EGFR-Mediated Chromatin Condensation Protects KRAS-Mutant Cancer Cells Against Ionizing Radiation

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Financial Support: Dana-Farber/Harvard Cancer Center SPORE in Lung Cancer, NCI P50 CA090578 (J.S., H.W.), NCI R01 CA142698 (D.C.), American Cancer Society 123420RSG-12-224-01-DMC (H.W.) and RSG-12-079-01 (D.C.), UK Wellcome Trust 086357 (C.H.B., J.S.), Deutsche Forschungsgemeinschaft DFG-PAK-190 (M.K., M.B.), Federal Share of program income earned by Massachusetts General Hospital on C06 CA059267, Proton Therapy Research and Treatment Center (J.A.E., H.W.).

Conflicts of Interest: Jeff Settleman, Ph.D., is employed by Genentech Inc., San Francisco, CA

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Running Title: EGFR protects KRAS-mutant cells

Keywords: KRAS, EGFR, Lung Cancer, Radiation

Word count: abstract 210, text with figure legends 4,672

References: 52

Figures: 5

Tables: 0

Supplementary Figures: 4
Abstract

Therapeutics that target the epidermal growth factor receptor (EGFR) can enhance the cytotoxic effects of ionizing radiation (IR). However, predictive genomic biomarkers of this radiosensitization have remained elusive. By screening 40 non-small cell lung cancer cell (NSCLC) lines, we established a surprising positive correlation between the presence of a KRAS mutation and radiosensitization by the EGFR inhibitors erlotinib and cetuximab. EGFR signaling in KRAS-mutant NSCLC cells promotes chromatin condensation in-vitro and in-vivo, thereby restricting the number of DNA double-strand breaks (DSB) produced by a given dose of IR. Chromatin condensation in interphase cells is characterized by an unexpected mitosis-like co-localization of serine 10 phosphorylation and lysine 9 trimethylation on histone H3. Aurora B promotes this process in a manner that is co-dependent upon EGFR and PKCα. PKCα, in addition to MEK/ERK signaling, is required for the suppression of DSB-inducible premature senescence by EGFR. Blockade of autophagy results in a mutant KRAS-dependent senescence-to-apoptosis switch in cancer cells treated with IR and erlotinib. In conclusion, we identify EGFR as a molecular target to overcome a novel mechanism of radioresistance in KRAS-mutant tumor cells, which stands in contrast to the unresponsiveness of KRAS-mutant cancers to EGFR-directed agents in monotherapy. Our findings may reposition EGFR-targeted agents for combination with DSB-inducing therapies in KRAS-mutant NSCLC.
Introduction

Exposure of cellular DNA to ionizing radiation (IR) generates various types of damage (1). A dose of 1 Gy produces 20-40 DNA double-strand breaks (DSB) in a mammalian cell (1, 2). Unrepaired or misrepaired DSB are the principal type of damage that may result in lethal chromosomal aberrations and cell death, or radiobiologically termed “cell inactivation”, within 1-3 division cycles (reviewed in ref. (3)). Molecular targeted anti-cancer agents have been tested extensively pre-clinically, and increasingly so in the clinic, to enhance the cytotoxic effects of IR. Pre-clinical data suggest that radiosensitization is frequently achieved by interfering with DSB repair, thereby increasing the levels of residual, unrepaired DSB (4-6). Unrepaired DSB may induce cellular senescence or apoptosis, but the latter outcome is generally more desirable as senescent cells remain viable and can even escape senescence (7, 8).

Non-small cell lung cancer (NSCLC) is difficult to control locally by IR due to the typically large tumor size at diagnosis and the proximity to critical normal organs, which limit the achievable dose of radiation (reviewed in ref. (9)). Therefore, radiation has been combined with radiosensitizing chemotherapeutics. However, this combination has yielded only a modest survival benefit and at the cost of significant toxicity in many patients. The epidermal growth factor receptor (EGFR) is a member of the erbB multi-gene family. Receptor activation is associated with phosphorylation of the intracellular tyrosine kinase domain and recruitment of signaling molecules that initiate the diverse signaling cascades that promote biological responses, including enhanced proliferation, cell survival, as well as radiation resistance (reviewed in ref. (10, 11)). As EGFR is expressed in up to ~80% of NSCLC, it constitutes a potentially important target in NSCLC therapy (12). Clinical trials have sought to integrate EGFR-targeted monoclonal antibodies (mAb) or selective tyrosine kinase inhibitors (TKI) into the treatment of NSCLC in order to achieve radiosensitization, with varying results (13-15). For NSCLC harboring wild-type EGFR, it is increasingly appreciated that these agents should be selected
based on the molecular profile of a given tumor rather than administered to all patients (16). However, predictive biomarkers of radiosensitization have been lacking.

The KRAS gene encodes a GTPase involved in relaying signals from the cell membrane to the nucleus. Upon the introduction of point mutations, most commonly at codons 12 and 13, the K-Ras protein becomes constitutively active and acquires oncogenic properties. KRAS mutations are found in approximately 30% of NSCLC (17) and are associated with resistance to EGFR-targeted agents in mono-therapy as well as poor prognosis (18-21). In KRAS-mutant cells, critical pro-survival and growth effector pathways are activated by K-Ras and consequently exhibit resistance to inhibition by TKIs such as erlotinib or mAbs such as cetuximab (22, 23). There is also evidence that KRAS mutations confer radioresistance, although this phenomenon is understudied in NSCLC (24-26).

Large scale screening of annotated cancer cell lines has been successfully employed to identify cell line subsets sensitive to single agent treatments and associated biomarkers (27-30). There is a need to adapt such screening platforms for the study of IR in conjunction with potentially radiosensitizing targeted compounds. We recently reported that the radiosensitizing effects of erlotinib and cetuximab seen in a short-term survival assay as used in screening platforms correlate well with results of the standard clonogenic survival assay (6). The degree of radiosensitization for single doses of IR was relatively small, i.e., 1.02-1.17 for short-term survival and 1.15-1.46 for clonogenic survival, consistent with data reported by others (6, 31, 32). However, it is thought that a small radiosensitizing effect incurred by a single dose of IR could be amplified when doses are repeated (33), as would be the case during a several-week clinical course of radiation consisting of 30 or more fractions of ~2 Gy each.

Here, we have carried out a screen of 40 human NSCLC-derived cell lines using 2 Gy IR together with erlotinib or cetuximab. We establish a surprising positive association between KRAS mutation and radiosensitization, and we have determined that EGFR-dependent chromatin condensation protects KRAS-mutant cancer cells against IR-induced DSB and premature senescence, thus revealing novel therapeutic opportunities to treat KRAS-mutant cancers.
Materials and Methods

Cell lines

Cell lines were selected from a previously published panel located in the Center for Molecular Therapeutics at Massachusetts General Hospital (30), except for A549 and Calu-6 which were purchased directly from ATCC, and DLD1 (KRAS G13D/wt) and DWT7 (del/wt) which were kind gifts from Dr. Bert Vogelstein (34). The identity of each of the cell lines in the panel had been tested as described previously (6), and additional cell line authentication was performed by Bio-Synthesis, Inc. No cell line was ever treated for mycoplasma and all lines tested mycoplasma free prior to the experiments (MycoAlert, Lonza). NCI-H1703 cells harboring wild-type KRAS were transfected with a pBABE-Puro vector encoding KRAS G12V or an empty control (kindly provided by Dr. David Barbie). Stably transfected clones were selected with 2 mg/mL puromycin (Sigma, P9620) and maintained at 1 mg/mL puromycin. shRNA KRAS transfection of A549 cells was performed as described previously (35). For 3D culture of tumor spheres, ~5,000 cells/well were grown in low-binding 96-well plates (Thermo, 145399) using serum-free medium composed of DMEM (Sigma-Aldrich), basic fibroblast growth factor (bFGF), and EGF (20 ng/mL each, Sigma-Aldrich), and B27 supplement (Life Technologies).

Xenograft experiments

A549 xenografts were grown and treated as described previously (6).

Treatments
Irradiation as well as treatments with erlotinib and cetuximab were described previously (6). NU7026 (EMD Millipore, 260961), hesperadin (Selleck, S1529), Gö6976 (LC Laboratories, G-6203), and AZD6244 (Selleck, S1008) were dissolved in dimethyl sulfoxide (DMSO) and chloroquine (Sigma, C6628) was dissolved in double distilled water. Drugs were added 1 hour prior to irradiation and maintained for the duration of each experiment. Chaetocin (Cayman, CAS 28097) and Ro-32-0432 (Merck, 557525) were dissolved in DMSO and incubated with cells for 1 hour before collecting for cell lysates. Thymidine (Sigma, T1895) was dissolved in PBS, and used at 2.5 mM concentration 16 hours before and 8 hours after releasing cells in normal cell culture medium.

**Cell proliferation and survival assays**

Clonogenic survival assays were performed as previously published (36). Determination of cell numbers 72 hours after irradiation with 2 Gy was performed by manual counting or by using a fluorescent nucleic acid stain Syto60 as described (30). For analyzing tumor spheres, the CellTiter-Glo® (CTG) luminescence assay was used (Promega, Madison, WI, USA). CTG reagents were applied to lysed spheres 3 days post-irradiation following the manufacturer’s protocol. Signals were read by the MultiLabel reader, 2140 Envision (Perkin Elmer, Waltham, MA, USA).

**RNA interference**

For depletion of PKCα, exponentially growing cells were transfected with validated siPKCα or a scrambled control siRNA (Ambion) using the X-tremeGENE transfection kit (Roche). Western blotting and subsequent experiments were performed 48 hours after transfection.

**Senescence staining**
Senescence-associated β-galactosidase staining was performed using a commercial kit (Cell Signaling, #9860) as described previously (6).

**Single-cell gel electrophoresis (comet) assay**

DSBs were measured at 15 minutes after 2 Gy irradiation by neutral single-cell gel electrophoresis as described previously (37).

**Flow cytometry**

Cell cycle distribution analysis and quantification of γ-H2AX signal were performed using flow cytometry as described previously (6).

**Immunofluorescence microscopy**

Staining and visualization of γ-H2AX and 53BP1 foci were performed as described (38). For visualization of EGFR and trimethyl (Lys9)-phospho (Ser10)-Histone H3 (EMD Millipore, 05-809) expression in vitro and in vivo, the same protocol was used. Following fixing and permeabilization, cells were incubated with γ-H2AX and 53BP1 (Abcam, ab22551 and ab21083, respectively), EGFR (Santa Cruz Biotechnology, sc-03) followed by incubation with an Alexa-488-conjugated secondary antibody (A11029, Invitrogen). All slides were counterstained with DAPI and examined by fluorescence microscopy (Olympus BX51).

**Western blotting**

Proteins in whole cell lysates from exponentially growing cell cultures were detected using standard methods. Specific antibodies against p-EGFR (Tyr992) (Invitrogen, 40-8250), trimethylated histone H3 lysine 9 (H3K9me3), dimethylated histone H3 lysine 9 (H3K9me2), monomethylated
histone H3 lysine 9 (H3K9me), acetylated histone H3 lysine 9 (H3K9ac), phosphorylation of histone H3 serine 10 (H3S10ph), total histone H3, total-PKCα, and p21 (Abcam, ab88988, ab1220, ab9045, ab12179, ab47297, ab1791, ab23276, and ab18209, respectively), trimethyl (Lys9)-phospho (Ser10)-Histone H3 (EMD Millipore, 05-809), phospho-PKC (pan) and Bcl-2 (Cell Signaling, #9371 and #2876), LC3 (MBL, M186-3), caspase 3 (Calbiochem, #235412) and β-actin (Sigma-Aldrich, A5441), and horseradish peroxidase–conjugated secondary antibody (Santa Cruz) were used. Protein bands were visualized with enhanced chemiluminescence (Invitrogen) followed by autoradiography.

**Transmission Electron Microscopy (TEM)**

Cells were treated by erlotinib for 1 hour and fixed using standard methods, and transmission electron microscopy was carried out at the MGH Microscopy Core, Program in Membrane Biology.

(http://www.partners.org/researchcores/microscopy/microscopy_brown_MGH.asp)
Radiosensitization of Cancer Cells by EGFR-Directed Agents is Dependent on KRAS Mutation

To identify a subset of NSCLC that can be radiosensitized by targeting EGFR, we employed a panel of 40 annotated NSCLC cell lines resistant to erlotinib alone (30). Cells were subjected to a clinically relevant dose of IR, 2 Gy, with or without erlotinib or cetuximab treatment (Fig. S1A). The number of persisting cells was determined at 72 hours post-IR, as described (6). The extent of drug-induced radiosensitization relative to the effect of IR alone was expressed as SRF_{2Gy} (Short-term Radiosensitization Factor at 2 Gy) (Fig. S1A).

Erlotinib and cetuximab caused radiosensitization with mean SRF_{2Gy} values of 1.10 and 1.13 in 40% and 48% of NSCLC cell lines, respectively (Fig. 1A, Fig. S1B). Strikingly, among the top eleven cell lines displaying the greatest radiosensitization by both EGFR-directed agents eight harbored a mutation in KRAS, even though all cell lines are resistant to erlotinib alone (fraction of cells, >0.5, Fig. S1C). When correlating KRAS status with SRF_{2Gy} for all cell lines in the panel we found a statistically significant increase in mean SRF_{2Gy} in mutant compared to wild-type cell lines for either drug (p<0.01) (Fig. 1B). Genetic manipulation of KRAS in isogenic NSCLC pairs and a colorectal cancer (CRC) pair (using mutant KRAS overexpression, knockdown, or allelic deletion (Fig. S1D) (35)) produced similar results (Fig. 1C). Mutant KRAS specific radiosensitization was even enhanced when drug incubation time pre-irradiation was extended (Fig. S1E). As a confirmation of the 72-hour assay, erlotinib radiosensitized KRAS-mutant but not wild-type NSCLC cells in a clonogenic survival assay (p<0.01) (Fig. 1D), which correlates with EGFR TKI-mediated radiosensitization of KRAS-mutant A549 xenografts (39). Similarly, radiosensitization was seen in KRAS-mutant but not wild-type tumor spheres (Fig. S1F).
EGFR Suppresses the Induction of DSB by Promoting Chromatin Condensation in KRAS-Mutant Cells

To elucidate the mechanisms of radiosensitization by targeting EGFR, we first examined the induction and removal of DSB using the established DSB marker γ-H2AX in irradiated KRAS-mutant A549 cells similar to previous studies (Fig. 2A, Fig. S2A) (5, 40). At 30 minutes post-irradiation with 1 Gy, EGFR inhibition by cetuximab or erlotinib increased the percentage of cells with > 20 foci per nucleus by 1.2- to 1.6-fold (p=0.02). This change correlated with an increase in the median number of IR-induced γ-H2AX foci per Gy per nucleus by 1.2- to 1.3-fold within 15-30 minutes of irradiation. The EGFR-dependent difference in γ-H2AX induction was confirmed by another DSB marker, 53BP1, and single-cell gel electrophoresis (Fig. 2B). Importantly, the difference in DSB numbers at 30 minutes was not due to a previously proposed effect of EGFR on NHEJ (41), as (a) pharmacological inhibition of DNA-PKcs did not abrogate the difference (Fig. 2C), (b) EGFR inhibition did not affect nuclear translocation previously hypothesized to represent an EGFR function in NHEJ (Fig. 2D, Fig. S2B) (40), and (c) EGFR activity did not affect NHEJ frequencies in a GFP-based I-SceI reporter assay (Fig. S2C). In addition, EGFR inhibition was required prior to irradiation but not immediately afterwards (Fig. 2E). A remarkably consistent and mutant KRAS-dependent increase in DSB upon EGFR inhibition was seen in several non-isogenic and isogenic comparisons, i.e., 1.2- to 1.5-fold difference (Fig. 2F).

We hypothesized that KRAS-mutant cells may harbor more condensed chromatin than wild-type cells, making them less susceptible to accumulate damage to their DNA (42). We, therefore, characterized the impact of erlotinib on chromatin structure using transmission electron microscopy in KRAS-mutant cells (Fig. 3A). EGFR inhibition for 1 hour reduced the fraction of cells with a high content of dense chromatin by 25%. Trimethylation of lysine 9 on histone H3 (H3K9me3) is a general marker of dense chromatin, found in heterochromatin as well as mitotic chromatin (43). Consistent
with the electron microscopy findings, EGFR inhibition led to a decrease in the H3K9me3 signal both in-vitro and in a xenograft model (Fig. 3B-D). To demonstrate the dependence of DSB induction on chromatin condensation, we pharmacologically inhibited histone methyltransferases (Fig. 3E). The resulting repression of H3K9me3 completely abrogated the effect of EGFR inhibition on DSB induction. Together, the data suggest that EGFR-mediated chromatin condensation protects mutant KRAS cells against IR-induced DSB.

**EGFR and PKCα are Co-Regulators of Aurora B Kinase-Mediated Chromatin Condensation in Interphase**

To identify the mechanism by which EGFR may modulate H3K9me3, we first determined the enzymatic activities of histone methyltransferases specifically targeting H3K9 in A549 cells, but there was no effect of EGFR on promoting methyltransferase activity (Fig. S3A). We next asked whether EGFR suppresses H3K9 demethylase activities. While EGFR inhibition did not cause an increase in di- or mono-methylation of H3K9, we observed an unexpectedly reduced phosphorylation on H3 serine 10 (p-H3S10) (Fig. 4A). Phosphorylation at H3S10 promotes mitotic condensation through H3K9me3 and is generally thought to be a marker of the G2 and M phases of the cell cycle (43, 44). Erlotinib treatment reduced the percentage of cells with co-localized p-H3S10 and H3K9me3 from 18.5% to 8.7% (Fig. 4B), and there was a pronounced signal reduction in-vivo as well (Fig. S3B). Interestingly, the reduced co-staining signal was almost exclusively due to a decrease in the interphase-type, punctuate staining pattern that has been previously described in G2-phase cells (43) (Fig. 4B). To determine whether the observed reduction was indeed specific for G2-phase cells or could be detected in other phases of the cell cycle as well, we performed a double thymidine block which essentially eliminated G2/M phase cells (Fig. 4C). Under these conditions we continued to observe cells with a co-localized H3K9me3 and p-H3S10 signal that was sensitive to erlotinib.
Aurora B kinase phosphorylates H3S10 to promote mitotic condensation, but the existence of this modification outside of the G2/M phase is unknown. Pharmacological inhibition of Aurora B kinase abrogated not only mitotic co-localization of p-H3S10 and H3K9me3 but also the interphase-type staining pattern (Fig. S3C). Furthermore, consistent with the data in Fig. 4C, Aurora B kinase inhibition increased the amount of IR-induced DSB in G1 phase cells, with no additional effect of EGFR inhibition on the DSB signal (Fig. 4D). DSB induction resulting from Aurora B kinase inhibition was more pronounced in KRAS mutant than in wild-type cells, with the latter effect likely representing a modification in mitotic cells (Fig. 4E). We conclude that Aurora B kinase and EGFR promote a mitosis-like chromatin condensation mechanism in a fraction of interphase KRAS-mutant cells, thereby suppressing DSB induction.

Lastly, we wanted to identify the EGFR signaling pathway that promotes the inhibition of DSB induction. We, therefore, screened KRAS-mutant A549 cells with a panel of pharmacological inhibitors directed at the key pathways known to function downstream of EGFR (Fig. S3D). Only inhibition of protein kinase C alpha (PKCα) was found to affect DSB induction, and the effect was epistatic with EGFR inhibition (Fig. 4F). The increase in DSB induction by a PKCα inhibitor was abolished in cells in which PKCα was depleted by RNA interference (Fig. 4G). As predicted, PKCα inhibition reduced H3K9me3 levels to a degree comparable to EGFR inhibition (Fig. 4H, Fig. S3E). Analogous to erlotinib, PKCα inhibition repressed the co-localized p-H3S10 and H3K9me3 signal and correspondingly increased DSB induction in NCI-H1703 cells expressing mutant KRAS, but not in isogenic wild-type cells (Fig. 4I, S3F).

A Senescence-To-Apoptosis Switch in Cells Expressing Mutant K-Ras

In an isogenic model, KRAS wild-type cells were sensitive to treatment with erlotinib alone (Fig. S4A), with the induction of premature cellular senescence accounting for at least some of the
In contrast, erlotinib-treated KRAS-mutant cells were resistant to senescence induction. However, upon additional irradiation with 2 Gy, the senescence response was reactivated (while IR alone did not cause senescence) (Fig. 5A). Consistent with our previous results (6), MEK-ERK inhibition caused a small degree of senescence in KRAS-mutant cells that could not explain all the effect of erlotinib treatment (Fig. 5B, Fig. S4B). However, combined inhibition of both MEK-ERK and PKCα produced the full senescence phenotype.

Therapy-induced premature senescence is not necessarily a desirable effect as senescent tumor cells remain viable and can secret growth factors. To convert senescence to apoptotic cell death, we considered that KRAS-mutant cells have been reported to exhibit increased autophagy activity and that autophagy may suppress apoptosis (45, 46). We inhibited autophagy in KRAS-mutant cells by blocking autophagosome-lysosome fusion with chloroquine (Fig. 5C). Chloroquine treatment combined with erlotinib but not by itself suppressed p21 induction and senescence in response to IR (Fig. 5C, D). In parallel, there was loss of BCL2 expression and induction of apoptosis as evidenced by caspase-3 cleavage and an increased sub-G1 fraction (Fig. 5D, S4C). This senescence-to-apoptosis switch was mutant KRAS specific (Fig. 5E). As a result, the radiosensitizing effect of combined erlotinib and chloroquine was observed only in the presence of KRAS mutation and was particularly pronounced in KRAS-mutant tumor spheres where the average SRF2Gy reached 2.0 (Fig. S4D,E, 5F).
Discussion

KRAS is the most commonly mutated oncogene in NSCLC. KRAS-mutant NSCLCs often display a poor prognosis and radioresistance, and have proven refractory to most targeted therapies including EGFR-directed agents. These cancers thus remain a major clinical challenge. Here, we report that in the context of a DSB-producing treatment EGFR assumes an important role in promoting the survival of KRAS-mutant cancer cells, thus rendering these cells susceptible to EGFR inhibitors (Fig. 1, 2). Interestingly, a previously understudied mechanism underlying the radiosensitizing effect of EGFR inhibition involves the modulation of the number of DSB generated by a given dose of IR, which is seen in all analyzed KRAS-mutant cell lines (Fig. 2F). It is generally accepted that the cytotoxic effects of IR result principally from damage to DNA (3). The majority of DNA breaks from X-rays or photon radiation as used in the clinic is caused by indirect action, meaning IR-induced free radicals that diffuse far enough to damage a DNA molecule. It can thus be postulated that regions of decondensed, open chromatin would make the exposed DNA helix more vulnerable to radiation ionizations, while condensed regions would be relatively less susceptible to DNA damage induction (42). Consistent with this concept, EGFR inhibition loses its effect on DSB induction in the setting of open chromatin (Fig. 3E).

Unexpectedly, the EGFR-mediated reduction of DSB induction was characterized by co-localized H3K9me3 and p-H3S10 (Fig. 4B), which is an established marker of mitotic chromatin condensation during late G2 and mitosis. Interestingly, we not only found that this chromatin mark is also present in the G1 phase in a subset of cells but also that Aurora B suppresses DSB induction in G1 (Fig. 4C,D). It was previously shown that among the key mitotic regulators Aurora B is degraded by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase at a relatively late stage and is still detectable in the G1 phase (47). Our results suggest that Aurora B remains active in G1 to maintain
condensed chromatin in a fraction of cells, a process that is co-regulated by an EGFR-PKCα-dependent pathway, thereby protecting KRAS-mutant cells against DSB through most of the cell cycle (Fig. 4D).

What might be the purpose of enhanced chromatin condensation in KRAS-mutant NSCLC cells? Oncogene-induced senescence is a crucial tumor suppressor mechanism controlling unchecked proliferation driven by oncogenic mutations such as KRAS (48). During carcinogenesis oncogene-driven cells can escape senescence by inactivation of the p53 or p16/RB pathways. However, these genetic events may not co-disrupt senescence responses that are inducible by DSB (i.e., “stress-induced premature senescence” (8)). It is important to note that while exogenous DSB can be induced by IR, endogenous DSBs arise as byproducts of normal intracellular metabolism. The spontaneous rate of endogenous DSBs may be as high as 50 DSB per cell per cell cycle (49), and conceivably could be even higher in cells with activated oncogenes such as mutant KRAS that drive cells through the cell cycle without sufficient time for damage repair. We observed that neither low doses of IR (2 Gy, causing 40-80 DSB) nor EGFR inhibition alone induced senescence in KRAS-mutant cells, but, unexpectedly, the combination of these treatments did (Fig. 5A). These data support a model in which KRAS-mutant cells employ EGFR-PKCα for suppression of a DSB-induced senescence response, which may be important during the process of carcinogenesis, yet represents a therapeutic target.

While there is currently great interest in exploiting stress-induced premature senescence for anti-cancer therapy it is important to realize that senescent cells remain alive and may even escape senescence (7). Interestingly, recent studies have suggested that autophagy may be a potential promoter or a precondition of stress-induced premature senescence (50, 51). At the same time, autophagy may inhibit apoptosis in tumor cells consistent with a cytoprotective role (46). Consistent with these data we discovered that autophagy inhibition by chloroquine converts the senescence response to a more desirable apoptotic outcome (Fig. 5C-E). Importantly, the combination of EGFR TKI and chloroquine has been already explored in the clinic and shows a favorable toxicity profile (52). Therefore,
combining IR with EGFR-directed therapy and an autophagy inhibitor such as chloroquine may warrant clinical investigation in KRAS-mutant cancers. In conclusion, our data challenge the current paradigm of EGFR inhibitor resistance in KRAS-mutant cancers and, by providing insight into the role of EGFR in chromatin modulation and senescence suppression, yield potential novel therapeutic opportunities.
Acknowledgments

We would like to thank Mary McKee (Microscopy Core, Center for Systems Biology/Program in Membrane Biology, Massachusetts General Hospital) for providing transmission electron micrographs, Drs. Bert Vogelstein and David Barbie for materials, and Chake Takadjian for technical assistance.
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**Figure legends**

**Figure 1.** Lung cancer cell line screening identifies a positive correlation between KRAS mutation and radiosensitization by EGFR-directed agents. A, Left panel, pie charts illustrate percentage of cell lines radiosensitized in the syto60 short-term assay by either erlotinib (2 μM) or cetuximab (100 nM) (see supplementary data, Fig. S1). Right panel, display of selected genomic profile of the top 11 cell lines radiosensitized by both agents, with mutations highlighted by a dark shade (curated from references Broad/Sanger). B, Scatter dot plots compare short-term radiosensitization factors (SRF$_{2Gy}$) for erlotinib or cetuximab in KAS mutant and wild-type NSCLC cell lines. Dots represent averages of at least 3 biological repeats and horizontal lines indicate the overall mean. Statistical comparisons were performed with the unpaired T-test (two-tailed). C, Analogous to panel B, SRF$_{2Gy}$ comparisons were performed in isogenic cell pairs with wild-type (wt) or mutant (mut) KRAS, or cells expressing shKRAS (+) or a scrambled control (0). D, Clonogenic survival of NCI-H1703 cells after single dose irradiation with or without erlotinib (2 μM) treatment initiated 1 hour before irradiation. Statistical comparison was carried out using the F-test.

**Figure 2.** EGFR suppresses the production of ionizing radiation (IR) induced DNA double-strand breaks in KRAS-mutant cells. A, A549 cells following irradiation with 1 Gy with or without cetuximab treatment (100 nM) initiated 1 hour before irradiation. Percentage of cells with ≥ 20 foci/nucleus over time post-irradiation. P-value (T-test) for data points at 0.5 hours (h). B, Left, percentage of A549 cells with ≥ 20 53BP1 foci/nucleus 30 minutes after 1 Gy +/- erlotinib (2 μM). Right, result of single-cell gel electrophoresis. C, Percentage of A549 cells with ≥ 20 IR-induced γ-H2AX foci +/- erlotinib and +/- DNA-PKcs inhibitor NU7026 (10 μM). D, Fraction of nuclei with IR-induced EGFR translocation as determined by immunofluorescence microscopy (15 minutes after 8 Gy). E, Percentage of A549 cells
with \( \geq 20 \) \( \gamma \)-H2AX foci with erlotinib treatment initiated 1 hour before irradiation (left) or immediately after irradiation (right) and in each case maintained for 1.5 hours before staining. F, Analogous comparison of \( \gamma \)-H2AX foci in non-isogenic and isogenic cell lines with or without mutant K-Ras expression. In all panels, bars represent mean +/- standard error based on typically 2-3 biological repeats with EGFR-dependent fold-suppression indicated. Where indicated, IR-induced foci were calculated by subtracting baseline foci in non-irradiated cells.

**Figure 3.** EGFR promotes chromatin condensation in KRAS-mutant cells in-vitro and in-vivo. A, Left, representative transmission electron microscopy images illustrating a reduction of dense chromatin by erlotinib. Right, fraction of nuclei with high, intermediate, or low chromatin density in A549 cells treated with or without erlotinib for 1 hour. B, Western blot for A549 cells treated for 1 hour with or without EGF (100 ng/ml), erlotinib (2 \( \mu \)M), or cetuximab (100 nM). Representative gel from 2 independent experiments is shown. C, Left, representative histological and anti-H3K9me3 immunofluorescence images (40X) from A549 xenografts following treatment of mice with erlotinib alone or no treatment. Right, quantification of H3K9me3 signal based on 10 images. Statistical comparison was performed with the T-test. D, Analogous to panel C, the nuclear H3K9me3 signal was assessed in A549 cells in-vitro. E, Percentage of A549 cells with \( \geq 20 \) IR-induced \( \gamma \)-H2AX foci with or without treatment with erlotinib or the histone methyltransferase inhibitor chaetocin. Western blot insert indicates the abrogation of H3K9me3 signal by chaetocin (100nM).

**Fig. 4.** Regulation of interphase chromatin condensation by Aurora B kinase and PKC\( \alpha \). A, Whole-cell lysates from A549 cells with or without treatment with EGF (100 ng/ml) or erlotinib (2 \( \mu \)M) were subjected to Western blotting with antibodies against proteins as shown. B, Left, representative immunofluorescence images showing co-localized phospho-H3S10 and H3K9me3 using a specific dual
antibody. Metaphase-type (m) and interphase-type (i) staining patterns are indicated. Right, percentage of cells with any phospho-H3S10/H3K9me3 signal. C, Percentage of nuclei with metaphase- or interphase-type phospho-H3S10/H3K9me3 staining with or without double thymidine block (TdR x2) to reduce the fraction of G2/M phase cells. D, Percentage of A549 cells with a high γ-H2AX staining intensity in G1- and G2/M phases based on FACS analysis 30 minutes after 8 Gy irradiation +/- erlotinib or +/- the Aurora B kinase inhibitor hesperadin. E, Percentage of NCI-H1703 cells with or without mutant K-Ras displaying ≥20 IR-induced γ-H2AX foci analogous to Fig. 2. F, Percentage of cells with γ-H2AX foci analogous to Fig. 2 +/- erlotinib or +/- the specific PKCα/β inhibitor Gö6976 (10 μM). G, Upper panel, Western blot of A549 cells transfected with scrambled control (CON) siRNA or siRNA against PKCα. Lower panel, analogous to Fig. 2B percentage of cells with 53BP1 foci +/- Gö6976 or siRNA transfections as shown. H, Upper panel, lysates from A549 cells following 1 hour treatment with or without EGF (100 ng/ml), erlotinib (2 μM), Gö6976 (10 μM), or the specific PKCα inhibitor Ro-32-0432 (100 nM) were subjected to Western blotting with antibodies against proteins as shown. Lower panel, percentage of cells with co-localized p-H3S10/H3K9me3 +/- erlotinib or +/- Gö6976. I, Percentage of H1703 cells with wild-type (wt) or mutant (mut) KRAS status displaying co-localized p-H3S10/H3K9me3 analogous to panel H. In all panels, bars represent mean +/- standard error based on typically 2-3 biological repeats.

**Figure 5.** A senescence-to-apoptosis switch in KRAS-mutant cancer cells. A, Representative images (40X) showing staining for DAPI and senescence-associated β-galactosidase (SA-β-gal) 3 days following 2 Gy irradiation in DLD1 (KRAS wt/mut) or DWT7 (wt/-) cells. B, Percentage of A549 cells staining for SA-β-gal staining 7 days following 2 Gy irradiation +/- erlotinib +/- the MEK inhibitor AZD6244 (250 nM), or Gö6976. C, Whole cell lysates from A549 cells 72 hours after irradiation (8 Gy) +/- erlotinib (2 μM) or +/- chloroquine (CQ; 25 mM) were subjected to Western blotting with
antibodies against proteins as shown. D, Representative images for DAPI and SA-β-gal staining obtained in parallel to the data shown in panel D. E, Percentage of sub-G1 cells as determined by FACS in KRAS mutant DLD-1 and wild-type DWT7 cells for the treatments indicated. F, SRF$_{2Gy}$ values for DLD-1 and DWT7 tumor spheres treated as indicated. Statistical comparisons by one-sample or unpaired T-test. In all panels, bars represent mean +/- standard error based on typically 2-3 biological repeats.
FIGURE 1 (2 columns)

A

Radiation + Erlotinib
60% 40%
Radiation + Cetuximab
52% 48%

resistant sensitized

B

Erlotinib
Mutant wild-type KRAS

C

Erlotinib
Mutant wild-type KRAS

D

NCI-H1703 KRAS wt

NCI-H1703 KRAS mut

control erlotinib
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Cancer Res Published OnlineFirst March 19, 2014.

Updated version
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Supplementary Material
Access the most recent supplemental material at:
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