Title
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Journal
Proceedings of the National Academy of Sciences of USA, 116(41)

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Publication Date
2019-10-08

DOI
10.1073/pnas.1906578116

Peer reviewed

CRIF1 as a potential target to improve the radiosensitivity of osteosarcoma

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Edited by James E. Cleaver, University of California San Francisco Medical Center, San Francisco, CA, and approved September 6, 2019 (received for review April 16, 2019)

Resistance to ionizing radiation (IR), which is a conventional treatment for osteosarcoma that cannot be resected, undermines the efficacy of this therapy. However, the mechanism by which IR induces radioresistance in osteosarcoma is not defined. Here, we report that CR6-interacting factor-1 (CRIF1) is highly expressed in osteosarcoma and undergoes nuclear-cytoplasmic shuttling of cyclin-dependent kinase 2 (CDK2) after IR. Osteosarcoma cells lacking CRIF1 show increased sensitivity to IR, which is associated with delayed DNA damage repair, inactivated G1/S checkpoint, and mitochondrial dysfunction. CRIF1 interacts with the DNA damage checkpoint regulator CDK2, and CRIF1 and CDK2 colocalize in the nucleus after IR. Nuclear localization of CDK2 is associated with phosphorylation changes that promote DNA repair and activation of the G1/S checkpoint. CRIF1 knockdown synergized with IR in an in vivo osteosarcoma model, leading to tumor regression. Based on these findings, we identify CRIF1 as a potential therapeutic target in osteosarcoma that can increase the efficacy of radiotherapy. More broadly, our findings may provide insights into the mechanism for other types of radioresistant cancers and be exploited for therapeutic ends.

CDK2 | CRIF1 | radiotherapy | osteosarcoma | radioresistance

Osteosarcoma, derived from mesenchymal stromal cells of an osteoblast origin, is the most common primary malignant bone tumor (1). Treatment for osteosarcoma is primarily dependent on tumor resection and systemic multiagent chemotherapy, which cures 65 to 70% of patients (2). For unresectable osteosarcoma or cases that are not completely resected, radiotherapy is one option for achieving local tumor control (3). While osteosarcoma initially shows a dose response to ionizing radiation (IR), it gradually develops radioresistance, eventually necessitating high doses of radiation to achieve an effective result (4). This is associated with unacceptable side effects and limits the efficacy of this treatment (5). Therefore, understanding the mechanisms of radioresistance in osteosarcoma could expand the therapeutic window and improve patient survival.

The gradual development of radioresistance to IR suggests that osteosarcoma cells have an unusual cellular repair ability following sublethal doses of radiation (6). This repair ability is closely related to the preferential activation of the DNA damage response (DDR), including repair of DNA and activation of the DNA damage checkpoint (7). Radiotherapy eliminates tumor cells by inducing DNA damage, while DDR sabotages radiotherapy (8). Inhibition of DDR enhances the radiosensitivity of tumor cells, and DDR-based therapies have been developed targeting molecules such as p21, WEE1, and NBS1 in osteosarcoma (9–11). Following DNA damage, p21 (12) and WEE1 (13) help activate the DNA damage checkpoint, probably by inhibiting the activity of cyclin-dependent kinase 2 (CDK2).

CDK2 is a critical regulator of cell cycle progression from G1 to S, and the activity of CDK2 is tightly regulated. The complete kinase activation of CDK2 needs to be translocated to the nucleus, phosphorylated at T160, and dephosphorylated at T14 and Y15 (14). Radiation increases the phosphorylation of CDK2 at T160 (15), which is crucial for the initiation of DNA repair. Inhibition of T160 phosphorylation might block phosphorylation at S432 of NBS1 and increase sensitivity to IR (16). Radiation can also inhibit the dephosphorylation of T14 and Y15 of CDK2 and lead to cell cycle arrest (12). Maintenance of T14 and Y15 dephosphorylation results in cells with DNA damage entering the cell cycle through the G1/S checkpoint (17). Therefore, factors regulating CDK2 phosphorylation are likely to play a role in radioresistance in osteosarcoma (*SI Appendix*, Fig. S1).

CR6-interacting factor-1 (CRIF1), with a nuclear localization signal (NLS), is a negative regulator of cell cycle progression (18). We have previously reported suggestive linkage of CRIF1 to IR responses in bone marrow mesenchymal stem cells (BMSCs). After radiation, the expression of CRIF1 is elevated, and CRIF1-knockdown BMSCs exhibited more apoptosis, implying that CRIF1 may have a role in radioresistance of these cells (19, 20). The osteosarcoma cells may originate from BMSCs and share some surface markers, including CD90, CD105, and CD106 (21). This prompted us to hypothesize that CRIF1 plays a

Significance

The mechanism of radioresistance in osteosarcoma is unknown. We analyze osteosarcoma patient tissues, combine in vitro and in vivo mouse osteosarcoma models, and determine that CR6-interacting factor-1 (CRIF1) drives radioresistance by regulating the activity of cyclin-dependent kinase 2 (CDK2). After ionizing radiation, CRIF1 promotes CDK2 nuclear translocation and phosphorylation on T14/T160, facilitating G1/S checkpoint activation and DNA damage repair. CRIF1 knockdown enhances the radiosensitivity of osteosarcoma in both in vitro and xenograft models. Our study sheds light on the role of tumor-specific biomarkers in identifying a therapeutic window to overcome radioresistance of osteosarcoma, pointing out a way to precision medicine for a particular patient.

Author contributions: Q.R., Y.Z., and Z.L. designed research; Q.R., F.J., Y. Xiang, L.X., Q.W., F.L., Li Chen, C.W., L.Z., Y. Xiao, Lili Chen, and J.W. performed research; Q.R., F.J., Y. Xiang, L.X., Q.W., F.L., Li Chen, Y.Z., J.F.Z., S.C.L., and Z.L. analyzed data; and Q.R., F.J., Y. Xiang, J.F.Z., S.C.L., and Z.L. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1906578116/-/DCSupplemental.

First published September 23, 2019.

role in the radioresistance of osteosarcoma. Here, we demonstrate that CRIF1 promotes radioresistance in osteosarcoma, and CRIF1 knockdown enhances the radiosensitivity of osteosarcoma cells. CRIF1 binds CDK2 and modulates its nuclear translocation and phosphorylation post-IR. We suggest that CRIF1-mediated CDK2 activates the G1/S checkpoint and enhances DNA repair, thus resulting in radioresistance.

Results

CRIF1 Is Highly Expressed in Osteosarcoma Tissues. The expression levels of CRIF1 were analyzed in 30 paraffin-embedded archived osteosarcoma tissues using immunohistochemistry (IHC). The results showed that CRIF1 levels were elevated in tumor tissues compared with the corresponding adjacent nontumor tissues, with strong staining of CRIF1 detected in 60% of the osteosarcoma tissues (Fig. 1*A*). Immunofluorescence (IF) further confirmed that CRIF1 was overexpressed in the cytoplasm of fresh osteosarcoma tissues (Fig. 1*B*). The *CRIF1* mRNA was highly expressed in 12 tumor tissues of 13 patients, and CRIF1 protein levels were all higher than those of corresponding adjacent nontumor samples by Western blotting (Fig. 1 *C* and *D*).

We next evaluated the association of the increased CRIF1 expression with clinicopathologic features in 30 osteosarcoma samples with IHC data. Kaplan–Meier survival analysis showed that patients with osteosarcoma and overexpression of CRIF1 in their tumors exhibited decreased overall survival compared with those with low expression of CRIF1 in their tumors ($P = 0.030$; Fig. 1*E*). We next performed a retrospective analysis of 4 patients who received radiotherapy from 30 archived osteosarcoma patients. Three of them who had a high level of CRIF1 developed metastases or recurrences within half a year. Another one who had not developed metastasis or recurrence for more than 3 y, since received radiotherapy expressed a low level of CRIF1 (Fig. 1*F*).

We further found that CRIF1 expression was higher in osteosarcoma cell lines than in nonosteosarcoma control cancer cells, except BMSCs (*SI Appendix*, Fig. S2 *A* and *B*). BMSCs are known to be radioresistant (22), and, consistent with our data, as previously shown, to express high levels of CRIF1 (20). Taken together, these results suggest that overexpression of CRIF1 was frequently detected in osteosarcoma, and its overexpression may contribute to radioresistance.

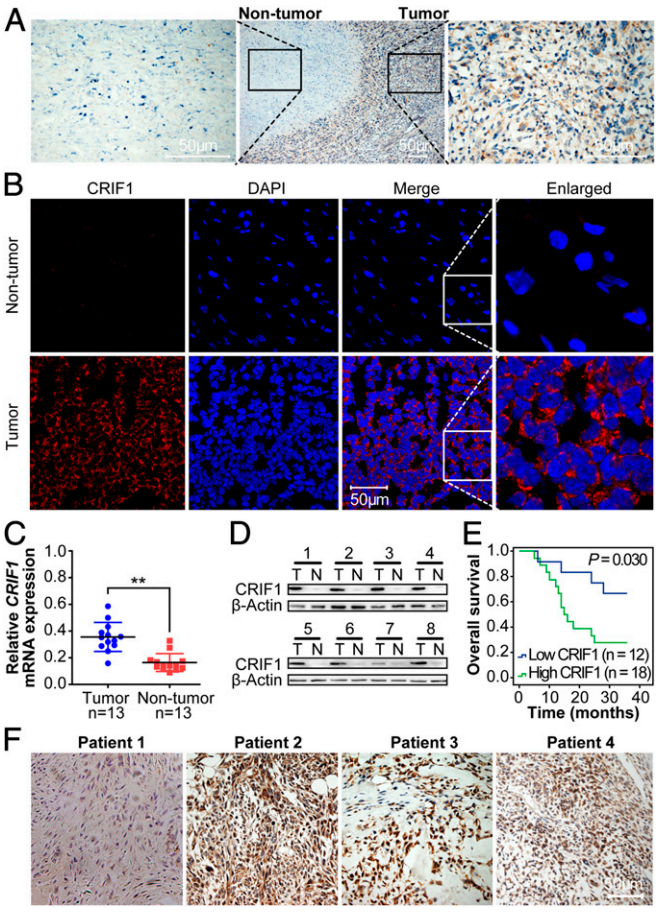

Fig. 1. CRIF1 is expressed at high levels in osteosarcoma tissues. The expression of CRIF1 in osteosarcoma tumor tissue (T) and adjacent nontumor tissue (N) from the same patient was assessed by immunohistochemistry (*A*), immunofluorescence (*B*), RT-PCR (*C*), and Western blotting (*D*). (*E*) Kaplan–Meier curves showing the overall survival of osteosarcoma patients with low versus high CRIF1 expression of CRIF1, $n = 30$. The CRIF1 expression was determined by IHC in 4 patients who received radiotherapy from 30 archived osteosarcoma patients (*F*). Error bars represent the SD, $^{**}P < 0.01$. (Scale bars, 50 μm.)

Decreased CRIF1 Expression Is Associated with Radiosensitivity in Osteosarcoma Cells. To explore the impact of CRIF1 on the radioresistance of osteosarcoma, we stably knocked down CRIF1 by transfecting U2OS cells with shRNA targeting CRIF1 (shCRIF1) or a nontargeting shRNA (NT shRNA) as a control (*SI Appendix*, Fig. S2*C*). We found that CRIF1-knockdown cells exhibited significantly more apoptosis than the control group post-IR (*SI Appendix*, Fig. S2 *D* and *E*), suggesting that CRIF1 expression might affect the radiosensitivity of osteosarcoma cells. CRIF1 also could affect radiosensitivity in some other cancer cells and normal cells (HuH-7, Jurkat, HF, and HUVEC) (*SI Appendix*, Fig. S2 *F*–*H*).

We next asked whether the effect that we observed of CRIF1 knockdown on radioresistance in osteosarcoma cells was due to CRIF1-mediated modulation of cell cycle progression, cell proliferation, DNA repair, or reactive oxygen species (ROS) level. CRIF1 knockdown did not affect the cell cycle; neither cell proliferation nor migration was affected in the absence of IR based on comparison with NT shRNA-U2OS cells (Fig. 2*A* and *SI Appendix*, Fig. S3 *A* and *B*). However, the shCRIF1 cells showed a significant increase in the proportion of cells in S phase and the proportion of EdU-positive cells 4 h post-IR compared with the NT shRNA cells (Fig. 2 *A* and *B*). Within 24 h after IR, the NT shRNA-U2OS cells exhibited a cell cycle distribution as they did before IR treatment, but 50% of the CRIF1-knockdown cells were still in G2/M phase (Fig. 2*A*). In conclusion, these results indicate that CRIF1 knockdown inactivated G1/S checkpoint after IR.

Our results showed that, in the absence of IR, the CRIF1 knockdown did not change the expression levels of γH2AX in osteosarcoma cells compared to NT shRNA-U2OS cells (*SI Appendix*, Fig. S3 *C* and *D*). In addition, knockdown of CRIF1 did not increase the proportion of cells with γH2AX-positive foci 2 h after IR compared to NT shRNA-U2OS cells (*SI Appendix*, Fig. S3*E*). In NT shRNA-U2OS cells, the number of γH2AX foci gradually decreased up to 6 h after IR, with a corresponding reduction in the γH2AX expression levels (Fig. 2 *C* and *D*). CRIF1 knockdown, however, resulted in an increase in the number of γH2AX foci at 6 h post-IR, and the high levels of γH2AX in osteosarcoma cells lasted up to 8 h post-IR (Fig. 2 *C* and *D*). Furthermore, a number of cells with γH2AX-positive foci were observed 24 h after radiation in a dose-responsive manner (0 to 12 Gy), suggesting that CRIF1 knockdown delays DNA repair (Fig. 2*C* and *SI Appendix*, Fig. S3*F*).

As CRIF1 is a member of mitoribosome (23), and CRIF1 regulates the production and clearance of ROS (24, 25), we hypothesized that reduction of CRIF1 can lead to excess ROS and thus impair the morphology and function of mitochondria after IR. To test this, we examined the morphology of mitochondria, mitochondrial membrane potential ($\Delta\Psi_m$), and ROS production in shCRIF1-U2OS and NT shRNA-U2OS cells. We found that,

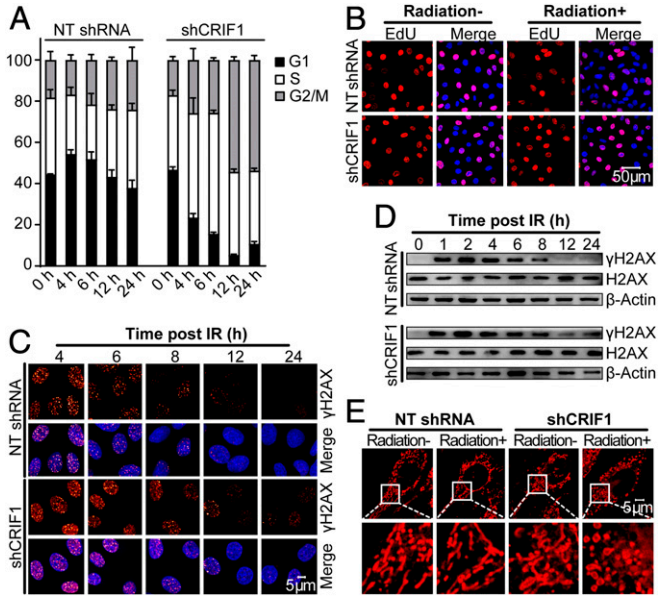

Fig. 2. CRIF1 expression responds to cellular radiation. (*A*) Time course of the distribution of NT shRNA-U2OS and shCRIF1-U2OS cells in different phases of the cell cycle after irradiation was detected by propidium iodide staining. Error bars represent the SD. (*B*) The 5-Ethynyl–2′-deoxyuridine (EdU) positivity of NT shRNA-U2OS and shCRIF1-U2OS cells post-IR. Time course showing the formation of γH2AX foci (*C*) and the total cellular levels of γH2AX protein (*D*) after IR in NT shRNA-expressing or shCRIF1-expressing U2OS cells. (*E*) Confocal microscopy analysis of mitochondrial morphology at 4 h post-IR in NT shRNA-U2OS and shCRIF1-U2OS cells. (Scale bars, 50 μm in *B*; 5 μm in *C* and *E*.) All IR treatments were with 9 Gy irradiation.

after IR, knockdown of CRIF1 led to the fragmentation of mitochondria (Fig. 2*E*) and significantly increased intracellular ROS levels compared with the NT shRNA-U2OS cells (*SI Appendix*, Fig. S4 *A* and *B*). CRIF1 knockdown also led to a reduction in $\Delta\Psi_m$ after IR in a dose-dependent fashion (0 to 15 Gy) (*SI Appendix*, Fig. S4 *C* and *D*). A decrease in ΔΨm is a marker of early apoptosis, suggesting that increased apoptosis in CRIF1-knockdown U2OS cells post-IR may partly initiate from mitochondria. In addition, shCRIF1-U2OS cells showed a significant reduction in their ATP concentration compared with NT shRNA-U2OS cells post-IR (*SI Appendix*, Fig. S4*E*). Moreover, U2OS cell viability after IR was significantly reduced in the CRIF1-knockdown group than in the control groups (*SI Appendix*, Fig. S3*A*). Thus, CRIF1 knockdown in U2OS cells enhanced radiosensitivity and was associated with inactivation of G1/S checkpoint, DNA repair delay, mitochondrial dysfunction, and oxidative stress.

CRIF1 Is Associated with CDK2 Nuclear Translocation after IR. CDK2 plays an essential role in double-strand break (DSB) induced apoptosis, G1/S checkpoint activation, and DNA repair (26, 27). We previously showed that G0/G1 arrest in Jurkat cells is related to the interaction of CRIF1 with CDK2 (28), and CRIF1-knockdown BMSCs exhibited more apoptosis post-IR (19). We therefore explored the localization of CRIF1 and CDK2 in osteosarcoma cell lines and found some colocalization of these 2 proteins (Fig. 3*A* and *SI Appendix*, Fig. S5*A*). In addition, we observed the colocalization of aggregated CRIF1 and CDK2 in the nucleus post-IR (Fig. 3*A*). We carried out immunoprecipitations and Western blotting and showed that CRIF1 was associated with CDK2 in osteosarcoma cells and BMSCs (Fig. 3*B*). To explore the impact of CRIF1 knockdown on the regulation of CDK2 post-IR, we first detected whether the expression levels of CRIF1 affected CDK2 expression. IR induced up-regulation of CRIF1 expression in NT shRNA-U2OS cells (*SI Appendix*, Fig. S5*B*). However, neither IR nor CRIF1 knockdown affected the levels of CDK2 or cyclin E, which is a key partner of CDK2 (*SI Appendix*, Fig. S5 *B–D*).

CDK2 nuclear translocation is dependent on binding to proteins with an NLS, and the NLS-containing protein CRIF1 can bind CDK2 (29). Therefore, we postulated that CRIF1 might modulate the nuclear translocation of CDK2 post-IR. To test this, we investigated the distributions of CRIF1 and CDK2 post-IR. After IR, the nuclear accumulation of CRIF1 was significantly increased in NT shRNA-U2OS cells, and CRIF1 colocalized with CDK2. CRIF1 knockdown led to reduced nuclear translocation of CDK2 after IR (Fig. 3*C*). In addition, the interaction between CDK2 and CRIF1 was enhanced in U2OS and BMSCs post-IR (*SI Appendix*, Fig. S5*E*). Nucleoproteins were extracted to allow the detection of CDK2 and CRIF1 using Western blotting. CRIF1 expression increased concurrently with CDK2 in the nucleus. CRIF1 knockdown delayed the nuclear translocation of both CDK2 and CRIF1 (Fig. 3*D*). These results suggested that CRIF1 might prompt the nuclear transport of CDK2 and thereby play an important role in IR-induced CDK2 nuclear aggregation.

CRIF1 Expression Promotes Post-IR Phosphorylation of CDK2 at T160. Given the colocalization of CRIF1 and CDK2 in the nucleus after IR, we postulated that CRIF1 may regulate the nuclear translocation of CDK2 and that this could be important for phosphorylation of CDK2 at T160. T160 is phosphorylated in the nucleus (30) and phosphorylation at T160 is essential for the function of radioprotective effect of CDK2 (16). To test this hypothesis, we examined the effect of decreasing CRIF1 expression on the accumulation of CDK2-pT160 after IR in U2OS cells. Although CRIF1 knockdown did not affect the levels of CDK2-pT160 in the absence

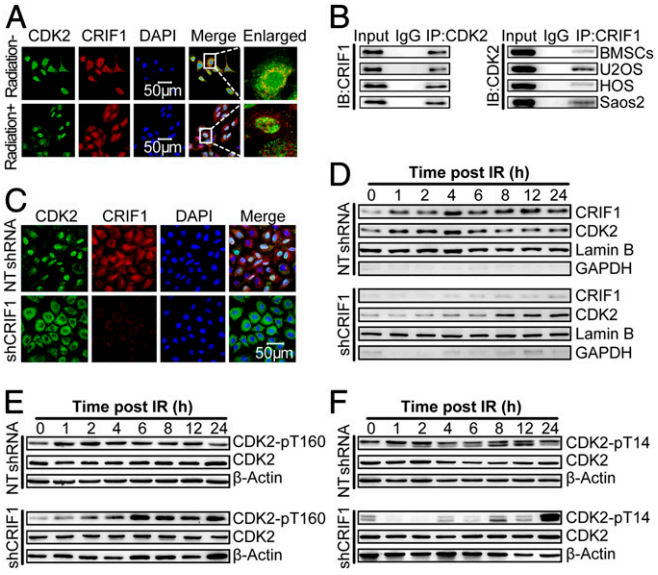

Fig. 3. CRIF1 colocalizes with CDK2 in the nucleus and is associated with phosphorylation of CDK2. (*A*) Confocal microscopy of U2OS cells showed that CRIF1 colocalizes with CDK2. (*B*) Association of CRIF1 with CDK2 in BMSCs, U2OS, HOS, and Saos2 cells was shown by immunoprecipitation and Western blotting. (*C*) Immunofluorescence of NT shRNA-U2OS and shCRIF1-U2OS cells after IR was evaluated using confocal microscopy. (*D*) Nuclear proteins from NT shRNA-U2OS and shCRIF1-U2OS cells were extracted after IR, and CRIF1 and CDK2 protein levels were detected by Western blotting. (*E* and *F*) Total protein was extracted from NT shRNA-U2OS and shCRIF1-U2OS cells after IR. Western blotting was used to evaluate the levels of CDK2-pT160 (*E*) and CDK2-pT14 (*F*). β-Actin was used as a loading control for total protein (*E* and *F*), and lamin B was used as a loading control for nuclear protein (*D*) in Western blotting. (Scale bars, 50 μm.) All IR treatments were with 9 Gy irradiation.

of IR (*SI Appendix*, Fig. S5*C*), the level of CDK2-pT160 was increased 1 h post-IR in the NT shRNA-U2OS cells (Fig. 3*E*). However, in the CRIF1-knockdown cells, an increase in the level of CDK2-pT160 was not observed until 4 h post-IR (Fig. 3*E*), suggesting that loss of CRIF1 delays the phosphorylation of CDK2 at T160. Like CDK2, nuclear CDK2-pT160 was increased 2 h post-IR in NT shRNA-U2OS cells. In contrast, in the CRIF1-knockdown U2OS cells, CDK2-pT160 was unaltered following IR within 2 h (*SI Appendix*, Fig. S6*A*). The Western blotting data showed that CRIF1 knockdown decreased the level of nuclear CDK2 and CDK2-pT160 relative to that in NT shRNA-treated cells after IR (*SI Appendix*, Fig. S6*B*).

The CDK-dependent phosphorylation of NBS1 S432 can promote DNA repair (31); thus, delaying or diminishing the phosphorylation of NBS1 at S432 leads to radiosensitivity (16). In U2OS cells transfected with NT shRNA, a rapid increase in the level of NBS1-pS432 was observed 1 h post-IR, peaking at 4 h and then gradually decreasing. In the CRIF1-knockdown cells, the increase in NBS1-pS432 levels was delayed, peaking at 8 h post-IR (*SI Appendix*, Fig. S6*C*). Taken together, these results suggest that CRIF1 knockdown delays the nuclear translocation of CDK2 and its phosphorylation on T160 after IR, resulting in the delayed phosphorylation of NBS1 S432.

CRIF1 Expression Increases Phosphorylation of CDK2 at T14 after IR. Given that CRIF1 knockdown led to inactivation of the G1/S checkpoint and that phosphorylation of T14 and Y15 on CDK2 is required for checkpoint activation, we therefore asked whether CRIF1 knockdown inhibited the dephosphorylation of these 2 residues. Dephosphorylation of these sites is regulated by CDC25A (12), and we therefore wished to investigate the role of CDC25A in this process. Compared with NT shRNA-U2OS cells, CRIF1 knockdown in U2OS cells reduced the phosphorylation of CDK2 at T14 but not Y15, either in the presence or absence of IR (Fig. 3*F* and *SI Appendix*, Figs. S5*C* and S6*D*). In NT shRNA-U2OS cells, the phosphorylation of CDK2 at T14 was increased 1 h post-IR. In contrast, in CRIF1-knockdown cells, T14 phosphorylation was low until 8 h post-IR (Fig. 3*F*). The results of both IF and Western blotting showed that CDK2-pT14 accumulated in the nuclei of U2OS cells post-IR, whereas CRIF1 knockdown reduced this accumulation (*SI Appendix*, Fig. S7 *A* and *B*).

We then examined the effect of CRIF1 knockdown on CDC25A nuclear localization. After IR, CDC25A was degraded in NT shRNA-U2OS cells, while the aggregation of CDC25A in the nucleus was found in shCRIF1-U2OS cells, and this localization of CDC25A might be responsible for the observed decrease in CDK2-pT14 (*SI Appendix*, Fig. S7 *C* and *D*). Since CDC25A degradation-mediated sustaining inhibitory phosphorylation of CDK2 blocks DNA replication after radiation (32), we next explored the impact of CRIF1 knockdown on DNA replication in DSBs unrepaired cells. We found that the proportion of EdU and γH2AX double-positive cells in CRIF1-knockdown U2OS cells was much higher than that in the NT shRNA group (*SI Appendix*, Fig. S7*E*), indicating that the dephosphorylation of T14 caused by CRIF1 knockdown might lead to G1/S checkpoint inactivation and DNA replication in cells with unrepaired DSBs.

CRIF1 Promotes Radioresistance of Osteosarcoma In Vivo. To examine whether the changes in CDK2 phosphorylation in U2OS cells were similar in osteosarcoma patient samples, we analyzed the expression of CDK2-pT14 and CDK2-pT160 in fresh samples and found that CDK2-pT14 levels were higher in osteosarcoma tissues than those in corresponding adjacent nontumor tissues, while the difference of CDK2-pT160 was not evident (Fig. 4*A*). In addition, CDK2-pT14 colocalized with CRIF1 in osteosarcoma tissues (*SI Appendix*, Fig. S8 *A* and *B*), further supporting our hypothesis that CRIF1 may regulate phosphorylation of CDK2 in osteosarcoma.

Our in vitro data suggested that up-regulation of CRIF1 could promote radioresistance in osteosarcoma cells; therefore we carried out experiments to test whether the reduction of CRIF1 expression could alter the radiosensitivity of osteosarcoma in vivo. Nude mice were s.c. injected with NT shRNA-U2OS or shCRIF1-U2OS cells to establish osteosarcoma tumors. The tumors were confirmed by H&E staining, and IHC analysis demonstrated that the expression of CRIF1 was down-regulated in the osteosarcoma formed by shCRIF1-U2OS cells (*SI Appendix*, Fig. S9*A*). The mice were treated with a single 5-Gy dose of radiotherapy when the tumor

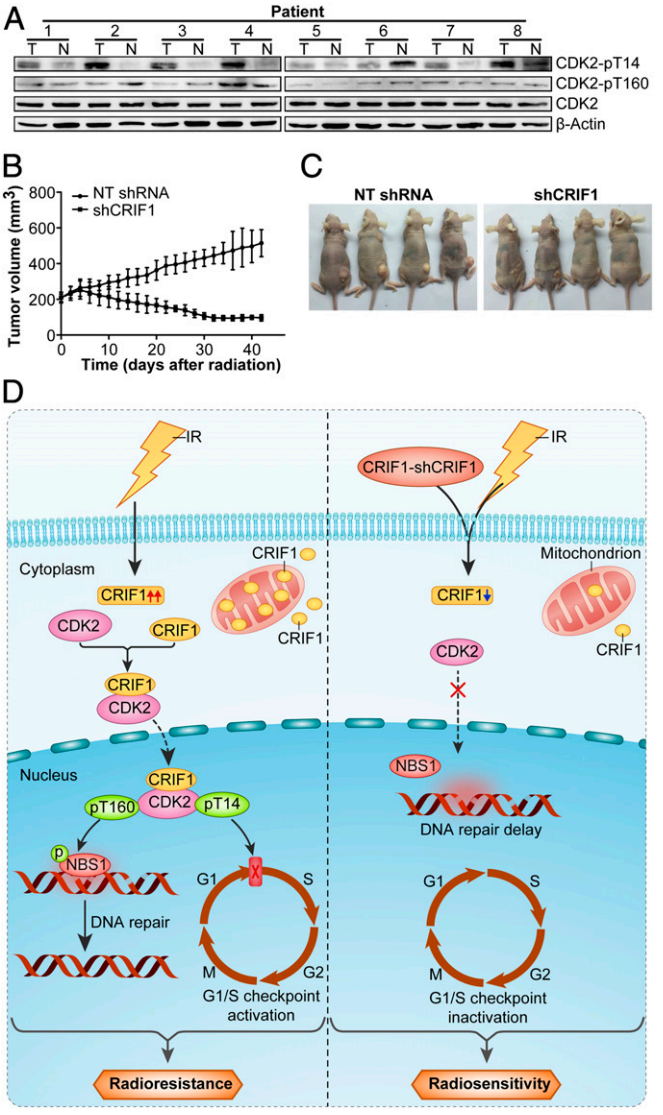

Fig. 4. CRIF1 knockdown synergizes with radiotherapy in a xenograft model of osteosarcoma. (*A*) Western blotting was carried out to detect CDK2-pT14, CDK2-pT160 in tumor (T) and nontumor tissues (N) from the same osteosarcoma patients. β-Actin was used as a loading control for Western blotting. (*B*) The tumor growth curves after IR in the xenograft mice are shown; tumor volumes were measured every other day. Error bars represent the SEs, $n = 6$. (*C*) Tumors in the NT shRNA and shCRIF1 groups 42 d after IR treatment. (*D*) Model for the suggested regulation of CDK2 nuclear localization and phosphorylation by CRIF1 after radiotherapy in osteosarcoma. In response to IR, CRIF1 up-regulation is associated with CDK2 nuclear translocation, which ultimately promotes activation of G1/S checkpoint and DNA repair, resulting in radioresistance (*Left*). CRIF1 knockdown prevents CDK2 nuclear translocation post-IR; subsequent delays of G1/S checkpoint activation and DNA repair lead to a radiosensitivity (*Right*).

volumes had reached 200 mm^3. Twelve hours after mice received radiation, tumor samples were collected and apoptosis was assessed using TUNEL. We found that, compared with the NT shRNA control group, the number of TUNEL-positive nuclei in the shCRIF1-U2OS group was increased (*SI Appendix*, Fig. S9*B*). This result suggested that the cells with CRIF1 knockdown seemed to be more sensitive to radiotherapy in vivo. We found that combination of IR and CRIF1 knockdown led to substantial tumor regression, whereas the tumors continued to grow in the NT shRNA group even after IR (Fig. 4 *B* and *C*). After 42 d, the remaining tumors were resected and measured, and the tumors in the shCRIF1-U2OS group were much smaller than in the NT shRNA control group (*SI Appendix*, Fig. S9*C*). Together, these results suggest that CRIF1 knockdown promotes radiosensitivity of osteosarcoma in vivo.

Discussion

Converging evidence indicates that CDK2 catalytic activity independent of cell cycle function is required to activate DNA repair proteins in the DDR (33). However, how CDK2 achieves this catalytic action in the inhibited state in response to DNA damage has not yet been elucidated. Here we show that, in osteosarcoma, CRIF1 overexpression is associated with CDK2 nuclear translocation and phosphorylation at T14 and T160 post-IR. We suggest that these modifications of CDK2 promote G1/S checkpoint activation and DNA repair and may contribute to the observed radioresistance of osteosarcoma. Our findings suggest a mechanistic model for the regulation of CDK2 by CRIF1 in osteosarcoma that may have consequences for the susceptibility of this tumor type to radiotherapy (Fig. 4*D*).

DDR, a highly orchestrated signaling cascade to recognize and repair DNA lesion, affects responses to DNA-damaging anticancer chemotherapy and radiotherapy (34). The DDR apparatus coordinates the repair of damaged DNA and the activation of checkpoint to block cell cycle progression. CDK2 is a central player of G1/S checkpoint activation and DNA repair by phosphorylating several DNA repair factors (33). While the kinase activation of CDK2 in cell cycle transition is well studied, comparatively little is known about the phosphorylation status regulation in CDK2-dependent DNA repair and G1/S checkpoint activation. Previous studies suggested that inhibitory phosphorylation (T14/Y15) is necessary for G1/S checkpoint activation (12, 17) and activating phosphorylation (T160) is required for DNA repair (15, 16), respectively, but the status of inhibitory and activating phosphorylation was not investigated simultaneously. Despite no apparent change at Y15, we found a clear and rapid increase in the phosphorylation of CDK2 both at T14 and T160 after IR. These observations indicate that rapid and asynchronous phosphorylation may facilitate G1/S checkpoint activation and DNA repair. Hence, it is fundamentally important to understand what factors govern the coordination between inhibitory and activating phosphorylation of CDK2 after IR. CDK2 is regulated spatially by cytoplasmic-nuclear shuttling, and the nuclear translocation of CDK2 is essential for the regulation of its activity by phosphorylation (35). The interaction between CRIF1 and CDK2 found in this study appears to be indispensable for CDK2 nuclear translocation post-IR. CRIF1 inhibition that disrupted the nuclear translocation post-IR delayed phosphorylation at T14 and T160, which hindered G1/S checkpoint activation and DNA repair. This strongly suggests that blocking CRIF1-mediated modulation of CDK2 activity can be applied to selectively enhancing the responses of cancer cells to DNA-damaging agents, such as cytotoxic chemotherapy and radiotherapy.

Numerous CDK-inhibiting drugs have been developed as potential cancer therapeutics and evaluated in a wide variety of cancer types, but only a few CDK2 inhibitors entered clinical trials due to their low selectivity in discriminating between cancerous and normal cells (36). Thus, the factors that regulate CDK2 activity might be another choice of therapeutic target, and some inhibitors targeting those regulators such as WEE1 and Chk1 have been demonstrated with antitumor activity in clinical trials (37). AZD1775, a specific inhibitor of WEE1 kinase, is currently studied in several clinical trials, and some studies showed encouraging antitumor activity involving radiosensitizing strategy (37, 38). Inhibition of CRIF1 is likely to play the same role of WEE1 inhibitor in CDK2 inappropriate activation, and we suggest that CRIF1 may also be a chemosensitizing and radiosensitizing target in elevated CRIF1 expression cancers.

In conclusion, we show that CRIF1 expression is up-regulated and translocated to the nucleus in response to radiation and that this may be important for the resistance of osteosarcoma cells to radiotherapy. This mechanistic understanding of radioresistant osteosarcoma may also provide insights into new therapeutic approaches for other types of radioresistant cancers.

Materials and Methods

Patient Samples. Osteosarcoma tissues and normal tissues adjacent to the tumors were collected from patients who underwent surgical resection. All experiments involving human subjects were approved by the Ethics Committee of the Third Military Medical University, and all patients provided informed consent.

Human Osteosarcoma Xenografts. Five-week-old female BALB/c nu/nu mice (Beijing Vital River Laboratories) were used for xenografts. The 5×10^6 U2OS cells expressing NT shRNA or shCRIF1 were s.c. injected into the dorsal lateral flank of nude mice to generate osteosarcoma tumors. The mouse procedures were performed in accordance with protocols approved by the Animal Welfare and Ethics Committee of the Third Military Medical University.

Details of the materials and methods are presented in *SI Appendix*, *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was supported by the National Natural Science Foundation of China (Grant 81472915), the National Natural Youth Science Foundation of China (Grant 81402634), the Youth Scientist Foundation of Chongqing (CSTC 2013JCYJJQ10001), the "1130" Project of Xinqiao Hospital, and clinical project of Xinqiao Hospital (2014YLC011).

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