Cell Research (2015) 25:225-236.

www.nature.com/cr

ORIGINAL ARTICLE

Tyrosine 370 phosphorylation of ATM positively regulates DNA damage response

Hong-Jen Lee[1, 5], Li Lan[6, 7], Guang Peng[4], Wei-Chao Chang[8, 9], Ming-Chuan Hsu[1], Ying-Nai Wang[1, 8], Chien-Chia Cheng[1], Leizhen Wei[6, 7], Satoshi Nakajima[6, 7], Shih-Shin Chang[1, 5], Hsin-Wei Liao[1, 5], Chung-Hsuan Chen[9], Martin Lavin[10], K Kian Ang[2, *], Shiaw-Yih Lin[3], Mien-Chie Hung[1, 5, 8, 11]

[1]Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA; [2]Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA; [3]Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA; [4]Department of Clinical Cancer Prevention, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA; [5]The University of Texas Graduate School of Biomedical Science at Houston, Houston, TX 77030, USA; [6]Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA 15213, USA; [7]Hillman Cancer Center, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, USA; [8]Graduate Institute of Cancer Biology, Center for Molecular Medicine, China Medical University, Taichung 404; [9]The Genomics Research Center, Academia Sinica, Taipei 115; [10]University of Queensland Centre for Clinical Research, Royal Brisbane Hospital Campus, Herston, Australia; [11]Department of Biotechnology, Asia University, Taichung 404

Ataxia telangiectasia mutated (ATM) mediates DNA damage response by controling irradiation-induced foci formation, cell cycle checkpoint, and apoptosis. However, how upstream signaling regulates ATM is not completely understood. Here, we show that upon irradiation stimulation, ATM associates with and is phosphorylated by epidermal growth factor receptor (EGFR) at Tyr370 (Y370) at the site of DNA double-strand breaks. Depletion of endogenous EGFR impairs ATM-mediated foci formation, homologous recombination, and DNA repair. Moreover, pretreatment with an EGFR kinase inhibitor, gefitinib, blocks EGFR and ATM association, hinders CHK2 activation and subsequent foci formation, and increases radiosensitivity. Thus, we reveal a critical mechanism by which EGFR directly regulates ATM activation in DNA damage response, and our results suggest that the status of ATM Y370 phosphorylation has the potential to serve as a biomarker to stratify patients for either radiotherapy alone or in combination with EGFR inhibition.

***Keywords*:** EGFR; ATM; tyrosine phosphorylation; DNA damage response

Cell Research (2015) **25**:225-236. doi:10.1038/cr.2015.8; published online 20 January 2015

Introduction

DNA damage response (DDR) is a critical and complex cellular protection event responsible for coordinating DNA repair systems and maintaining chromosome integrity and stability in mammalian cells [1]. Ataxia telangiectasia mutated (ATM) is one of the major serine/threonine kinases mediating numerous downstream signaling pathways, including apoptosis, cell cycle arrest, irradiation-induced foci (IRIF) formation, and DNA repair, in response to various DNA damage stimuli such as ionizing radiation (IR), H_2O_2, and cytotoxic agents [2, 3]. Upon formation of DNA double-strand breaks (DSBs), inactive homodimeric ATM dissociates into active monomers for autophosphorylation at S367, S1893, S1981, and S2996 [4, 5]. The Mre11-Rad50-NBS1 (MRN) complex then recruits activated ATM to DSBs to rapidly phosphorylate downstream effectors, such as H2AX, NBS1, or CHK2, to facilitate DNA repair process and maintain genomic integrity [2, 6, 7]. Inhibition of ATM has been shown to sensitize tumor cells to DNA damage-inducing therapies

Correspondence: Mien-Chie Hung
E-mail: mhung@mdanderson.org
*Ang KK regretably passed away.

Received 25 April 2014; revised 25 September 2014; accepted 22 October 2014; published online 20 January 2015

[8]. In addition, primary cells derived from A-T patients (whose ATM protein is missing or defective due to *ATM* gene mutations) and from ATM-knockout mice were more sensitive to radiation or chemotherapy reagents that induce DSBs [9, 10]. Although ATM serves as a central node in DDR and restrains susceptibility to tumor development, it remains largely unclear how upstream signaling regulates ATM upon DNA damage stimulation.

Epidermal growth factor receptor (EGFR), a membrane-bound receptor tyrosine kinase and well-documented oncogene, functions in regulating the mitogen-activated protein kinase, phospholipase C, signal transducer and activator of transcription (STAT), and phosphatidylinositol-3 kinase pathways in cancer cells [11]. In addition to its role in traditional signaling pathway, several lines of evidence indicate that EGFR translocates from cell membrane to the nucleus in response to growth factors, H_2O_2, UV, therapeutic agents, and IR to play a role in cell proliferation, tumor progression, DNA repair, and chemo- or radioresistance [12-16]. For instance, nuclear EGFR associates with STAT3 [17], STAT5 [18], DNA-PK [14, 19], and polynucleotide phosphorylase [20], and also transactivates *iNOS* (inducible nitric oxide synthase) [17], *CCND1* (cyclin D1) [12], and *AURKA* (Aurora-A kinase) [18] to mediate cancer cell proliferation, tumor progression, and radioresistance. In addition, nuclear EGFR directly phosphorylates proliferative cell nuclear antigen (PCNA), a chromatin-associated DNA replication factor, at Y211, and increased Y211 phosphorylation that is associated with cell proliferation [21]. Blocking Y211 phosphorylation of PCNA was recently demonstrated as a potential therapeutic approach for cancer treatment [22, 23].

In this study, we reveal a novel mechanism by which nuclear EGFR regulates ATM through association with and phosphorylation of ATM at Tyr370 (Y370) upon IR stimulation. We also demonstrate that nuclear EGFR co-localizes with ATM or γ-H2AX at DSBs. Inactivation of EGFR blocks the interaction between EGFR and ATM, attenuates ATM tyrosine phosphorylation, impairs ATM-mediated DDR, and increases radiosensitivity. Together, our data suggest that EGFR-mediated ATM Y370 phosphorylation regulates DDR, contributes to IR resistance, and has the potential to serve as a biomarker to stratify patients for either radiotherapy alone or in combination with EGFR inhibition.

Results

ATM is phosphorylated at tyrosine 370 upon IR stimulation

Several lines of evidence have demonstrated that autophosphorylation of ATM at S367, S1893, S1981, and S2996 are individually required for ATM activation and ATM-mediated DDR in human. However, more recent studies have indicated that mutation of either the prominent S1987 autophosphorylation site (corresponds to S1981 in human) or the three conserved autophosphorylation sites S367/S1899/S1987 (correspond to S367/S1893/S1981 in human) of ATM in mice had no effect on ATM-dependent response [24, 25]. These findings suggest that other mechanisms may be involved in DNA damage-induced activation of ATM in addition to S367/S1893/S1981 autophosphorylation. We performed a mass spectrometry analysis and identified additional IR-triggered ATM phosphorylation at S85, Y370, T1885, S1891, and S2592 (Supplementary information, Figure S1A). Among them, S85, T1885, and S1891 have been reported by other groups [26-28], which further substantiates the reliability of this mass spectrometry analysis. Between the two novel phosphorylation sites Y370 and S2592 identified, Y370 (Figure 1A) appeared to be evolutionarily conserved from yeast to mammals (but not frog or fruit fly) (Supplementary information, Figure S1B). Since ATM is a serine/threonine kinase, the results suggest that ATM Y370 phosphorylation would require a tyrosine kinase.

EGFR phosphorylates and associates with ATM after IR treatment

To determine which tyrosine kinase might be responsible for ATM tyrosine phosphorylation after IR stimulation, we first tested a series of tyrosine kinase inhibitors (TKIs) for their effect on ATM tyrosine phosphorylation by immunoprecipitation (IP) using 4G10, a pan anti-phosphotyrosine antibody. Pretreatment with EGFR kinase inhibitors (gefitinib and AG1478) significantly decreased ATM tyrosine phosphorylation levels (Figure 1B). In contrast, pretreatments with other TKIs, including imatinib, crizotinib, SU4312, PD173074, masitinib, and picropodophyllin, did not have any substantial effects on ATM tyrosine phosphorylation (Figure 1B). These results suggest that EGFR is a potential tyrosine kinase of ATM. Indeed, results from *in vitro* kinase assay indicated that EGFR wild type (WT) phosphorylated purified Flag-tagged ATM but the EGFR kinase-dead (Dead) mutant failed to do so (Figure 1C). In addition, purified human EGFR kinase domain directly phosphorylated a recombinant GST-ATM2 fragment (residues 250-522) [29] but not the one containing the Y370F mutation (Supplementary information, Figure S1C and S1D), which strongly suggests that EGFR is a *bona fide* kinase of ATM. While the *in vitro* kinase assay detected certain non-specific phosphorylation, our data certainly support Y370 as the major site phosphorylated by EGFR.

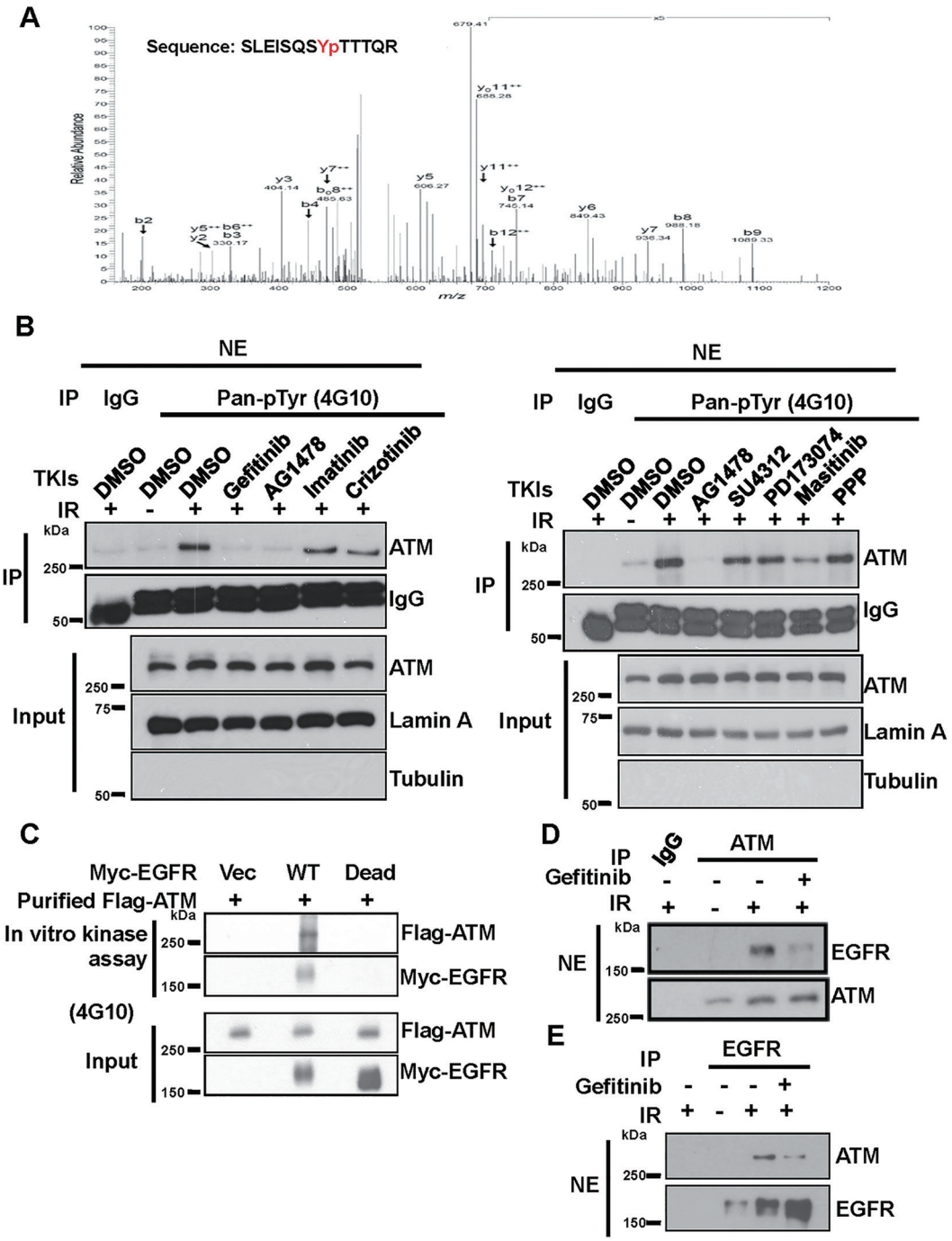


Figure 1 ATM is phosphorylated at tyrosine 370 (Y370) and associated with EGFR after ionizing radiation (IR) stimulation. **(A)** Mass spectrum image showed that ATM is phosphorylated at Y370 (labeled as red) after 10 Gy IR. **(B)** Western blot analysis of ATM tyrosine phosphorylation in nuclear extracts (NE) of HeLa cells pretreated with vehicle (DMSO) or tyrosine kinase inhibitors (TKIs) as indicated, followed by IR stimulation and IP with pan-pTyr antibody (4G10). Lamin A and tubulin served as nuclear and cytosolic markers, respectively. Imatinib, Bcr-Abl kinase inhibitor; crizotinib, lymphoma kinase, c-ros oncogene 1 receptor tyrosine kinase (ROS1), and c-Met inhibitor; SU4312, vascular endothelial growth factor (VEGF) receptor protein tyrosine kinase 1/2 and platelet-derived growth factor (PDGF) receptor inhibitor; PD173074, fibroblast growth factor receptor inhibitor; masitinib, stem cell growth factor receptor (c-kit) and PDGF receptor inhibitor; picropodophyllin (PPP), insulin-like growth factor-1 (IGF-1) receptor inhibitor. **(C)** Purified Flag-tagged ATM proteins were incubated with immunoprecipitated vector alone (Vec), Myc-tagged EGFR wild type (WT), or kinase-dead (Dead) mutant *in vitro* and analyzed by western blotting with Pan-pTyr antibody. **(D**, **E)** Western blot analyses of endogenous ATM **(D)** or EGFR **(E)** IP products from NE of HeLa cells with the indicated antibodies.

EGFR has been shown to translocate to the nucleus in response to various stimuli, including IR [14, 30, 31]. We demonstrated that IR not only provoked EGFR nuclear translocation (Supplementary information, Figure S1E) but also induced endogenous EGFR association with ATM in the nucleus as determined by IP and reciprocal IP assays using anti-EGFR and anti-ATM antibodies against nuclear extracts from HeLa cells (Figure 1D and 1E). Similar results were observed in MDA-MB-468 breast cancer cells (data not shown). Meanwhile, pretreatment with gefitinib reduced the interaction between EGFR and ATM (Figure 1D and 1E). Among various functional domains (Supplementary information, Figure S1F), the C-terminal regulatory (CR) domain of EGFR seemed to be required for its association with ATM (Supplementary information, Figure S1G). Together, these results indicate that EGFR translocates to the nucleus and phosphorylates ATM and that the CR domain of EGFR is required for ATM interaction upon IR stimulation.

EGFR co-localizes with ATM at DSBs upon IR treatment

To investigate whether EGFR is recruited to DSBs in response to DNA damage stimuli, we first carried out chromatin IP combined with PCR in DR-GFP reporter integrated U2OS cell system (Supplementary information, Figure S2A) [32]. We found that exogenous I-*Sce*I expression induced the recruitment of endogenous EGFR to the DSBs (Figure 2A) similar to activated ATM (p-ATM S1981; Figure 2B). Then, using a KillerRed light activation system (Supplementary information, Figure S2B; detailed information is described in Materials and Methods) that generates DSBs, we also showed in Figure 2C that GFP-tagged EGFR signal (indicated by yellow arrowheads in the lower panels) overlapped with tetR-KillerRed (orange color in the merged inset), which was not observed in the control (upper panels), further substantiating the localization of EGFR at DSBs upon laser activation. Together, these data suggest that EGFR, like ATM, is recruited to DSBs upon DNA damage stimulation.

To examine whether EGFR co-localizes with ATM or other DDR proteins such as γ-H2AX at DSBs, we treated U2OS or HeLa cells by laser microirradiation [33, 34] and found overlapping signals indicative of co-localization between GFP-tagged (yellow arrowheads, Figure 2D) or endogenous EGFR (yellow arrowheads, Figure 2E) and activated ATM (p-ATM S1981) or γ-H2AX (Supplementary information, Figure S3A). As ATM is recruited to DSBs by MRN complex [35] and activated by MRN *in vitro* [36, 37], we showed that EGFR IP can pull down the MRN complex after IR treatment (Figure 2F). Depletion of endogenous ATM abrogated this phenomenon, suggesting that EGFR indirectly associates with the MRN complex. These results suggest that EGFR may play a role in DDR by associating with ATM and the MRN complex at DSBs upon IR stimulation.

EGFR is required for ATM-mediated DDR and DNA repair

To determine whether EGFR is required for ATM downstream functions, such as IRIF formation and DNA repair ability, we generated pooled stable clones with EGFR knockdown using lentiviral-based shRNA targeting against EGFR. Silencing EGFR (Supplementary information, Figure S3B) impaired p-ATM S1981 and downstream p-CHK2 and p-KAP1 IRIF formation (Figure 3A, 3B and Supplementary information, Figure S3C). Chromatin-enriched fractionation also showed that ATM S1981 phosphorylation levels were lower in EGFR-knockdown stable clones from two different shRNAs than control cells (Supplementary information, Figure S3D). Importantly, pretreatment with EGFR kinase inhibitor gefitinib also abolished p-ATM S1981 IRIF induced by laser microirradiation (Supplementary information, Figure S3E). IRIF of p-ATM S1981 is known to activate or recruit DDR proteins such as NBS1 and BRCA1 to DSBs to execute DNA repair [38-40]. We next attempted to establish the link between EGFR and DNA repair by carrying out a neutral comet assay, which detects DNA damage level as indicated by comet tail movement. The results showed that EGFR-silenced cells had four times higher DNA damage levels than the control cells, suggesting that EGFR deficiency reduces DNA repair ability (Figure 3C). To substantiate this finding, we compared the number of EGFR-knockdown and control cells that contained γ-H2AX foci as previous reports have indicated that the kinetics of γ-H2AX foci clearance correlates with mammalian cell radiosensitivity [41, 42]. Indeed, EGFR-depleted cells demonstrated delayed DNA repair as indicated by a higher percentage of cells containing γ-H2AX foci compared with control cells at 24 and 48 h after IR stimulation (Figure 3D). To further determine whether EGFR is involved in ATM-mediated homologous recombination (HR) repair [43, 44], we generated control and EGFR-depleted U2OS cells integrated with DR-GFP reporter (Supplementary information, Figure S2A) [32]. We found that DSBs induced by exogenous I-*Sce*I expression was efficiently repaired, as indicated by the number of GFP-expressing cells, in control but not in EGFR-depleted cells (Figure 3E). All together, these results suggest that EGFR is required for ATM-mediated IRIF formation and DNA repair.

EGFR regulates DDR via ATM Y370 phosphorylation

Next, we asked whether EGFR phosphorylates ATM

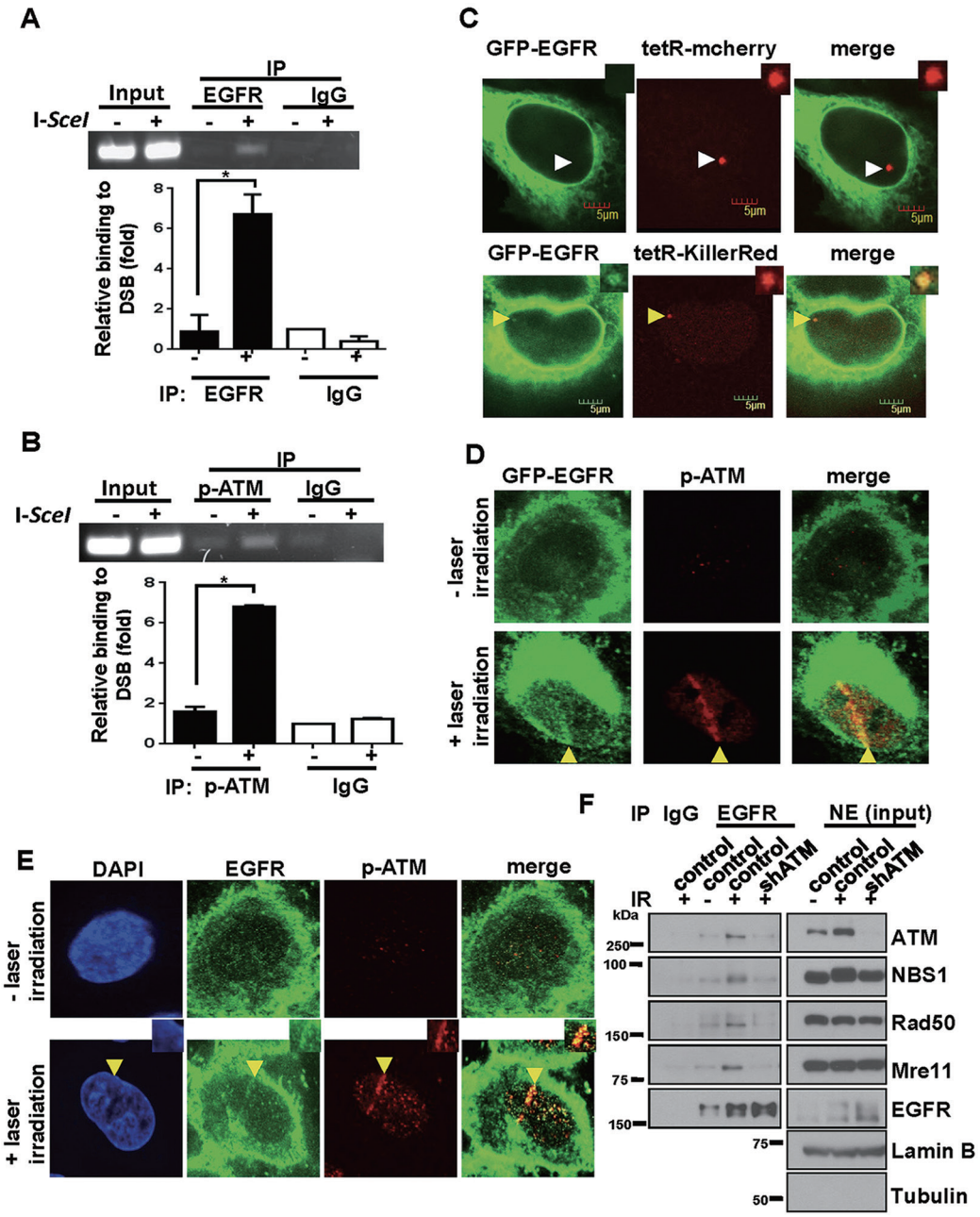
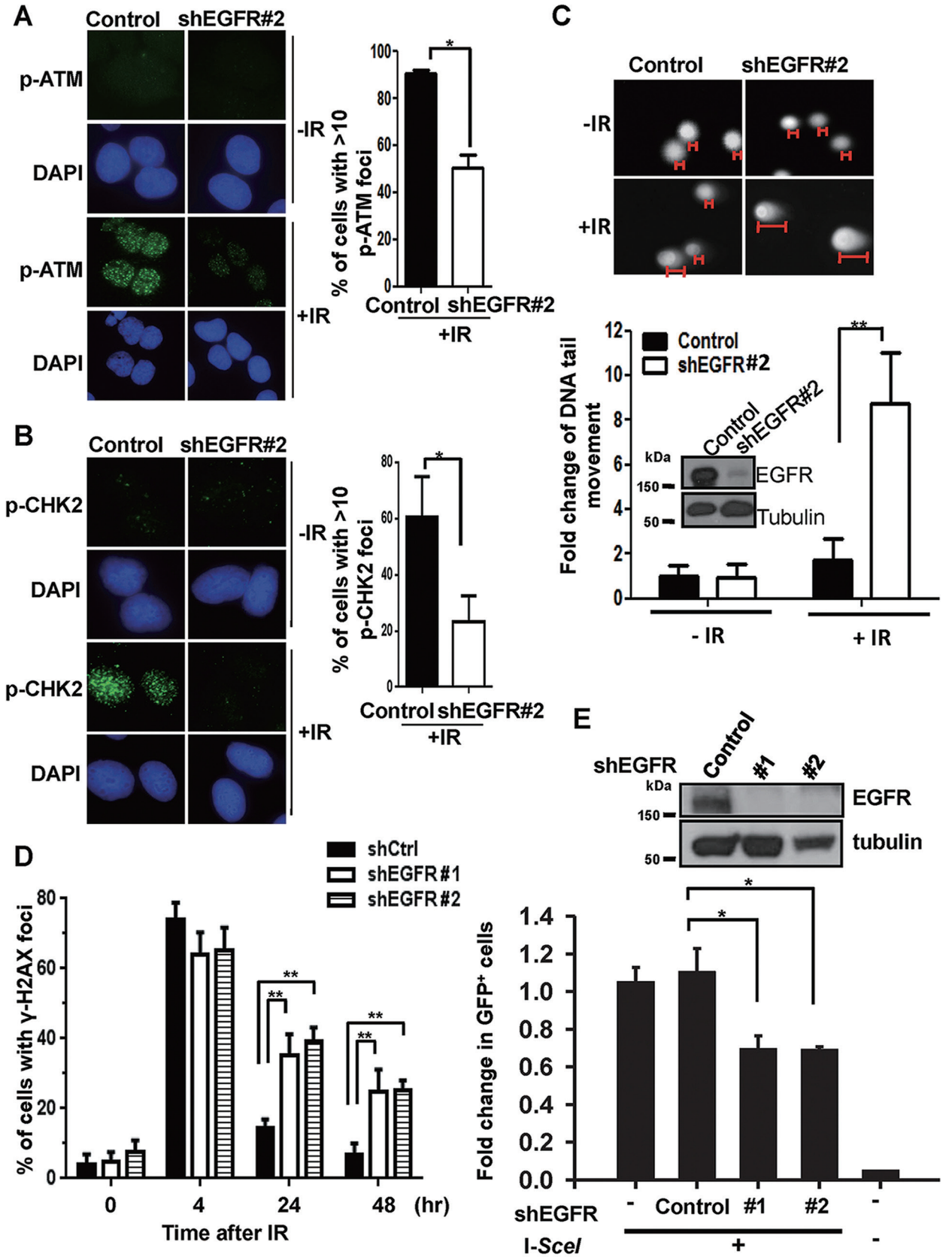


Figure 2 EGFR is recruited to and co-localizes with ATM at DSBs upon IR stimulation. **(A**, **B)** ChIP assay was performed with anti-EGFR antibody, p-ATM S1981 (pATM) antibody or IgG in DR-GFP-integrated U2OS cells with I-*SceI*-induced DSBs. Specific primers flanking I-*SceI* site were used in PCR to detect activated ATM and EGFR localized at DSBs. Quantitation of DSB recruitment fold change is presented as mean ± SD. $n = 3$. $^*P < 0.05$. **(C)** GFP-tagged EGFR and tetR-mcherry or tetR-KillerRed were transfected into U2OS TRE cells. The KillerRed spot was activated with 559 nm laser to induce DNA damage. Representative images after DNA damage induced by KillerRed activation are shown in the lower panels. White arrowheads (also shown in enlarged insets): a tet-repressor fused monomer cherry (tetR-mcherry) binds to a TRE cassette integrated at a defined genomic locus in U2OS cells without laser light-activated DNA damage. Yellow arrowheads (also shown in enlarged insets): DNA damage sites induced by a tet-repressor fused KillerRed (tetR-KillerRed) expression and light activation as described in Supplementary information, Figure S2B. **(D)** GFP-tagged EGFR-transfected U2OS cells were irradiated with 405 nm laser for 100 ms. After irradiation, cells were fixed and stained with p-ATM S1981 antibody. Laser microirradiation-induced DSBs are indicated by yellow arrowheads. **(E)** HeLa cells are irradiated with 405 nm laser for 60 ms. After irradiation, cells were fixed and stained with antibodies against EGFR and p-ATM S1981. Laser microirradiation-induced DSBs are indicated by yellow arrowheads (also shown in enlarged insets). **(F)** Western blotting analysis of endogenous EGFR IP products from nuclear extract (NE) of control or ATM-depleted (shATM) HeLa cells with or without IR stimulation. Lamin B, nuclear fraction marker. Tubulin, cytosolic fraction marker.


A
Control
shEGFR#2
p-ATM
DAPI
-IR
+IR
% of cells with >10 p-ATM foci
Control
shEGFR#2
+IR
B
p-CHK2
% of cells with >10 p-CHK2 foci
C
-IR
+IR
Fold change of DNA tail movement
kDa
150
50
EGFR
Tubulin
- IR
+ IR
D
shCtrl
shEGFR#1
shEGFR#2
% of cells with γ-H2AX foci
Time after IR
0
4
24
48
(hr)
E
shEGFR
#1
#2
Fold change in GFP+ cells
I-SceI

at the conserved Y370 and mediates ATM function through this phosphorylation event. Co-IP of Myc-tagged EGFR with Flag-tagged ATM in HEK293T cells showed that ectopic expression of WT but not kinase-dead (Dead) mutant EGFR enhanced ATM tyrosine phosphorylation *in vivo* (Figure 4A). Consistent with the above finding in which gefitinib pretreatment reduced the association between endogenous EGFR and ATM (Figure 1D and 1E), we found that only EGFR WT but not the kinase-dead mutant interacted with Flag-tagged ATM (Figure 4A). This suggests that the kinase activity of EGFR is required for its binding with ATM. In addition, the ATM Y370F mutant decreased its ability to bind to EGFR as well as its tyrosine phosphorylation level, further supporting that Y370 serves as a major EGFR phosphorylation site. To investigate the role of Y370 phosphorylation *in vivo*, we generated a specific antibody against ATM Y370 phosphorylation and validated the specificity of this antibody by which only the phospho-Y370 peptide but not the non-phospho-Y370 peptide or other phospho-Y peptides was recognized (Supplementary information, Figure S4A). Using this antibody, we showed that phospho-Y370 level was decreased when Flag-tagged Y370F but not WT ATM was co-immunoprecipitated with Myc-tagged EGFR (Supplementary information, Figure S4B). Moreover, the levels of phospho-Y370 increased upon IR but reduced when cells were pretreated with gefitinib (Figure 4B), suggesting that IR-induced ATM Y370 phosphorylation relies on EGFR kinase activity.

To further explore whether binding of EGFR to ATM is required for EGFR-mediated phosphorylation of ATM Y370, we performed co-IP of Flag-tagged ATM with Myc-tagged EGFR WT and EGFR mutants in 293T cells (Supplementary information, Figure S4C and S4D). ATM Y370 phosphorylation was observed only when co-immunoprecipitated with EGFR WT but not the other mutants or when cells were pretreated with gefitinib. Thus, the kinase activity and the CR domain of EGFR are both required for ATM-EGFR association and ATM Y370 phosphorylation. To validate that ATM Y370 phosphorylation responds to IR and orchestrates DDR, Flag-tagged ATM WT or Y370F mutant was restored in ATM-depleted HeLa cells to examine its effect on p-ATM S1981, S367, and S2996 IRIF formation. As shown in Figure 4C and Supplementary information, Figure S4E and S4F, re-expression of ATM WT but not of Y370F mutant rescued p-ATM S1981, S367, and S2996 IRIF. In addition, compared with ATM WT, recruitment of ATM Y370F to chromatin induced by IR was significantly reduced as demonstrated by chromatin-enriched cell fractionation assay (Figure 4D). These findings suggest that EGFR-mediated ATM Y370 phosphorylation facilitates p-ATM S1981, S367, and S2996 IRIF formation.

Activated ATM is known to rapidly phosphorylate protein kinase CHK2 at T68 upon IR stimulation to regulate cell cycle arrest [45-48]. To decipher whether ATM Y370 phosphorylation plays a role in mediating downstream DDR proteins like CHK2, we examined p-CHK2 IRIF in ATM-depleted HeLa cells. Re-expression of Flag-tagged ATM WT but not Y370F mutant rescued p-CHK2 IRIF (Figure 4E), indicating that ATM Y370 phosphorylation is also involved in p-CHK2 IRIF formation. Similar results were observed that only restoring expression of Flag-tagged ATM WT but not the Y370F mutant rescued ATM-mediated p-KAP1 [49, 50] IRIF in ATM-depleted HeLa cells (Supplementary information, Figure S4G). We further investigated whether ATM Y370 phosphorylation is also involved in S343 phosphorylation of NBS1, as ATM serves as an upstream kinase in the ATM-NBS1-SMC1 signaling for cell cycle checkpoint [51, 52]. Only ectopic expression of Flag-tagged ATM WT but not Y370F mutant in ATM-depleted HeLa cells reactivated NBS1 S343 phosphorylation (Supplementary information, Figure S4H). Together, these results suggest that EGFR-mediated ATM Y370 phosphorylation is essential for ATM activation, downstream CHK2 and KAP1 IRIF

Figure 3 EGFR is required for ATM-mediated DDR. **(A**, **B)** Immunofluorescent (IF) staining of irradiation-induced foci (IRIF) of control or EGFR-depleted (shEGFR #2) U2OS cells with the indicated antibodies. EGFR knockdown efficiency was shown by western blot in Supplementary information, Figure S3B. DAPI: 4,6-diamidio-2-phenylindole. Quantitation of ther percentage of cells with p-ATM S1981 and p-CHK2 IRIF is presented as mean ± SD. $n = 103$. $^{*}P < 0.05$. **(C)** Comet assay of EGFR-depleted HeLa cells were carried out with or without 15 Gy IR. Top: four representative images showing comet tail movements (indicated by red bars) in EGFR-knockdown or vector control HeLa cells after IR treatment for 6 h. Bottom: quantitation from three independent experiments with or without IR exposure. Cells were measured by CometScore software in each experiment. $n = 50$. $^{**}P < 0.01$. Western blot analysis of EGFR in control or EGFR-knockdown HeLa cells used in comet assay. **(D)** Quantification of the percentage of cells with γ-H2AX foci after IR in control (shCtrl) or two EGFR-depleted (shEGFR#1 and #2) U2OS cells. Cells were exposed to 5 Gy IR, fixed after 0, 4 , 24 and 48 h and stained with DAPI and antibodies against γ-H2AX. Percentage of γ-H2AX foci staining-positive cells was quantitated among various fields. $^{**}P < 0.01$. **(E)** Homologous recombination efficiency in DR-GFP reporter-integrated U2OS cells with control or EGFR silencing (shEGFR #1 and #2) was determined by flow cytometry after ectopic expression of I-*SceI*. Top: western blotting showing knockdown efficiency of two EGFR-targeting shRNA #1 and #2 in DR-GFP-integrated U2OS cells. Bottom: quantitation of three independent experiments. $^{*}P = 0.02$.

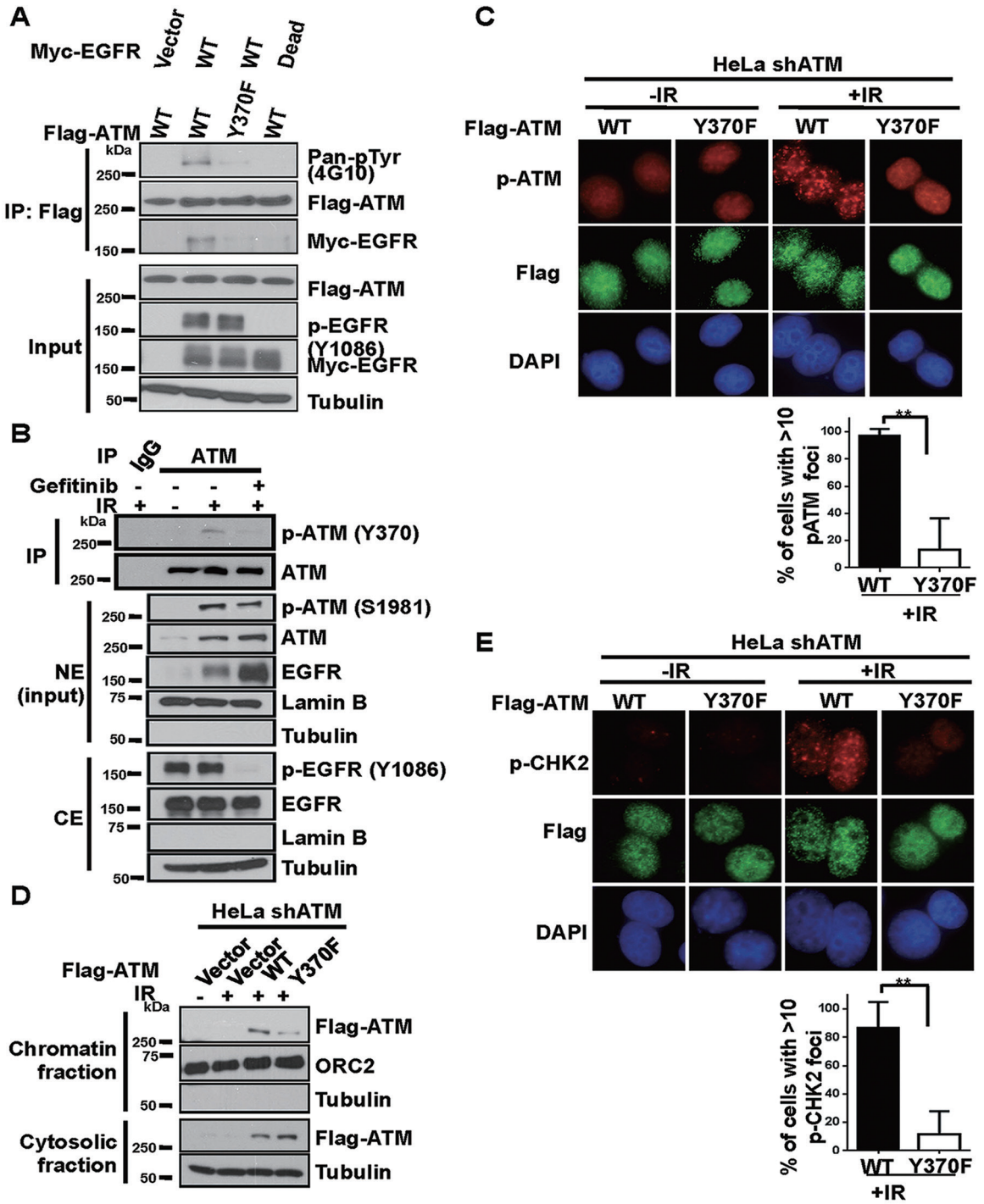


Figure 4 EGFR-mediated ATM Y370 phosphorylation facilitates its activation upon IR. **(A)** HEK 293T cells transfected with the indicated plasmids were treated by IR stimulation. The resulting cells were harvested for co-immunoprecipitation followed by western blot analysis. **(B)** Western blot analysis of endogenous ATM IP products from HeLa cell NE treated with or without IR. Gefitinib: EGFR kinase inhibitor. **(C)** p-ATM S1981 IRIF staining of ATM-depleted (shATM) HeLa cells with reconstitution of Flag-tagged ATM wild type (WT) or Y370F mutant. Quantitation of the percentage of cells with p-ATM S1981 IRIF is presented as mean ± SD. $n = 50$. $^{**}P < 0.01$. **(D)** Vector control, Flag-tagged ATM WT, or Y370F was restored in ATM-depleted HeLa cells. After treatment with or without IR and recovery for 4 h, chromatin-enriched fractionation was carried out, followed by western blot analysis with the indicated antibodies. **(E)** p-CHK2 IRIF staining of ATM-depleted HeLa cells with reconstituted Flag-tagged ATM WT or Y370F mutant. Quantitation of the percentage of cells with p-CHK2 IRIF is presented as mean ± SD. $n = 50$. $^{**}P < 0.01$.

formation, and NBS1 S343 phosphorylation.

Clinically, EGFR inhibitors synergistically sensitize response to radiation therapy in patients with head and neck squamous cell carcinoma [53]. Since EGFR-mediated ATM Y370 phosphorylation is required for ATM activation, downstream signaling, and DNA repair, we hypothesized that ATM Y370 phosphorylation plays a role in radiotherapy resistance. Indeed, HeLa cell colony formation was reduced in ATM-depleted cells compared with control cells after IR stimulation, which can be rescued by reconstitution of only ATM WT but not the Y370F mutant (Supplementary information, Figure S5A and S5B), suggesting that ATM Y370 phosphorylation is essential for ATM-mediated DNA repair and radiotherapy resistance. To show that EGFR regulates radiotherapy resistance through EGFR's kinase activity, we performed colony formation assay by using various doses of EGFR kinase inhibitor gefitinib. Consistent with previous findings [54-56], gefitinib combined with IR also produced a synergistic effect in radiosensitivity (Supplementary information, Figure S5C). Collectively, our findings uncovered an underlying mechanism by which gefitinib enhances radiosensitivity through EGFR-mediated ATM Y370 phosphorylation to facilitate ATM activation and subsequent DDR.

Discussion

The current report reveals a tyrosine phosphorylation of ATM at Y370 by EGFR after IR stimulation. The ATM Y370 phosphorylation event facilitates not only ATM activation but also ATM-mediated downstream DDR (Supplementary information, Figure S6). Our data indicate that EGFR-mediated ATM Y370 phosphorylation confers radiotherapy resistance in cancer cells and suggest that ATM phospho-Y370 could serve as a marker to stratify patients for rational combinational therapy of IR and TKI treatment. It would be important to further validate ATM Y370 phosphorylation in prospective tumor tissues in the near future. Two different groups have previously reported that erlotinib and gefitinib pretreatment increases radiosensitivities of triple-negative breast cancer (TNBC) and non-small cell lung cancer (NSCLC) cells, respectively [54, 57]. The data presented in these studies substantiate the role of EGFR kinase activity in radiotherapy resistance by demonstrating that inactivation of EGFR kinase activity enhances radiosensitivities of TNBC and NSCLC cells. In fact, Das *et al*. [58] demonstrated that EGFR tyrosine kinase domain mutantion in NSCLC cells impaired radiation-induced EGFR nuclear translocation and significantly delayed DSB repair, further providing evidence to support the role of EGFR in DDR in our study.

In 2013, Jackson and Kaidi demonstrated that c-Abl regulates ATM signaling through Y44 phosphorylation of the protein acetyltransferase KAT5 (also known as TIP60), which increases the acetylation levels of ATM [59]. We showed that pretreatment of c-Abl kinase inhibitor imatinib did not reduce ATM tyrosine phosphorylation as opposed to EGFR kinase inhibitors, gefitinib and AG1478 (Figure 1B), indicating that c-Abl may not regulate ATM signaling by direct phosphorylation of ATM. To determine the effect of EGFR on ATM acetylation, we examined the level of acetylated ATM in EGFR-depleted HeLa cells. Our data indicated that while IR stimulation triggered ATM acetylation, and as expected, EGFR silencing by two different EGFR shRNAs did not reduce the level of ATM acetylation (Supplementary information, Figure S5D). Thus, ATM activity can be regulated by upstream tyrosine kinases either through direct phosphorylation by EGFR or indirectly by c-Abl via KAT5 phosphorylation.

Since EGF stimulation also provokes EGFR nuclear translocation [12], we also examined whether EGF stimulation induces EGFR binding with ATM and found that only IR but not EGF treatment can induce association between EGFR and ATM (data not shown). Currently, there is no evidence indicating that EGF can induce ATM monomerization under IR stimulation. It is possible that EGFR only associates with monomeric ATM (stimulated by IR) but not with homodimeric ATM (stimulated by EGF treatment).

Interestingly, we observed increased levels of nuclear EGFR in cells pretreated with gefitinib, which is further enhanced by IR stimulation (Figure 4B and Supplementary information, Figure S1E). Wang *et al*. [60] previously demonstrated that EGFR dimerization rather than kinase activation controls its endocytosis. Later, Bjorkelund *et al*. [61] reported that gefitinib treatment induces EGFR dimerization [61]. It is possible that IR combined with gefitinib pretreatment elevates nuclear EGFR levels by enhancing EGFR dimerization and endocytosis.

EGFR has been reported to associate with DNA-PK, one of the major serine/threonine kinases in DDR, to mediate non-homologous end joining after IR, but the detailed mechanism remains unclear [14, 19]. Chen *et al*. [62] also demonstrated that ATM is essential for DNA-PK activation as ATM-depleted (shRNA) or A-T cells had decreased DNA-PK T2609 phosphorylation, a critical event required for DSB repair and radiation resistance. Further experiments will be required to determine whether EGFR-mediated ATM Y370 phosphorylation regulates DNA-PK T2609 phosphorylation. Taken together, our findings indicate that EGFR plays a critical

role in DDR and that phospho-ATM-Y370 has the potential to serve as a biomarker in radiotherapy or chemotherapy combined with targeted EGFR inhibitors in cancer treatments.

Materials and Methods

Laser microirradiation

The Olympus FV1000 confocal microscopy system was employed (Cat# F10PRDMYR-1, Olympus, UPCI facility) and FV1000 software was used for acquisition of images. For induction of DNA damage, cells are irradiated with 405 nm laser irradiation. The output power of the laser (original 50 mW) passed through the lens was 5 mW/scan. Laser light was passed through a PLAPON 60× oil lens (Cat# FM1-U2B990). Cells were incubated at 37 °C on a thermo-plate (MATS-U52RA26 for IX81/71/51/70/50; metal insert, HQ control, Cat# OTH-I0126) in Opti-MEM during observation to avoid pH changes.

Mass spectrometry analysis

Exogenously overexpressed Flag-tagged ATM was isolated from HeLa cell nuclear extracts by IP using anti-Flag antibody and analyzed by SDS-PAGE. To identify novel phosphorylation sites on ATM, mass spectrometry analysis was carried out as previously described [63].

HR repair analysis

To generate EGFR-knockdown stable clones, U2OS cells containing a single copy of the HR repair reporter substrate DR-GFP were infected by lentiviral shRNAs targeting EGFR or vector control. After 48-h transfection with mock or I-*Sce*I plasmids followed by 16-h sodium butyrate (5 mM) treatment, flow cytometric analysis was carried out to determine the number of HR-repaired GFP-positive cells.

See Supplementary information, Data S1 for additional details.

Acknowledgments

We thank Drs Michael B Kastan and Cheryl L Walker for providing Flag-tagged ATM plasmid and Dr Jennifer L Hsu for editing the manuscript. This study was funded in part by the following: National Institutes of Health (CA109311, CA099031, and CCSG CA16672); The University of Texas MD Anderson-China Medical University and Hospital Sister Institution Fund (to M-C H); Ministry of Health and Welfare, China Medical University Hospital Cancer Research Center of Excellence (MOHW103-TD-B-111-03); Program for Stem Cell and Regenerative Medicine Frontier Research (NSC102-2321-B-039-001); International Research-Intensive Centers of Excellence (NSC103-2911-I-002-303); Center for Biological Pathways; Competitive Medical Research Fund (CMRF) of the University of Pittsburgh Medical Center (to LL); and National Institutes of Health (AG045545-01 to LL). This work is in memoriam of Mr Tiong Loi Ang for his courageous fight against cancer.

References

1 Harper JW, Elledge SJ. The DNA damage response: ten years after. *Mol Cell* 2007; **28**:739-745.
2 Lee JH, Paull TT. Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. *Oncogene* 2007; **26**:7741-7748.
3 Lavin MF. Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. *Nat Rev Mol Cell Biol* 2008; **9**:759-769.
4 Kozlov SV, Graham ME, Peng C, *et al*. Involvement of novel autophosphorylation sites in ATM activation. *EMBO J* 2006; **25**:3504-3514.
5 Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003; **421**:499-506.
6 Lavin MF, Kozlov S. ATM activation and DNA damage response. *Cell Cycle* 2007; **6**:931-942.
7 Lavin MF. ATM and the Mre11 complex combine to recognize and signal DNA double-strand breaks. *Oncogene* 2007; **26**:7749-7758.
8 Jiang H, Reinhardt HC, Bartkova J, *et al*. The combined status of ATM and p53 link tumor development with therapeutic response. *Genes Dev* 2009; **23**:1895-1909.
9 Chun HH, Gatti RA. Ataxia-telangiectasia, an evolving phenotype. *DNA Repair (Amst)* 2004; **3**:1187-1196.
10 Tribius S, Pidel A, Casper D. ATM protein expression correlates with radioresistance in primary glioblastoma cells in culture. *Int J Radiat Oncol Biol Phys* 2001; **50**:511-523.
11 Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001; **2**:127-137.
12 Lin SY, Makino K, Xia W, *et al*. Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* 2001; **3**:802-808.
13 Cao H, Lei ZM, Bian L, *et al*. Functional nuclear epidermal growth factor receptors in human choriocarcinoma JEG-3 cells and normal human placenta. *Endocrinology* 1995; **136**:3163-3172.
14 Dittmann K, Mayer C, Fehrenbacher B, *et al*. Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. *J Biol Chem* 2005; **280**:31182-31189.
15 Lo HW, Hung MC. Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. *Br J Cancer* 2006; **94**:184-188.
16 Wang YN, Yamaguchi H, Hsu JM, *et al*. Nuclear trafficking of the epidermal growth factor receptor family membrane proteins. *Oncogene* 2010; **29**:3997-4006.
17 Lo HW, Hsu SC, Ali-Seyed M, *et al*. Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* 2005; **7**:575-589.
18 Hung LY, Tseng JT, Lee YC, *et al*. Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating Aurora-A gene expression. *Nucleic Acids Res* 2008; **36**:4337-4351.
19 Bandyopadhyay D, Mandal M, Adam L, *et al*. Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. *J Biol Chem* 1998; **273**:1568-1573.
20 Yu YL, Chou RH, Wu CH, *et al*. Nuclear EGFR suppresses ribonuclease activity of polynucleotide phosphorylase through

DNAPK-mediated phosphorylation at serine 776. *J Biol Chem* 2012; **287**:31015-31026.
21 Wang SC, Nakajima Y, Yu YL, *et al*. Tyrosine phosphorylation controls PCNA function through protein stability. *Nat Cell Biol* 2006; **8**:1359-1368.
22 Yu YL, Chou RH, Liang JH, *et al*. Targeting the EGFR/PCNA signaling suppresses tumor growth of triple-negative breast cancer cells with cell-penetrating PCNA peptides. *PLoS One* 2013; **8**:e61362.
23 Zhao H, Lo YH, Ma L, *et al*. Targeting tyrosine phosphorylation of PCNA inhibits prostate cancer growth. *Mol Cancer Ther* 2011; **10**:29-36.
24 Pellegrini M, Celeste A, Difilippantonio S, *et al*. Autophosphorylation at serine 1987 is dispensable for murine Atm activation *in vivo*. *Nature* 2006; **443**:222-225.
25 Daniel JA, Pellegrini M, Lee JH, *et al*. Multiple autophosphorylation sites are dispensable for murine ATM activation *in vivo*. *J Cell Biol* 2008; **183**:777-783.
26 Matsuoka S, Ballif BA, Smogorzewska A, *et al*. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 2007; **316**:1160-1166.
27 Kozlov SV, Graham ME, Jakob B, *et al*. Autophosphorylation and ATM activation: additional sites add to the complexity. *J Biol Chem* 2011; **286**:9107-9119.
28 Bennetzen MV, Larsen DH, Bunkenborg J, *et al*. Site-specific phosphorylation dynamics of the nuclear proteome during the DNA damage response. *Mol Cell Proteomics* 2010; **9**:1314-1323.
29 Khanna KK, Keating KE, Kozlov S, *et al*. ATM associates with and phosphorylates p53: mapping the region of interaction. *Nat Genet* 1998; **20**:398-400.
30 Wang SC, Hung MC. Nuclear translocation of the epidermal growth factor receptor family membrane tyrosine kinase receptors. *Clin Cancer Res* 2009; **15**:6484-6489.
31 Dittmann K, Mayer C, Kehlbach R, *et al*. Radiation-induced caveolin-1 associated EGFR internalization is linked with nuclear EGFR transport and activation of DNA-PK. *Mol Cancer* 2008; **7**:69.
32 Peng G, Yim EK, Dai H, *et al*. BRIT1/MCPH1 links chromatin remodelling to DNA damage response. *Nat Cell Biol* 2009; **11**:865-872.
33 Lan L, Nakajima S, Komatsu K, *et al*. Accumulation of Werner protein at DNA double-strand breaks in human cells. *J Cell Sci* 2005; **118**:4153-4162.
34 Lan L, Nakajima S, Oohata Y, *et al*. *In situ* analysis of repair processes for oxidative DNA damage in mammalian cells. *Proc Natl Acad Sci USA* 2004; **101**:13738-13743.
35 Uziel T, Lerenthal Y, Moyal L, *et al*. Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* 2003; **22**:5612-5621.
36 Lee JH, Paull TT. Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* 2004; **304**:93-96.
37 Lee JH, Paull TT. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* 2005; **308**:551-554.
38 Kastan MB, Lim DS. The many substrates and functions of ATM. *Nat Rev Mol Cell Biol* 2000; **1**:179-186.
39 Cortez D, Wang Y, Qin J, *et al*. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* 1999; **286**:1162-1166.
40 Lukas J, Lukas C, Bartek J. More than just a focus: the chromatin response to DNA damage and its role in genome integrity maintenance. *Nat Cell Biol* 2011; **13**:1161-1169.
41 MacPhail SH, Banath JP, Yu TY, *et al*. Expression of phosphorylated histone H2AX in cultured cell lines following exposure to X-rays. *Int J Radiat Biol* 2003; **79**:351-358.
42 Taneja N, Davis M, Choy JS, *et al*. Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy. *J Biol Chem* 2004; **279**:2273-2280.
43 Iijima K, Ohara M, Seki R, *et al*. Dancing on damaged chromatin: functions of ATM and the RAD50/MRE11/NBS1 complex in cellular responses to DNA damage. *J Radiat Res* 2008; **49**:451-464.
44 Morrison C, Sonoda E, Takao N, *et al*. The controlling role of ATM in homologous recombinational repair of DNA damage. *EMBO J* 2000; **19**:463-471.
45 Zhou BB, Chaturvedi P, Spring K, *et al*. Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. *J Biol Chem* 2000; **275**:10342-10348.
46 Matsuoka S, Rotman G, Ogawa A, *et al*. Ataxia telangiectasia-mutated phosphorylates Chk2 *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* 2000; **97**:10389-10394.
47 Ahn JY, Schwarz JK, Piwnica-Worms H, *et al*. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Res* 2000; **60**:5934-5936.
48 Ward IM, Wu X, Chen J. Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. *J Biol Chem* 2001; **276**:47755-47758.
49 White DE, Negorev D, Peng H, *et al*. KAP1, a novel substrate for PIKK family members, colocalizes with numerous damage response factors at DNA lesions. *Cancer Res* 2006; **66**:11594-11599.
50 Ziv Y, Bielopolski D, Galanty Y, *et al*. Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. *Nat Cell Biol* 2006; **8**:870-876.
51 Lim DS, Kim ST, Xu B, *et al*. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* 2000; **404**:613-617.
52 Kitagawa R, Bakkenist CJ, McKinnon PJ, *et al*. Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. *Genes Dev* 2004; **18**:1423-1438.
53 Bernier J, Bentzen SM, Vermorken JB. Molecular therapy in head and neck oncology. *Nat Rev Clin Oncol* 2009; **6**:266-277.
54 Park SY, Kim YM, Pyo H. Gefitinib radiosensitizes non-small cell lung cancer cells through inhibition of ataxia telangiectasia mutated. *Mol Cancer* 2010; **9**:222.
55 Lin HQ, Meriaty H, Katsifis A. Prediction of synergistic antitumour effect of gefitinib and radiation *in vitro*. *Anticancer Res* 2011; **31**:2883-2888.
56 Kang KB, Zhu C, Wong YL, *et al*. Gefitinib radiosensitizes stem-like glioma cells: inhibition of epidermal growth factor receptor-Akt-DNA-PK signaling, accompanied by inhibition of DNA double-strand break repair. *Int J Radiat Oncol Biol Phys* 2012; **83**:e43-e52.

57 Lee MJ, Ye AS, Gardino AK, *et al*. Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell* 2012; **149**:780-794.
58 Das AK, Chen BP, Story MD, *et al*. Somatic mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) abrogate EGFR-mediated radioprotection in non-small cell lung carcinoma. *Cancer Res* 2007; **67**:5267-5274.
59 Kaidi A, Jackson SP. KAT5 tyrosine phosphorylation couples chromatin sensing to ATM signalling. *Nature* 2013; **498**:70-74.
60 Wang Q, Villeneuve G, Wang Z. Control of epidermal growth factor receptor endocytosis by receptor dimerization, rather than receptor kinase activation. *EMBO Rep* 2005; **6**:942-948.
61 Bjorkelund H, Gedda L, Barta P, *et al*. Gefitinib induces epidermal growth factor receptor dimers which alters the interaction characteristics with (1)(2)(5)I-EGF. *PLoS One* 2011; **6**:e24739.
62 Chen BP, Uematsu N, Kobayashi J, *et al*. Ataxia telangiectasia mutated (ATM) is essential for DNA-PKcs phosphorylations at the Thr-2609 cluster upon DNA double strand break. *J Biol Chem* 2007; **282**:6582-6587.
63 Liu M, Lee DF, Chen CT, *et al*. IKKalpha activation of NOTCH links tumorigenesis via FOXA2 suppression. *Mol Cell* 2012; **45**:171-184.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)