chambers, allowed to spread for 3 h, and exposed to various doses of IR. Six hours after IR, cells were fixed, stained, and counted as described for the invasion assays.

MTS Cell Viability Assay

Cells were transfected with the desired siRNAs and 48 h after transfection, 5×10^3 cells were seeded into 6-cm dishes and exposed to IR. The irradiated cells were seeded into a 96-well assay plate and incubated in tissue culture incubator for 24 h. The reconstituted MTS/PMS solution was added to the wells following the protocol suggested by the manufacturer (Promega, Madison, WI, USA) and 30 min later the absorbance at 490 nm was recorded using an ELISA plate reader.

Statistical and Densitometrical Analysis

The statistical significance of differences between the means of two groups was evaluated by the two-tailed *t* test and the level of significance was determined and reported as indicated in the figure legends. Experiments were performed 3–8 times as described in the respective figure legends. Quantification of the intensity of protein bands on western blots was performed with the ImageJ software (NIH).

Results

MRK is Required for Glioblastoma Invasion Stimulated by IR

Sublethal doses of IR promote migration and invasiveness of glioblastoma cells (Wild-Bode et al. 2001). We have extended this result to both the SNB19 and the U87 cells. To study cell invasion, we adopted a highly reproducible three-dimensional invasion assay in which the cells are imbedded into Matrigel and then deposited in the insert of a transwell chamber. We found that IR (3 Gy) enhances invasion as well as migration of glioblastoma cells by about twofold compared to the non-irradiated control (0 Gy) (Figs. 1, 2). A higher dose (6 Gy) did not further increase cell invasion (Fig. 1a), possibly because of increased toxicity as previously shown by Wild-Bode et al. (2001). Therefore, subsequent invasion and migration experiments were performed at 3 Gy. This is a low dose, similar to the single dose received by glioblastoma patients treated with fractionated radiotherapy in the clinic (Marcu 2010; Niyazi et al. 2011).

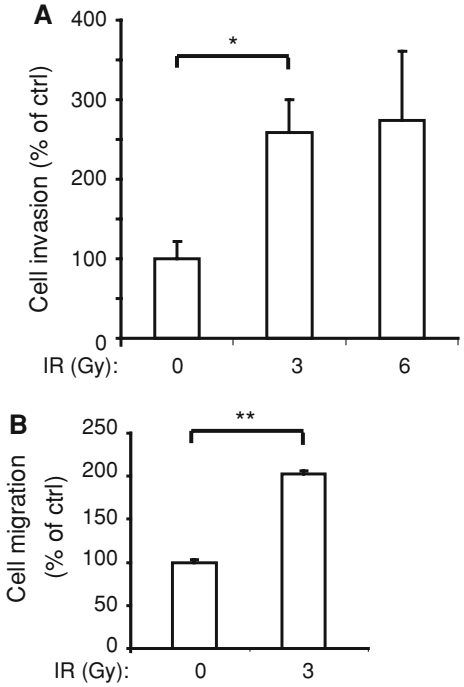

Fig. 1 IR induces migration and invasion of glioblastoma cells. SNB19 cells were exposed to the indicated doses of IR and tested for invasion (**a**) and for migration (**b**). Cells were imbedded in Matrigel for the invasion assay or seeded directly onto the transwell filter of migration chambers, allowed to spread for 3 h and exposed to the indicated doses of IR. Invaded cells were quantified 24 h later and cell migration was quantified 6 h after plating and normalized for controls. Histograms represent the mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.001$, two-tailed *t* test

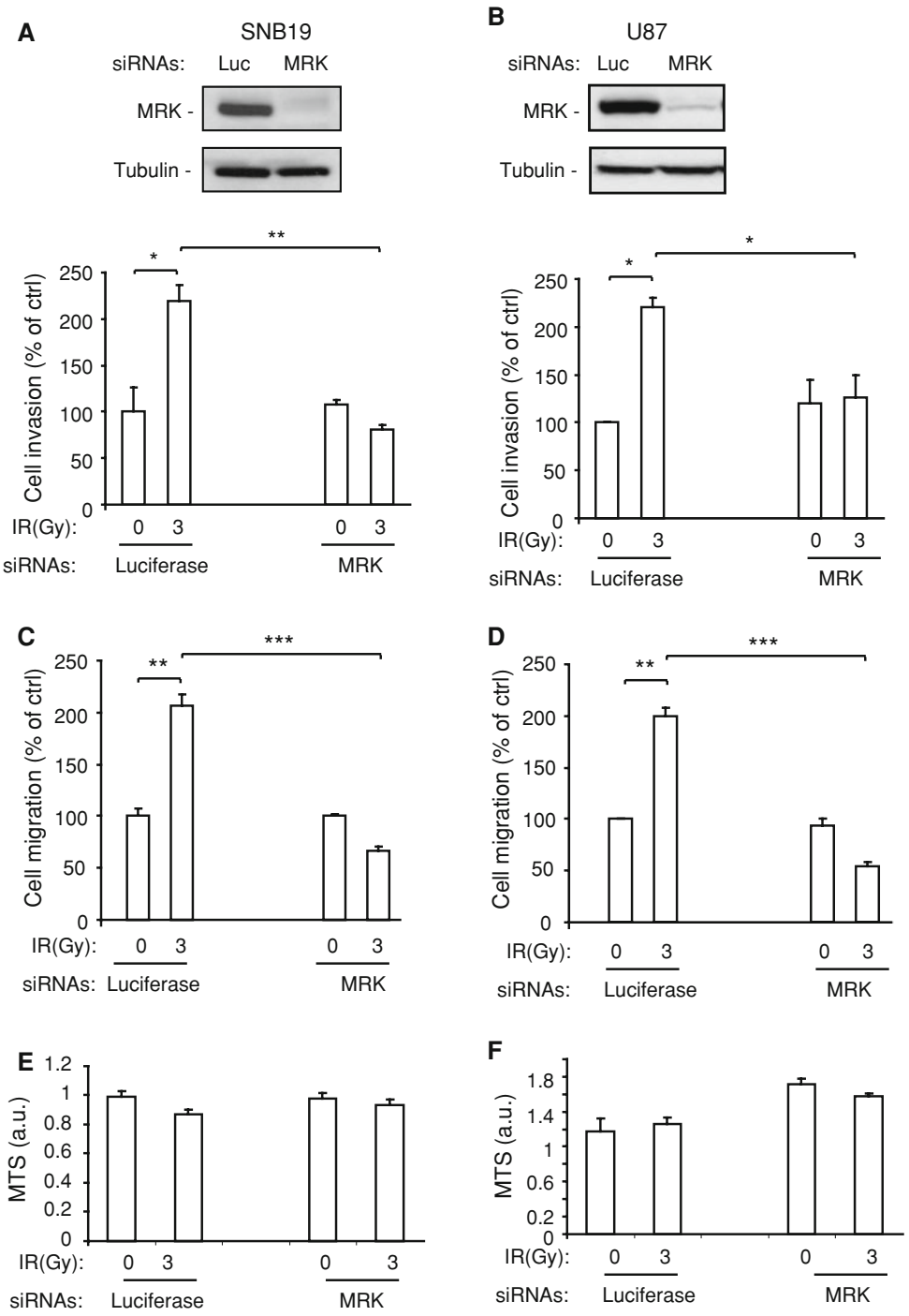

Fig. 2 MRK depletion inhibits IR-induced glioblastoma cell invasion and migration. SNB19 (**a**, **c**, and **e**) and U87 (**b**, **d**, and **f**) cells were transfected with luciferase control or MRK siRNAs, seeded as described in Fig. 1 for invasion (**a**, **b**) or migration (**c**, **d**) and exposed to the indicated doses of IR. MTS assays (**e**, **f**) were performed as described in "Materials and methods" at a time corresponding to the end of the invasion assays. Histograms are mean ± SEM of three independent experiments. Samples comparisons for which a statistically significant difference was observed are indicated by the *edges* of each *square bracket*.*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, two-tailed *t* test

We have previously shown that MRK is activated by IR and mediates checkpoint responses to DNA damage (Gross et al. 2002). Because IR activates growth factor receptors and downstream MAP kinase pathways (Valerie et al. 2007) involved in the regulation of migration, we asked whether MRK, which is a MAP3K protein, also could mediate the migratory and invasive properties of glioblastoma cells after IR treatment. Figure 2 shows that downregulation of MRK completely inhibits IR-stimulated invasion and migration of glioblastoma cells. Under non-irradiated conditions, MRK downregulation did not affect basal invasion or migration. This is consistent with the observation that under these conditions MRK activity is very low and thus its contribution to basal invasion and migration is negligible. Importantly, we did not observe any effect of depleting MRK on cell viability by MTS assay during the time frame of the invasion assay (Fig. 2e, f), indicating that the decrease in cell invasion is not caused

by cell death. Thus, in addition to controlling cell cycle arrest after IR, MRK is required for IR-induced migration and invasion of glioblastoma cells.

MRK Functions Downstream of Nbs1 in IR-Induced Glioblastoma Cell Migration

As the signaling elements that mediate the MRK activation by DNA damage are not known, we next investigated whether the IR-stimulated activation of MRK requires MDC1 and Nbs1, the most upstream elements in the cascade of checkpoint proteins. Thus, we depleted Nbs1 or MDC1, using specific siRNAs, and measured IR-induced MRK activation using a phospho-MRK specific antibody, which we previously showed to detect active MRK (Tosti et al. 2004). To compensate for the weak signal produced by this antibody, we used a Tet-On U2-OS osteosarcoma cell system that can be induced to express high levels of MRK by treatment with doxycycline (Gossen and Bujard 1992). As this was a short-term experiment, we increased the radiation dose to 6 Gy to detect an appreciable signal for MRK activation. Figure 3 shows that depletion of Nbs1, or its upstream adaptor protein MDC1, completely inhibited IR-stimulated MRK activation.

We also observed that siRNA-mediated depletion of Nbs1 completely inhibits IR-induced migration of glioblastoma cells down to base-line levels (Fig. 4), indicating that MRK functions downstream of Nbs1 in IR-induced glioblastoma cell migration. Thus, these observations identify a novel pathway, comprising Nbs1 and MRK, which mediates IR-induced glioblastoma cell migration.

Cell Cycle Arrest is Necessary for IR-Stimulated Glioblastoma Cell Migration

Chk2 is one of the checkpoint kinases that are important for the DNA damage response, in particular following double-strand DNA damage caused by IR (Bartek and Lukas 2003). We have shown previously that MRK is necessary for full activation of Chk2 after IR in osteosarcoma cells (Tosti et al. 2004). To extend these observations to glioblastoma cells, we examined the requirement of MRK for IR-stimulated Chk2 phosphorylation in SNB19 cells. MRK depletion reduced the extent of Chk2 phosphorylation stimulated by IR in SNB19 cells (data not shown), confirming the role of MRK in checkpoint regulation in glioblastoma cells.

Chk2 functions as one of the key control elements in the regulation of IR-induced cell cycle arrest (Bartek and

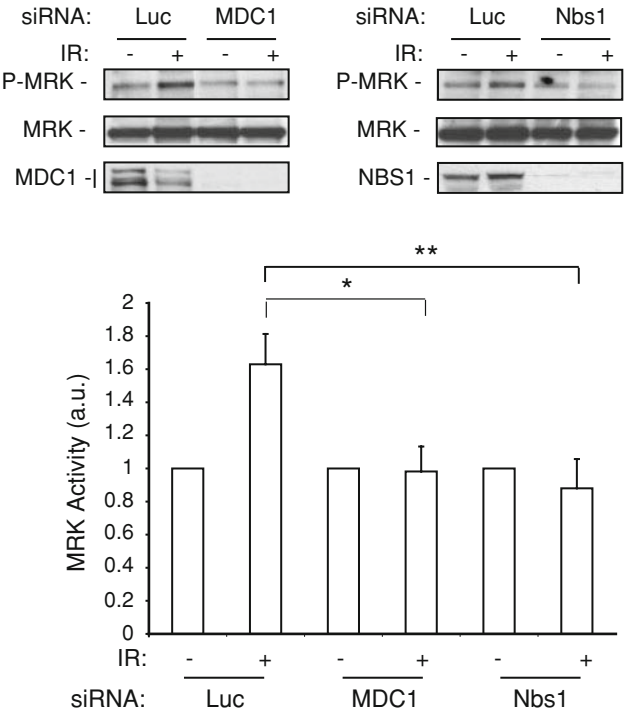

Fig. 3 Nbs1 and MDC1 are necessary for MRK activation by IR. M28 cells, expressing recombinant MRK, were transfected with the indicated siRNAs and 24 h after transfection they were induced overnight with doxycycline (0.25 μg/ml) to promote KT3-tagged recombinant MRK expression. Cells were then exposed to 6 Gy of IR, incubated at 37 °C for 15 min and harvested. Proteins were immunoprecipitated with the KT3 antibody and tested by western blot analysis first with the phospho-MRK antibody (P-MRK) and then with total MRK antibody to test for loading. Histograms represent the mean ± SEM from 6–8 independent experiments. $*p < 0.05$, $**p < 0.005$, two-tailed t test

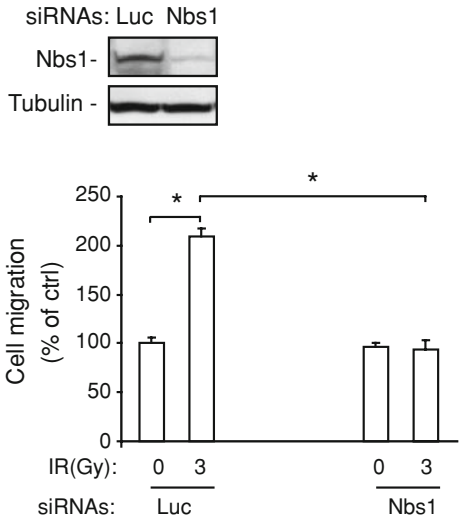

Fig. 4 Nbs1 downregulation inhibits IR-induced glioblastoma cell migration. SNB19 cells were transfected with siRNAs directed at luciferase (Luc) control or Nbs1. Sixty hours later they were seeded on migration chambers and exposed to the indicated doses of IR. *Top panel* A western blot analysis to confirm downregulation. Histograms are mean ± SEM of three independent experiments. $*p < 0.001$, two-tailed t test

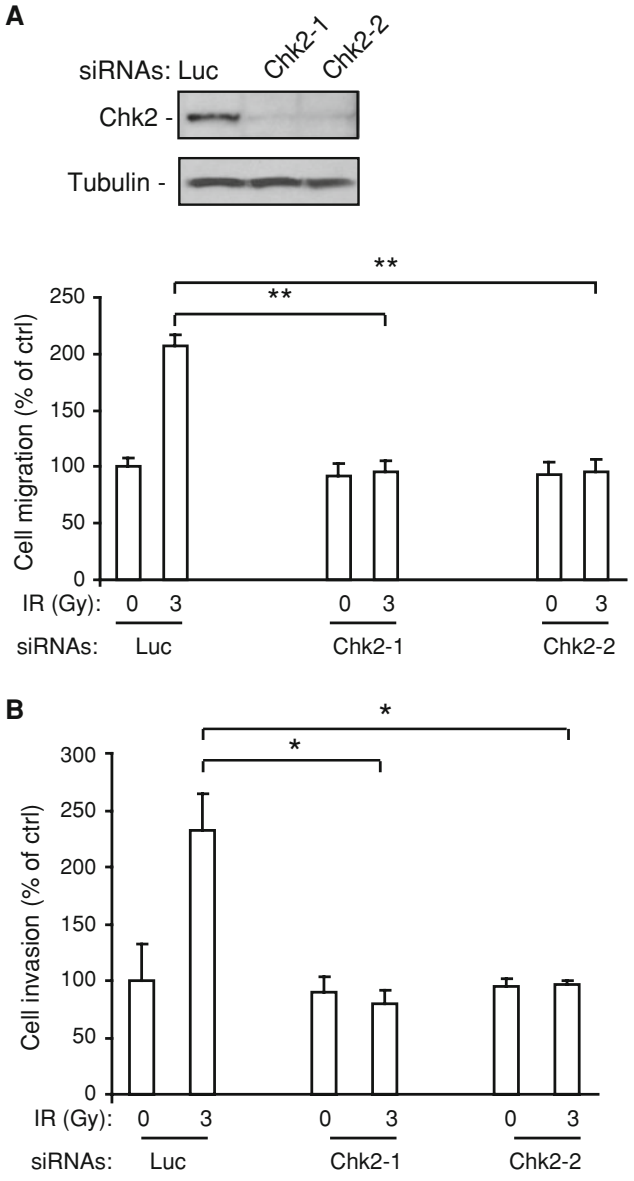

Fig. 5 Chk2 depletion abolishes IR-induced glioblastoma cell migration and invasion. SNB19 cells were transfected with control (Luc) or two independent siRNAs oligos targeting Chk2 and migration (**a**) and invasion (**b**) assays were carried out as in Fig. 1. Western blot shows protein knockdowns. Histograms are mean ± SEM of three independent experiments with three replicates each. $*p < 0.05$, $**p < 0.005$, two-tailed *t* test

Lukas 2003), and to date, no role for Chk2 in cell migration has been described. We therefore examined the effect of depleting Chk2 on IR-stimulated cell migration and invasion. We found that downregulation of Chk2 by two different siRNAs inhibited both the IR-stimulated SNB19 migration and the invasion (Fig. 5), suggesting that Chk2 also functions downstream of MRK to mediate the IR-stimulated migratory behavior of glioblastoma cells.

To investigate the mechanism through which Chk2 controls IR-stimulated invasion, we considered two possibilities. One is that Chk2 could control cell migration and cell cycle arrest via two independent pathways. Alternatively, Chk2 could regulate cell migration indirectly, via controlling cell cycle arrest (Fig. 6a). In support of the latter hypothesis, there is accumulating data in the literature that suggest that the cell cycle can negatively regulate cell migration (Giese et al. 1996). To distinguish between these two scenarios, we depleted MRK or Chk2, thereby preventing IR-induced cell cycle arrest (Falck et al. 2001; Tosti et al. 2004), and enforced cell cycle arrest, independently of MRK or Chk2, by using CGP74514A, a selective inhibitor of CDK1/2 (Imbach et al. 1999). CGP74514A on its own did not affect IR-stimulated glioma cell migration, but it largely restored migration of MRK-depleted cells and completely restored migration in Chk2-depleted cells (Fig. 6b). The same pattern was observed for IR-stimulated cell invasion (Fig. 6c). Thus, these results strongly suggest that MRK and Chk2 control IR-stimulated cell migration and invasion by mediating cell cycle arrest.

To eliminate the possibility that these results were caused by off-target effects of the CDK inhibitor, we also used siRNA-mediated depletion of CDC25A, a mediator of cell cycle progression (Zhou and Elledge 2000). Similar to the CDK inhibitor treatment, depletion of CDC25A did not affect the response to IR of control cells, but it completely reverted the inhibition of migration and invasion caused by depletion of either MRK or Chk2 (Fig. 7). Thus, collectively these results indicate that MRK and Chk2 control IR-stimulated cell migration and invasion via cell cycle regulation. In addition, these studies indicate that cell cycle arrest is necessary for the IR-stimulated invasive behavior of glioblastoma cells and that IR-induced cell cycle arrest removes an inhibitory function on this behavior.

Discussion

In this study, we have identified Nbs1, MRK, and Chk2 as elements of a novel signaling pathway that mediates IR-stimulated cell migration and invasion. Moreover, we found that cell cycle progression, caused by depletion of either MRK or Chk2 in the context of IR-stimulation, interferes with IR-stimulated glioblastoma cell migration and invasion, providing strong evidence that cell cycle progression can modulate the migratory behavior of cells.

A number of studies have indicated the existence of an antagonistic relationship between cell migration and proliferation (reviewed in Giese et al. 2003 and Evdokimova et al. 2009). For instance, glioblastoma cells seeded on extracellular matrix components that promote their migratory behavior are less proliferative, while the opposite is observed for cells seeded on non-motogenic substrates (Giese et al. 1996). One mechanism that has been

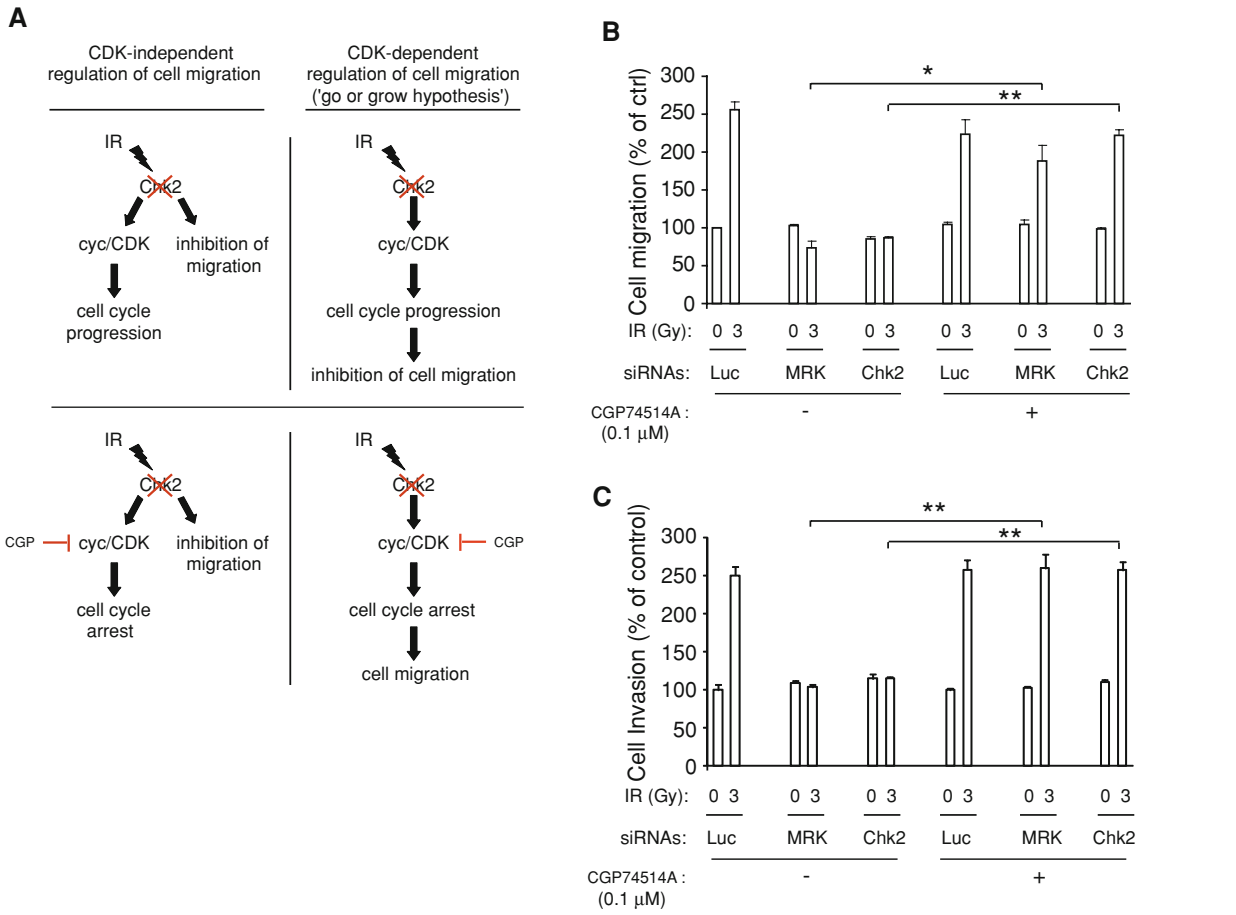

Fig. 6 The CDK1/2 inhibitor CGP74514A reverts inhibition of IR-induced migration in cells depleted of MRK or Chk2. **a** Models representing two possible mechanisms of regulation of cell migration downstream of Chk2. *Left panels* A model in which Chk2 directly stimulates cell migration, independently of the inhibitory effect of Chk2 on cell cycle progression. *Right panels* A model in which Chk2 promotes cell migration via its inhibitory effect on the cell cycle. *Top panels* The result that depletion of Chk2 inhibits IR-induced cell migration. *Bottom panels* The consequence of forcing cell cycle arrest with the CDK1/2 kinase inhibitor in the absence of Chk2 in the respective scenarios: in the CDK-independent regulation of cell migration model, the CDK inhibitor arrests the cell cycle without impacting inhibition of cell migration. In the CDK-dependent model, the CDK inhibitor causes cell cycle arrest, thereby restoring cell migration. SNB19 cells were transfected with the indicated siRNAs and, at the time of seeding for migration (**b**) or invasion (**c**), the CDK1/2 kinase inhibitor was added to the media in the top chamber as well as in the bottom well. Migration and invasion assays were carried out as in Fig. 1. Data are mean ± SEM from 3–6 independent experiments. $*p < 0.005$, $**p < 0.001$, two-tailed t test

proposed to mediate the cell migration–proliferation switch involves miR-451, which is down regulated in migrating glioma cells (Godlewski et al. 2010). On the one hand, miR-451 inhibits cell migration by targeting the LKB1 kinase complex, which in turn activates pro-migratory MARKs proteins; while on the other hand, miR-451 stimulates proliferation by targeting AMPK, a kinase that inhibits cell proliferation. Another example is the transcription/translation regulatory protein YB-1, which is overexpressed in a number of human cancers and inhibits cell proliferation, while inducing an epithelial-to-mesenchymal transition (Evdokimova et al. 2009). However, the relationship between cell cycle progression and cell migration is complex and may depend on how the cell is stimulated. Most growth factors promote both the cell cycle progression and the cell migration.

It is important to note that a few core cell cycle proteins, including the Cip/Kip family members and Rb and E2F3, also have been implicated in the regulation of neuronal cell migration (McClellan et al. 2007). The Cip/Kip proteins have also been shown to regulate cell migration in other cell types (Besson et al. 2008). In all these cases, these effects are independent of cell cycle regulation. It is possible that MRK and Chk2 also can control the invasive properties of cells via signaling mechanisms that are distinct from those that regulate cell cycle progression. However, the observation that inhibition of IR-stimulated migration and invasion caused by loss of these proteins is

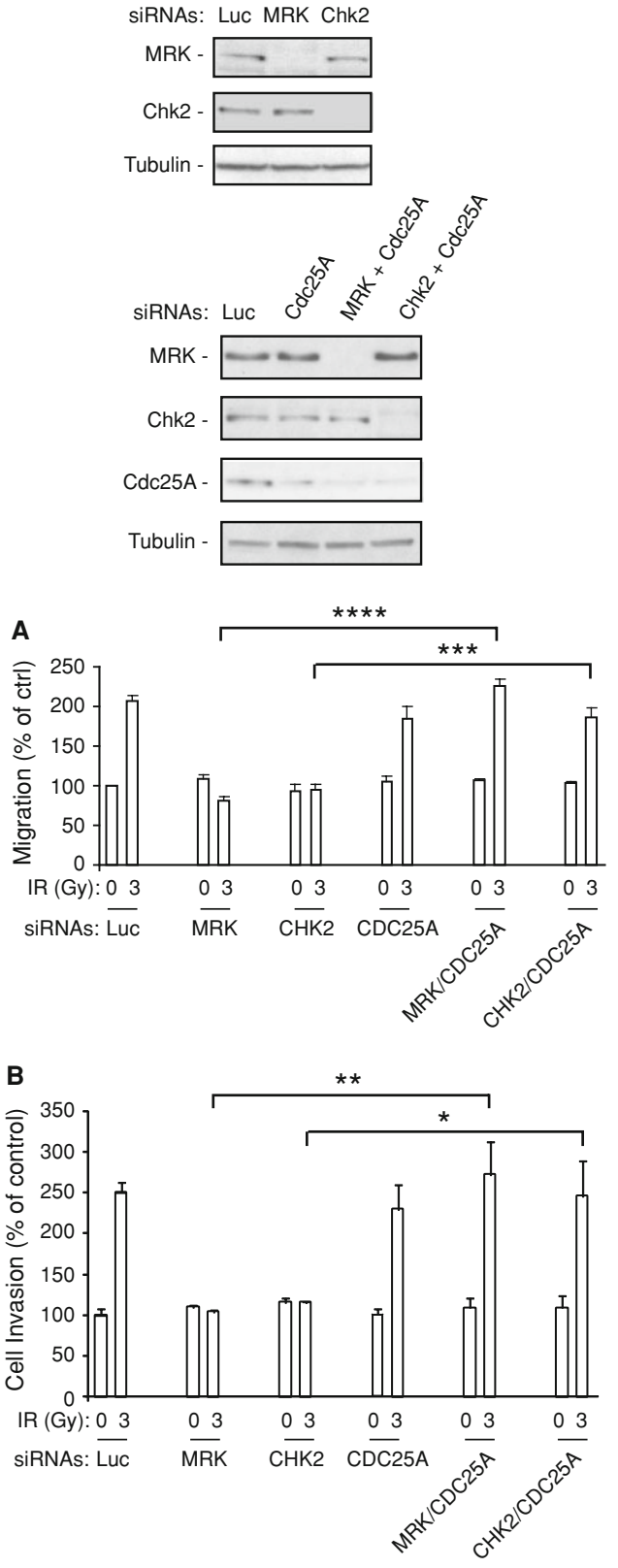

Fig. 7 Downregulation of Cdc25A restores IR-induced migration in cells depleted of MRK or Chk2. SNB19 cells were transfected with the indicated siRNAs and migration (**a**) and invasion (**b**) assays were carried out as explained in Fig. 1. Western blots show downregulation of the respective proteins. Histograms are mean ± SEM from three independent experiments done in triplicates. $*p < 0.05$, $**p < 0.02$, $***p < 0.005$, $****p < 0.001$, two-tailed *t* test

restored by blocking cell cycle progression provides strong evidence that MRK and Chk2 mediate IR-stimulated invasive cell behavior by inducing cell cycle arrest.

The involvement of CDK proteins in the regulation of the invasive properties of cells has not been reported to date, although cyclin D1 has been shown to be important for cell migration and invasion of breast cancer cells via p27 and filamin A (Li et al. 2006; Zhong et al. 2010). The observation that the CDK inhibitor restores migration and invasion in cells depleted of either MRK or Chk2 indicates that the CDK1/2 proteins provide inhibitory signals for cell migration in the context of IR, presumably via their stimulatory effect on the cell cycle. How cell cycle progression negatively regulates IR-stimulated invasive cell behavior is a critical question that remains to be investigated.

In this study, we also identified signaling elements that are essential for IR-induced MRK activation. We showed that the activation of MRK by IR critically depends on MDC1 and Nbs1. IR is known to stimulate the production of reactive oxygen species, which in turn leads to the activation of several motogenic growth factor receptors (including EGFR and IGF-1R) and downstream MAP kinases (Valerie et al. 2007). Thus, because MRK acts as a MAP3K protein (Gross et al. 2002), one possible mechanism of MRK activation by IR could be downstream of growth factor receptors. However, the finding that loss of MDC1 and Nbs1 completely abolished IR-stimulated activation of MRK suggests that the contribution of growth factor receptors to MRK activation in the context of IR is negligible.

In summary, we have shown that elements of the DNA damage checkpoint pathway are necessary for IR-induced tumor cell migration and invasion and that they operate via inhibiting cell cycle progression, presumably by relieving inhibitory signals on migration downstream of CDK proteins. Inhibition of checkpoint regulators has been recognized to enhance the sensitivity of tumor cells to genotoxic stress since more than a decade (Shapiro and Harper 1999) and several drugs specifically targeting checkpoint proteins are under development and are being tested in clinical trials (Ashwell et al. 2008; Bolderson et al. 2009). Recently, a specific inhibitor of Chk2 has been reported to potentiate the cytotoxicity of PARP inhibitors, which interfere with DNA repair (Anderson et al. 2011).

Our findings have important clinical implications, as targeting checkpoint proteins may provide a double benefit in conjunction with radiotherapy: increased radiosensitivity and inhibition of IR-induced invasive behavior of tumor cells. This dual effect is likely to be of particular relevance

for glioblastoma, where tumor cell migration remains a substantial obstacle to effective therapy and relapses are commonly observed in the vicinity of the initial tumor mass (Hess et al. 1994). If, as suggested by Wild-Bode et al. (2001), sub-lethal doses of radiation allow escape of tumor cells from the target irradiation area, concomitant use of inhibitors of checkpoint regulatory proteins could also significantly improve the efficacy of radiotherapy. Thus, MRK represents a new therapeutic target in this context. Future studies will determine the efficacy of inhibiting MRK in conjunction with IR treatment in animal models of glioblastoma.

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Conflict of interest The authors declare that they have no conflict of interest.

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