EGFR-Activating Mutations Correlate with a Fanconi Anemia–like Cellular Phenotype That Includes PARP Inhibitor Sensitivity

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Abstract

In patients with lung cancer whose tumors harbor activating mutations in the EGF receptor (EGFR), increased responses to platinum-based chemotherapies are seen compared with wild-type cancers. However, the mechanisms underlying this association have remained elusive. Here, we describe a cellular phenotype of cross-linker sensitivity in a subset of EGFR-mutant lung cancer cell lines that is reminiscent of the defects seen in cells impaired in the Fanconi anemia pathway, including a pronounced G2-M cell-cycle arrest and chromosomal radial formation. We identified a defect downstream of FANCD2 at the level of recruitment of FAN1 nuclease and DNA interstrand cross-link (ICL) unhooking. The effect of EGFR mutation was epistatic with FANCD2. Consistent with the known role of FANCD2 in promoting RAD51 foci formation and homologous recombination repair (HRR), EGFR-mutant cells also exhibited an impaired RAD51 foci response to ICLs, but not to DNA double-strand breaks. EGFR kinase inhibition affected RAD51 foci formation neither in EGFR-mutant nor wild-type cells. In contrast, EGFR depletion or overexpression of mutant EGFR in wild-type cells suppressed RAD51 foci, suggesting an EGFR kinase-independent regulation of DNA repair. Interestingly, EGFR-mutant cells treated with the PARP inhibitor olaparib also displayed decreased FAN1 foci induction, coupled with a putative block in a late HRR step. As a result, EGFR-mutant lung cancer cells exhibited olaparib sensitivity in vitro and in vivo. Our findings provide insight into the mechanisms of cisplatin and PARP inhibitor sensitivity of EGFR-mutant cells, yielding potential therapeutic opportunities for further treatment individualization in this genetically defined subset of lung cancer.

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Introduction

Lung cancer is the most common cause of cancer-related death worldwide. Five-year overall survival of patients remains at a rate of only approximately 15%, stressing the need for the development of novel treatment approaches (1). The identification in recent years of molecular lung cancer subsets characterized by targetable oncogenic driver mutations has revolutionized therapy. For example, activating mutations in the EGF receptor (EGFR) are associated with tumor response rates to small-molecule tyrosine kinase inhibitors (TKI) of around 70% (2). Despite often-impressive tumor responses, however, virtually all patients eventually experience progression. Notably, EGFR-mutant lung cancers seem to be more responsive to platinum-based chemotherapy than wild-type tumors in clinical trials (3–5), but the mechanisms underlying this finding remain to be elucidated.

The EGFR has been implicated in the repair of DNA double-strand breaks (DSB) via DNA-PKcs–dependent nonhomologous end joining (NHEJ; refs. 6–8). However, NHEJ is not required for the removal of platinum-induced DNA damage from the genome (9, 10). Homologous recombination repair (HRR) is a pathway critical for several cellular processes including the error-free repair of DSB and the recovery of stalled or collapsed DNA replication forks (11). HRR-defective cells are hypersensitive to DNA lesions that block replication forks, such as DNA interstrand cross-links (ICL) produced by cisplatin or mitomycin C (MMC; refs. 12–15). In addition, impaired HRR is synthetically lethal with inhibitors of PARP1/2 (13, 16–20). There is currently great interest in exploring the clinical use of PARP inhibitors in multiple cancer types including lung cancer (11). It is clear that predictive
Biomarkers of treatment sensitivity will be needed to select patients most likely to benefit. However, in human cancers, HRR may be altered by various genetic, epigenetic, or other mechanisms, which makes it challenging to assess the functional HRR status in a given tumor (11). We recently identified HRR defects in human lung cancer cell lines and tumors, although whether such defects are more frequent in EGFR-mutant cancers has remained unknown (13).

HRR has evolved to be tightly regulated to promote precise DNA repair and limit genomic alterations. This is achieved through cell-cycle phase coordination, posttranslational modifications, and many accessory factors that either promote or inhibit protein interactions (11). Thus, for cancers, there exists ample opportunity to deregulate this process. How exactly selection pressure arises during carcinogenesis to disrupt HRR pathways is currently unknown. Given the crucial role of HRR for replication fork restart and repair and the possibility of widespread genomic instability if this process fails, it is conceivable that replication-associated HRR is specifically targeted when premalignant cells accumulate oncogenic stress and associated DNA damage (11).

Stalled replication forks activate the Fanconi anemia (FA) pathway, which is composed of 15 identified genes, FANCA through FANCP, known to cause Fanconi anemia in patients when mutated in both alleles (except FANCJ; refs. 21–24). The Fanconi anemia proteins together with BRCA1 cooperate in a common biochemical “FA/BRCA” pathway, which is believed to function mainly in the detection, stabilization, and repair of stalled DNA replication forks (15). In response to fork-blocking ICLs, monoubiquitinated FANCD2 relocates into chromatin and colocalizes with BRCA2, RAD51, and other DNA damage response proteins; and these protein accumulations can be visualized as subnuclear foci (11). The FANCD2/FANCI complex and associated factors promote nucleolytic incision near an ICL, for example via the recently discovered FAN1 nuclease (25–27). The Fanconi anemia proteins are closely linked to HRR via multiple mechanisms and Fanconi anemia defects can be associated with reduced homology-mediated repair of DSB and impaired RAD51 foci formation (13, 28–30). Cross-linker sensitivity is a hallmark of defects in the FA/BRCA pathway (12, 14, 15).

Here, we describe an unexpected Fanconi anemia-like cellular phenotype in a subset of cisplatin-treated lung cancer cell lines with mutant EGFR. We find that EGFR mutation is closely linked to altered FAN1 function and RAD51 subnuclear localization downstream of FANCD2, leading not only to cisplatin and MMC sensitivity but also sensitivity to the PARP inhibitor olaparib, thus yielding a potential therapeutic opportunity.

**Materials and Methods**

**Cell lines and cell culture**

Cell lines were selected from a published panel (31, 32). The identity of each of the cell lines in the panel was described previously (31). A549, NCI-H1650, and HCC4006 were purchased from the American Type Culture Collection. NIH3T3 mouse embryonic fibroblasts (MEF) stably transfected with a plabe puromycin resistance expression vector–encoding human wild-type EGFR, mutant E746_A750, or mutant L858R (33) were maintained in Dulbecco’s Modified Eagle Medium (DMEM). NCI-H1650, NCI-H1703, NCI-H1792, NCI-H1975, NCI-H2228, and PC3 were maintained in RPMI-1640. A549 were grown in DMEM and PC3 in DMEM/F12. PD20 human fibroblasts with mutant or wild-type FANCD2 were described previously (28). All media were supplemented with 10% bovine growth serum (HyClone), 20 mmol/L L-glutamine, and 1% penicillin–streptomycin (all Sigma-Aldrich unless indicated otherwise). Cell lines were assigned arbitrary passage numbers upon receipt in the laboratory and passaged less than 20 times (i.e., typically 2 months). No cell line was ever treated for Mycoplasma and all lines tested Mycoplasma-free before the experiments (Mycoplasma Alert; Lonza).

**Treatments**

Cisplatin and MMC were obtained from Sigma-Aldrich and olaparib and erlotinib from LC Laboratories. Cisplatin was dissolved at 4 mmol/L in 0.9% NaCl and protected from light at 4°C for 2 weeks. MMC was dissolved in double-distilled water (ddH2O) and protected from light at 4°C for 2 weeks. All other drugs were dissolved in dimethyl sulfoxide, aliquoted and protected from light at −20°C for 6 weeks. X-irradiation was carried out as described previously (31).

**Cell survival assays**

Clonogenic survival assays were conducted as described previously (31). Cells were treated with varying concentrations of cisplatin or MMC for 1 hour or olaparib for 72 hours. Following aspiration of drug, cells were washed once and media was replaced. Depending on doubling time, cells were left to form colonies for 9 to 29 days. Nonclonogenic cell survival was assessed by fixing and staining cells with a fluorescent nucleic acid stain (syto60) 72 hours after treatment as described (31).

**Immunofluorescence microscopy**

Foci staining and analysis were conducted as described previously (13, 28, 31). Briefly, cells were incubated with primary antibodies for 2 hours at room temperature (γ-H2AX: 1:200 in 2% bovine serum albumin, BSA/PBS; ab22551; Abcam), for 3 hours at 37°C (FANCD2: 1:500 in 2% BSA/PBS, NB100–182, Novus Biologicals) or overnight at 4°C (RAD51, 1:500 in 5% FBS/PBS; GTX70230; GeneTex), and subsequently incubated with secondary antibodies for 1 hour at room temperature (Alexa Fluor 488 goat anti-mouse or Alexa Fluor 488 chicken anti-rabbit, Invitrogen). Subnuclear foci were scored by fluorescence microscopy (Olympus BX51).

Fresh tumor tissues from patients with EGFR mutant or wild-type tumors were collected on protocols approved by Institutional Review Boards and subjected to ex vivo treatment as described previously (13, 34). Tumor samples were aliquoted and placed in RPMI medium, and either mock-treated or treated with 10 μmol/L olaparib for 24 hours, followed by snap-freezing in optimum cutting temperature formulation (OCT; Sigma-Aldrich). Cryosections were thawed at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, and subsequently fixed with 2% PFA for 15 minutes. This was followed by another permeabilization step with 0.5% Triton X-100.
Triton X at room temperature for 5 minutes. Blocking ensued by covering the tissue with 5% goat serum/PBS in a humid chamber at room temperature for 1 hour. Incubation with primary antibody was done at 4°C overnight. Primary antibody solution contained mouse monoclonal anti-γ-H2AX antibody (Abcam; ab22551, 1:500 dilution) and rabbit polyclonal anti-PCNA (Abcam; ab2426, 1:200) in 3% goat serum/PBS. Incubation with secondary antibody was done at room temperature for 1 hour in a humid chamber for 1 hour. Secondary antibody solution contained goat anti-mouse immunoglobulin G (IgG; Alexa Fluor 488; Invitrogen #A11029; 1:1,000 dilution) and goat anti-rabbit IgG (Alexa Fluor 555; Invitrogen #A21429; 1:1,000) in 3% goat serum/PBS. Nuclei were stained with DAPI (1 μg/mL in ddH2O) for 2 minutes.

Flow cytometry
Cells were treated with MMC (25 ng/mL) for 24 hours. Cells were harvested, fixed with ethanol, and stained with propidium iodide according to the standard protocols. Cell-cycle distribution was analyzed at the Ragon Imaging Core as described previously (31).

Western blotting
Proteins in whole-cell lysates from exponentially growing cell cultures were detected using standard methods. Specific antibodies against human EGFR (2239S; Cell Signaling Technology) and β-actin (Sigma-Aldrich) were used, except for siRNA experiments, which used a rabbit polyclonal anti-EGFR antibody (sc-03; Santa Cruz Biotechnology). Horseradish peroxidase–conjugated secondary antibodies were used (sc-2031; Santa Cruz Biotechnology). Protein bands were visualized with enhanced chemiluminescence (Invitrogen) followed by autoradiography.

Transfections
Full-length human EGFR expression constructs encoding wild-type or mutant (L858R) proteins were described previously (35). A lentiviral expression vector encoding GFP-flagged FAN1 (PHAGE CMV N-EGF-FP-FL FAN1) was a gift from Stephen Elledge, Ph.D. (Dept. of Genetics, Harvard Medical School, Boston, MA; ref. 26). Plasmid pEGFP-N1 was obtained from Invitrogen. All transfections were carried out using Metafectene Pro (Biontex) according to the manufacturer’s protocol. For siRNA transfections, exponentially growing cells were transfected with validated siEGFR or a scrambled control siRNA (Ambion) using the X-tremeGENE Transfection Kit (Roche). Western blotting and subsequent experiments were carried out 48 hours after transfection.

Modified alkaline comet Assay
To monitor ICL repair, a modified alkaline comet assay (8) was deployed (CometAssay; #4250-050-K; Trevigen). Tail moment was analyzed using TriTek CometScore (see Supplementary Data for details).

Chromatid aberrations
Following treatment of cells with 0.5 μmol/L of MMC for 1 hour, metaphases were scored to determine the fraction of cells with tri- and quadri-radials as described previously (36).

Results

**EGFR mutation is associated with a Fanconi anemia–like cellular phenotype**

To determine the cellular cisplatin sensitivity associated with EGFR mutation, we first studied isogenic MEFs expressing either human wild-type EGFR or EGFR harboring–activating mutations (Fig. 1A). For cisplatin concentrations of 8 to 16 μmol/L, mutant EGFR was associated with a 1.3- to 2.2-fold lower cell-survival fraction compared with exogenous wild-type protein. Similarly, overexpressing mutant or wild-type EGFR in lung cancer cells with wild-type or mutant EGFR, respectively, changed cisplatin sensitivity by at least 1.5-fold (Supplementary Fig. S1A–S1D). In a panel of lung cancer cell lines, the cell lines with mutant EGFR showed a logarithmic mean survival fraction of 25.8% at 8 μmol/L cisplatin, compared with 73.6% for the wild-type lines (difference of 2.9-fold; Fig. 1B). In addition, as a surrogate endpoint of cisplatin sensitivity, we observed increased numbers of γ-H2AX foci at 24 hours after cisplatin treatment in the cisplatin-sensitive EGFR-mutant cell lines (Fig. 1C and Supplementary Fig. S1E), consistent with persistent, unrepaired DNA damage (13). Importantly, EGFR-mutant PC9 clones with acquired TKI resistance maintained their cisplatin sensitivity relative to EGFR wild-type cells (Supplementary Fig. S1F and S1G).

![Figure 1](https://example.com/figure1.png)
Cisplatin sensitivity can be associated with genetic or epigenetic defects in the Fanconi anemia pathway (11). Strikingly, we observed the hallmarks of the Fanconi anemia phenotype in cells with mutant EGFR. In MEFs expressing mutant EGFR, there was increased MMC sensitivity compared with isogenic cells with wild-type EGFR (Fig. 2A). Furthermore, EGFR-mutant lung cancer cell lines were on average 3.9-fold more MMC sensitive than wild-type lines, based on a logarithmic mean survival fraction (at 0.5 μg/mL) of 8.0% (n = 8) versus 30.8% (n = 4), respectively. Follow-up analysis in EGFR-mutant PC9/PC14 cells revealed a pronounced damage-induced G2-M cell-cycle arrest (Fig. 2B and Supplementary Fig. S2A) and chromosomal radial formation (Fig. 2C), comparable with the effects of FANCD2 mutation (Fig. 2C and Supplementary Fig. S2A; ref. 36). In addition, as Fanconi anemia cells have been reported to show increased Ataxia telangiectasia mutated (ATM) activity (37), we observed an elevated number of phosphorylated ATM foci (Supplementary Fig. S2B).

**FANCD2 downstream defect in EGFR-mutant cells**

To screen for defects in the Fanconi anemia pathway, we determined the ability of EGFR-mutant cells to form subnuclear foci of FANCD2. Foci formation in EGFR-mutant cells was normal or even elevated compared with wild-type cells (Fig. 3A and Supplementary Fig. S3A). In conjunction with intact FANCD2 monoubiquitination (Supplementary Fig. S3B), this argues against a defect in the nuclear Fanconi anemia core complex or BRCA1, which are upstream of FANCD2 foci formation. Consistent with the latter, BRCA1 foci formed normally (Supplementary Fig. S3C). To investigate downstream components, we first determined the ability of EGFR-mutant cells to unhook ICLs using a modified alkaline comet assay (Fig. 3B; ref. 8). These cells were dramatically impaired in ICL unhooking at 3 to 5 hours after cisplatin exposure (Fig. 3B and Supplementary Fig. S4A). There was also an increase in foci of replication protein A, which accumulates on single-stranded DNA exposed at stalled or collapsed replication forks (Supplementary Fig. S4B).

To screen for candidate nucleases involved in ICL incision that might be defective in EGFR-mutant lung cancer cells, we selectively examined the gene expression of SLX4/FANCP, MUS81, EME1, FAN1, and ERCC1 (Supplementary Fig. S4C). Interestingly, EGFR mutation was correlated with reduced expression of the FANCD2-associated nuclease FAN1 (Supplementary Fig. S4C and S4D). We, therefore, wished to determine if FAN1 function was altered in the presence of mutant EGFR. As FAN1 is known to form subnuclear foci in response to ICLs (26), we transfected GFP-tagged FAN1 into isogenic MEFs expressing mutant or...
wild-type EGFR. Strikingly, the ability of cells to form FAN1 foci was essentially abrogated in EGFR-mutant cells (Fig. 3C). Whether downregulated FAN1 expression was the cause or a result of impaired FAN1 localization and function was not established. Taken together, the findings in Fig. 3A–C suggested a functional defect at the level of ICL unhooking downstream of FANCD2.

We, therefore, predicted that the repair defect associated with mutant EGFR is epistatic with FANCD2. MMC treatment of FANCD2-deficient cells in the presence of wild-type EGFR caused increased $\gamma$-H2AX foci, i.e., on average 57.3%, compared with 32.5% for FANCD2 wild-type complemented cells (Fig. 3D and Supplementary Fig. S4E). Transfection of mutant EGFR into FANCD2 wild-type complemented cells also increased MMC-induced $\gamma$-H2AX foci, i.e., to 59.7%, but there was no further increase in the context of mutant FANCD2 status (52.7%).

**RAD51 foci formation defect in EGFR-mutant cells treated with cross-linking drugs**

Disruption of the FANCD2-controlled pathway impairs aspects of HRR that can be visualized by reduced RAD51 foci formation at 5 to 24 hours after MMC or cisplatin treatment (Supplementary Fig. S5A; data not shown; refs. 13, 28). Interestingly, EGFR-mutant cancer cells also exhibited a RAD51 foci defect in response to cisplatin but not to DSB caused by ionizing radiation (Fig. 4A and Supplementary Fig. S5B and S5C), whereas RAD51 protein levels were normal (Supplementary Fig. S5D). In addition, homology-dependent repair of DSB generated by the I-SceI endonuclease was intact (Supplementary Fig. S5E), indicating that there is no global HRR defect associated with mutant EGFR. Remarkably, the ability of EGFR-mutant and wild-type cell lines to form RAD51 foci correlated closely with cisplatin survival ($P = 0.01$; Fig. 4B).

With regard to the mechanisms by which mutant EGFR may affect the FANCD2 pathway, it has been suggested that in response to cisplatin wild-type but not mutant EGFR translocates into the nucleus where it promotes DSB repair, possibly through DNA-PKcs (6–8). However, we observed that only approximately 6% of cisplatin-treated EGFR wild-type cells contain nuclear EGFR (Supplementary Fig. S6A), which is not consistent with the magnitude of the FAN1 and RAD51 foci defects seen in Fig. 3C and Fig. 4A. Furthermore, DNA-PKcs had no role in the cisplatin sensitivity of EGFR-mutant cells (Supplementary Fig. S6B).

Next, we wished to determine whether signal transduction pathways downstream of mutant EGFR were involved in blocking RAD51. For example, Akt has been reported to suppress HRR (38). However, we did not observe rescue of RAD51 foci formation upon pharmacologic inhibition of the PI3K-Akt axis (Supplementary Fig. S7A and S7B). Similarly, there was no stimulation of cisplatin-induced RAD51 foci in PC9 cells by short-term (2 hours) or long-term (19 hours) EGFR tyrosine kinase inhibition with erlotinib (Supplementary Fig. S7C and Fig. 4C). Vice versa, erlotinib neither sensitized EGFR wild-type A549 cells to cisplatin nor suppressed RAD51 foci (Supplementary Fig. S7D and S7E).
suggesting that EGFR affects the FANCD2/RAD51 pathway through a kinase-independent function. In support of this notion, overexpression of wild-type EGFR in PC9 cells was able to rescue RAD51 foci formation (Fig. 4D), whereas transfection of mutant EGFR into A549 wild-type cells reduced RAD51 foci (Supplementary Fig. S7E), which is consistent with competition between wild-type and mutant EGFR proteins (Supplementary Fig. S7F, and see Discussion). Lastly, as predicted from these data, depletion of wild-type EGFR in A549 cells by siRNA disrupted RAD51 foci formation (Fig. 4E) and caused cisplatin sensitivity (Fig. 4F).

PARP inhibitor sensitivity of EGFR-mutant cells

In search for novel treatment approaches in EGFR-mutant lung cancer, we considered that FANCD2-deficient cells are PARP inhibitor sensitive and that FAN1 may have functions in HRR beyond ICL unhooking, such as Holliday junction resolution (17, 25). We thus reasoned that EGFR-mutant cells may be impaired in their ability to form FAN1 foci in response to the PARP inhibitor olaparib and this is shown in Fig. 5A. Unexpectedly, olaparib-treated EGFR-mutant PC9 cells formed RAD51 foci at levels comparable with wild-type A549 cells up to 24 hours (Fig. 5B). However, at later time

Figure 4. RAD51 foci formation as a function of EGFR status. A, representative images of nuclei with RAD51 foci induced 5 hours after treatment with cisplatin (cis; 8 μmol/L) or ionizing radiation (IR; 8 Gy; left). Fraction of cells with ≥10 RAD51 foci (right). B, correlation of clonogenic cisplatin survival with fraction of cells with induced RAD51 foci. Line represents result of linear regression analysis. C, RAD51 foci formation in erlotinib-treated EGFR-mutant PC9 cells. Cells were exposed to erlotinib for 2 hours (illustrated by arrows in the left figure inset) or 19 hours (right inset) before adding cisplatin. D, protein levels of EGFR in EGFR-mutant PC9 following transfection of 1 or 5 μg expression vector encoding wild-type EGFR (left). Fraction of cells with ≥10 RAD51 foci 5 hours after cisplatin treatment and 48 hours after transfection with either 5 μg of wild-type EGFR vector or an empty control (right). Data not corrected for approximately 50% transfection efficiency (Supplementary Fig. S1B). E, Western blot of A549 cells transfected with scrambled control (CON) siRNA or siRNA against EGFR (left). Fraction of cells with RAD51 foci analogous to D (right). F, cell survival determined by syto60 staining for the cell lines indicated on the x-axis. All data represent mean ± SE based on two to four biologic repeats.
points (here shown for 34 hours, Fig. 5B), there was a pronounced persistence of RAD51 foci in EGFR-mutant but not wild-type cells, mirroring the effect of mutant FANCD2 in the PD20 cell pair and suggesting the presence of a block in a late step of HRR.

The EGFR-mutant cell lines (except PC3) exhibited olaparib sensitivity to a varying degree, with an average of 8.5% (SE ± 2.8%) survival at 10 μmol/L olaparib, compared with 29.0% (± 8.6%) for the wild-type cell lines (difference of 3.4-fold; Fig. 5C). The survival difference in isogenic MEFs with/without mutant EGFR was 3.8-fold (Fig. 5C) and 1.2-fold in A549 cells with/without EGFR deletion (Supplementary Fig. S8A; at 10 μmol/L olaparib). EGFR-mutant and wild-type lines could also be separated on the basis of estimates of IC50 (inhibitory concentration of drug to achieve 50% survival; Fig. 5C). To determine whether olaparib sensitivity can also be observed in vivo, we considered that PARP inhibition causes increased γ-H2AX staining. In our olaparib-sensitive EGFR-mutant cell lines, we observed considerably elevated levels of γ-H2AX foci after 24 hours of olaparib treatment, thus further validating this surrogate endpoint (Fig. 5D and Supplementary Fig. S8B). Note that untreated EGFR-mutant PC9 cells have already higher levels of γ-H2AX signal compared with other cell lines, which is consistent with baseline genomic instability, but there is strong damage induction upon PARP inhibition.

For comparison, fresh lung cancer tissues from patients were exposed to olaparib in the laboratory using a previously established ex vivo foci protocol (Supplementary Fig. S8C; refs. 13, 34). Because DSB form in S-phase and the S-phase fraction of lung cancers in patients is much lower than in cell lines (13), we used costaining with PCNA to identify cells with an olaparib-specific γ-H2AX signal. As predicted, PCNA-positive cells from an EGFR-mutant tumor exhibited substantially more γ-H2AX staining than wild-type cells, i.e., 21% versus 4% (P = 0.03, Fisher exact; Fig. 5D and Supplementary Fig. S8D), suggesting that this kind of assay could be adapted to guide the identification of EGFR-mutant patients.

![Figure 5](https://example.com/figure5.png)
with non–small cell lung cancer (NSCLC) who may benefit from PARP inhibitor treatment.

**Discussion**

We report that mutant EGFR is correlated with a cellular Fanconi anemia phenotype characterized not only by the hallmark of cross-linker sensitivity (cisplatin, MMC; Figs. 1A and B, 2A), but also PARP inhibitor sensitivity (Fig. 5C) and impaired RAD51 foci formation (Fig. 4A and Supplementary Fig. S5C). Mutant EGFR is epistatic with FANCED2 (Fig. 3D), which is a central chromatin-associated regulator of ICL repair, recruiting and coordinating several repair proteins at stalled replication forks (24). While our current knowledge of FANCED2 function and regulation remains limited and the role of the Fanconi anemia proteins in ICL unhooking is not without controversy (23, 24), it is clear that FANCED2 promotes RAD51 recruitment specifically in response to ICLs (Supplementary Fig. S5A; refs. 13, 28) and acts upstream of several structure-specific endonucleases such as FAN1 and SLX1-SLX4/FANCP (27, 39). Accordingly, EGFR-mutant cells display a profound ICL unhooking defect as well as impaired RAD51 foci formation after cisplatin treatment (Figs. 3B, 4A). Although we show impaired FAN1 foci formation (Fig. 3C), we acknowledge that the magnitude of the ICL unhooking defect (Fig. 3B) suggests that other endonucleases may be affected as well.

The extent of RAD51 foci formation at 24 hours in olaparib-treated EGFR-mutant PC9 cells was comparable with cells with wild-type EGFR (Fig. 3B), in contrast with the lack of RAD51 foci induction following cisplatin treatment (Fig. 4A). A possible explanation is that mutant EGFR only disrupts FANCED2-dependent endonuclease recruitment but not RAD51 foci formation, so that after cross-linker treatment RAD51 foci do not form (Fig. 4A) because a proper one-ended DSB substrate at the stalled fork is not being produced. Because RAD51 foci formation seems intact after depletion of either FAN1 or SLX4/FANCPC (25), this model predicts the impairment of more than one endonuclease involved in ICL incision. On the other hand, RAD51 can load onto stalled replication forks even before DSB formation, at least in *Xenopus* extracts (40).

Alternatively, the olaparib data in Fig. 5B can be interpreted as showing a relative RAD51 foci formation defect in EGFR-mutant cells at 24 hours, considering the substantially increased amount of DNA damage at that olaparib concentration compared with wild-type EGFR cells (Fig. 5D and Supplementary Fig. S8B), which should have led to much higher numbers of RAD51 foci. Thus, RAD51 foci recruitment may in fact be impaired after both cross-linker and PARP inhibitor treatment. However, in contrast with other data (8, 41, 42), our findings do not imply that wild-type EGFR promotes HRR of two-ended DSBs such as caused by ionizing radiation or I-SceI endonuclease breaks (Fig. 4A and Supplementary Fig. S5E).

Interestingly, RAD51 foci persisted in EGFR-mutant as well as FANCED2-mutant cells after more than 24 hours of olaparib treatment (Fig. 5B). We hypothesize that EGFR and FANCED2 may promote endonucleases, such as FAN1, which not only function in ICL unhooking but also in a Holliday junction resolvase complex, disruption of which will impair the disassembly of RAD51 foci. Whether impaired RAD51 foci formation or disassembly is more important for olaparib toxicity remains to be determined. Altogether, the data suggest a complex model in which EGFR may affect a FANCED2-dependent pathway of replication fork maintenance and HRR through multiple mechanisms. As upstream FANCED2 foci formation and monoubiquitination is intact in EGFR-mutant cells (Fig. 3A and Supplementary Fig. S3B), we speculate that EGFR targets yet unknown binding partners of chromatin-associated monoubiquitinated FANCED2.

Our data indicate that the impaired ability of EGFR-mutant cells to remove ICLs is not genetically fixed. Either expression of mutant EGFR in a wild-type background or the reverse can modify cross-linker sensitivity (Figs. 1A, 2A and Supplementary Fig. S1A–S1D). Similarly, expression of wild-type EGFR in PC9 cells but not erlotinib treatment rescues RAD51 foci formation (Fig. 4C and D), whereas mutant EGFR expression in A549 or depletion of endogenous wild-type protein, suppresses the RAD51 foci response (Fig. 4E and Supplementary Fig. S7E), implying that there is competition between wild-type and mutant proteins. Transfection of human mutant EGFR into MEFs with endogenous wild-type EGFR is thought to reflect the same mechanism (Fig. 1A). We, therefore, hypothesize that wild-type EGFR promotes ICL repair through an EGFR kinase-independent function and that mutant protein disrupts this function in a dominant-negative fashion (model in Supplementary Fig. S7F). However, the precise biochemical mechanism linking EGFR function to the FANCED2 pathway remains to be established.

To this end, we note that it has been reported that in response to DNA-damaging agents, including cisplatin, wild-type but not mutant EGFR translocates into the nucleus where it may promote DNA repair (6–8). However, we found that only approximately 6% of nuclei contain wild-type EGFR in response to cisplatin (Supplementary Fig. S6A), which clearly is not consistent with the magnitude of the repair defect we observe (Figs. 3B, 4A). It has also been suggested that EGFR-mutant NSCLCs have low ERCC1 expression (43). However, we do not see this on our cell lines (Supplementary Fig. S4C) and it is unclear how ERCC1 deficiency would abrogate RAD51 foci formation or lead to PARP inhibitor sensitivity (44).

A link between altered FA/BRCA function and EGFR signaling is not without precedent. Anecdotally, EGFR-mutant lung cancer has been linked to *BRCA* germline mutations (45). In addition, *BRCA1*-mutant breast cancers exhibit increased EGFR expression (46). It is tempting to speculate that altered repair of replication fork-blocking DNA damage may produce an environment favorable to oncogenic EGFR signaling. At least in a glioblastoma model, EGFRVIII was associated with an increased level of oxidative stress and there is evidence that the FA/BRCA pathway is required for the response to oxidative DNA lesions that pose a barrier to replication fork progression (28, 47, 48). We hypothesize that oxidative DNA damage accumulating in premalignant bronchial epithelial cells harboring mutant EGFR may generate selection pressure for impaired FA/BRCA function and perturbed HRR by producing...
continuous fork stalling and collapse, a concept that mirrors several features of the previously described oncosensued-induced DNA damage model in carcinogenesis (49).

We acknowledge that the potential clinical significance of the association of EGFR mutation with cisplatin and PARP inhibitor sensitivity remains to be fully determined as the survival differences in the isogenic models are relatively small and several of our EGFR-mutant lung cancer cell lines seem to have restored HHR and display little or no drug sensitivity. Still, EGFR mutation may be a useful biomarker in a clinical trial to enrich a study population treated with a PARP inhibitor +/chemo- or radiotherapy. In addition, the ex vivo foci assay that we have described in Fig. 5D and whether a given EGFR-mutant lung cancer is sensitive to PARP inhibition.

Disclosure of Potential Conflicts of Interest
J. Settleman is a Senior Director in (other than primary affiliation; e.g., consulting) Genentech. L.V. Sequist is a consultant/advisory board in Boehringer Ingelheim. Uncompensated: Merrimack Pharmaceuticals and compensated in Chovis Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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**EGFR-Activating Mutations Correlate with a Fanconi Anemia–like Cellular Phenotype That Includes PARP Inhibitor Sensitivity**

Heike N. Pfäffle, Meng Wang, Liliana Gheorghiu, et al.

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