Supplementary Information for:

EGFR fusions as novel therapeutic targets in lung cancer

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Supplementary Methods

EGFR plasmid construction

A cDNA encoding the *EGFR-RAD51* fusion was synthesized by Life Technologies based on the combined Refseq sequences of *EGFR* (NM_ 005228) and *RAD51* (NM_002875) (**Supplementary Fig. S10**). The pMSCV-puro vector backbone (Clontech) was used to construct all retroviruses. Assembly of pMSCV-puro-EGFR-WT and pMSCV-puro-EGFR-L858R was previously described(1). EGFR-RAD51 was subcloned from the pMA synthesis vector (Life Technologies) into the HpaI site of pMSCV-puro using blunt end ligation. All plasmids were sequence verified in the forward and reverse directions.

Ba/F3 and NR6 cell line generation

The empty pMSCV-puro retroviral vector or pMSCV-puro vectors encoding EGFR (either EGFR-WT, EGFR-L858R, or EGFR-RAD51) were transfected, along with the envelope plasmid pCMV-VSV-G (Addgene), into cells Plat-GP packaging cells (CellBioLabs). Viral media was harvested 48 hours after transfection, spun down to remove debris, and supplemented with 2 μg/mL polybrene (Santa Cruz). 2.5×10^6 Ba/F3 cells (or 1×10^6 NR6 cells) were re-suspended in 10 mL viral media. Transduced cells were selected for 1 week in 2 μg/mL puromycin (Invivogen), and Ba/F3 cells were selected for an additional week in the absence of IL-3. Stable polyclonal populations were used for experiments and routinely tested for expression of EGFR constructs.

Compounds

Erlotinib, afatinib, and osimertinib (AZD9291) were purchased from Selleck Chemicals. Cetuximab was purchased from ImClone.

Antibodies

The following antibodies were obtained from Cell Signaling Technology: phospho-EGFR tyrosine 845 (#2231, 1:1000 dilution), phospho-EGFR tyrosine 992 (#2235, 1:500 dilution), phospho-EGFR tyrosine 1068 (#2234, 1:1000 dilution), phospho-EGFR tyrosine 1173 (#4407, 1:1000 dilution), C-term EGFR

clone D38 (#4267, 1:2000 dilution), phospho-AKT serine 473 (#9271, 1:500 dilution), AKT (#9272, 1:1000 dilution), phospho-ERK threonine 202/tyrosine 204 (#9101, 1:2000 dilution), ERK (#9102, 1:2000 dilution), HRP-conjugated anti-mouse (#7076, 1:5000 dilution), and HRP-conjugated anti-rabbit (#7074, 1:5000 dilution). β-actin antibody (#A2066, 1:5000 dilution) was purchased from Sigma-Aldrich. N-term EGFR clone H11 (MA-13070, 1:500 dilution) and HRP-conjugated anti-goat (PA-129617, 1:5000 dilution) were purchased from Thermo Fisher. N-term EGFR clone 528 (#120, 1:500 dilution) and C-term RAD51 clone C20 (#6862, 1:1000 dilution) were purchased from Santa-Cruz Biotechnologies. Anti-phosphotyrosine clone 4G10 (#05-321; 1:2000 dilution) was purchased from Millipore.

Soft agar assays

1.5 mL of 0.5% agar/DMEM was layered in each well of a 6-well dish. A total of 5,000 NR6 cells in 1.5 mL of 0.33% agar/DMEM were seeded on top of the initial agar and allowed to grow for three weeks. Each cell line was plated in triplicate. Colonies were counted using GelCount (Oxford Optronix) with identical acquisition and analysis settings.

EGFR turnover analysis

Quantitative turnover of EGFR in Ba/F3 cells was performed as previously described (2,3) . Briefly, 1×10^7 Ba/F3 cells expressing EGFR-WT, -L858R, or -RAD51 were serum starved for 16 hours and then treated with the combination of 50 ng/mL EGF and 50 μg/mL cyclohexamide. Cells were harvested at various timepoints and subjected to western blot analysis as described above. For quantification of signal, desaturated western blot images were opened with FiJi (ImageJ). The density of individual bands was measured using the Gel Analysis method outlined in the ImageJ documentation (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels). Values were normalized relative to N-EGFR density at t=0 and the actin density at equivalent timepoints. The normalized band densities were plotted into Prism and the receptor half-life determined by asymmetric sigmoidal interpolation.

Supplementary References

1. Red Brewer M, Yun C-H, Lai D, Lemmon MA, Eck MJ, Pao W. Mechanism for activation of mutated epidermal growth factor receptors in lung cancer. Proceedings of the National Academy of Sciences. National Acad Sciences; 2013;110:E3595–604.

2. Sorkin A, Duex JE. Quantitative Analysis of Endocytosis and Turnover of Epidermal Growth Factor (EGF) and EGF Receptor. Hoboken, NJ, USA: John Wiley & Sons, Inc; 2001;30:1–20.

3. Heuckmann JM, Balke-Want H, Malchers F, Peifer M, Sos ML, Koker M, et al. Differential Protein Stability and ALK Inhibitor Sensitivity of EML4-ALK Fusion Variants. Clin Cancer Res. 2012;18:4682–90.

Supplementary Table Legends

Supplementary Table S1: Summary of *EGFR* alterations in NSCLC identified by FoundationOne. The Foundation Medicine database of annotated clinical cases was interrogated for the presence of various *EGFR* genomic alterations known to recur in lung cancer. The 'X' in G719X denotes any of several amino acids which may be substituted for glycine in this variant. *EGFR* fusions events were defined by a genomic breakpoint in *EGFR* exons 23 through intron 25.

Supplementary Table S2: Summary of genomic coordinates for the kinase fusions identified in this study. The sample number is depicted in the far left column. For tumor samples in which a kinase fusion was identified, the genomic coordinates for each of the fusion genes is indicated. Additional alterations are noted. Amp = amplification. N/A = Not Applicable *breakpoint not clear

Supplementary Table S3: Results of MTT curve fitting from Prism. These data correspond to the graphs shown in Figure 3a.

Supplementary Figure Legends

Supplementary Figure S1: Additional information for Patient 1.

(**A**) Platelet counts in peripheral blood samples from patient 1 before and after the initiation of erlotinib therapy. ULN= upper limit of normal for platelet count. LLN= lower limit of normal for platelet count.

(**B**) Serial CT scans from patient 1 documenting response to the EGFR TKI, erlotinib. This figure includes the images from Figure 1C (as denoted on the left side of the image) and as well as additional images showing response to erlotinib at other disease sites.

Supplementary Figure S2: Additional information for Patient 2.

(**A**) Platelet counts in peripheral blood samples from patient 2 before and after the initiation of erlotinib therapy. ULN= upper limit of normal for platelet count. LLN= lower limit of normal for platelet count.

(**B**) Serial CT scans from patient 2 documenting response to the EGFR TKI, erlotinib. This figure includes the images from Figure 1C (as denoted on the left side of the image) and as well as additional images showing response to erlotinib at other disease sites.

Supplementary Figure S3: Additional information for Patient 3.

(**A**) Scaled representation of *EGFR-PURB* depicting the genomic structure of the fusion. ATG = translational start site. Blue = EGFR. Orange = PURB.

(**B**) Putative representations of EGFR-PURB protein structures. Numbers correspond to amino acid residues. Y = tyrosine residue. ECD = extracellular domain. TM = transmembrane domain. KD = kinase domain. The three different EGFR-PURB protein variants (v1, v2, v3) are based on the different potential reading frames of the fusion sequence.

(**C**) Serial CT scans from patient 3 documenting response to the EGFR TKI, erlotinib. This figure includes the images from Figure 1C (as denoted on the left side of the image) and as well as additional images showing response to erlotinib at other disease sites.

Supplementary Figure S4: Additional clinical information for Patient 4.

Serial PET scans from patient 4 documenting response to the EGFR TKI, erlotinib. This figure images showing response to erlotinib at disease sites other than those shown in Figure 1C.

Supplementary Figure S5: Characterization of EGFR-RAD51 in NR6 cells.

(**A**) NR6 lines stably expressing pMSCV (vector only), EGFR-WT, EGFR-L858R or EGFR–RAD51 were subjected to western blot analysis with the indicated antibodies. EGFR-RAD51 fusion is detected with both the N-terminal EGFR antibody [EGFR(N)] and with the RAD51 antibody. There is no cross reactivity between wild-type RAD51 protein, which has MW ~35kD, and the EGFR-RAD51 fusion.

(**B**) NR6 expressing EGFR variants were serum starved for 16 hours, treated with 100 nM afatinib for 1 hour followed by 50 ng/mL EGF for 5 minutes, and subjected to western blot analysis with the indicated antibodies.

(**C**) NR6 cells stably expressing the indicated constructs (empty = no cells; pMSCV = vector only) were plated in triplicate in soft agar, grown for three weeks, and quantified for colony formation. *** = $p < 0.0001$.

(**D**) Representative 4x microphotographs of soft agar assays showing empty wells (no cells) and NR6 cells transduced with pMSCV vector, EGFR-WT, EGFR-RAD51, and EGFR-L858R after three weeks of growth in soft agar.

Supplementary Figure S6: Relative stability of EGFR-WT, -L858R, and -RAD51.

(**A**) Ba/F3 cells harboring indicated EGFR variants were serum starved overnight and then treated with the combination of 50 ng/mL EGF and 50 μg/mL cyclohexamide (CHX) at time = 0. Cells were lysed for western blot analysis with indicated antibodies at various timepoints.

(**B**) Quantification of N-EGFR western blot signal (using FiJi) relative to time = 0 (and normalized to actin) for each of the EGFR variants. Intensity was plotted into Prism and the receptor half-life determined by asymmetric sigmoidal interpolation.

Supplementary Figure S7: Structural model of EGFR-RAD51 filaments.

Space-filling model of the EGFR-RAD51 kinase domains illustrating hypothetical filament based on the RAD51 filament structure. Purple = first EGFR kinase domain; green = second EGFR kinase domain; red = first RAD51 partner; blue = second RAD51 partner.

Supplementary Figure S8: On-target inhibition of EGFR-RAD51 by EGFR TKI.

Ba/F3 cells stably expressing EGFR-WT, EGFR-L858R and EGFR-RAD51 were serum starved for 16 hours, treated with 100 nM afatinib for 1 hour followed by 50 ng/mL EGF for 5 minutes, and subjected to western blot analysis with the indicated antibodies.

Supplementary Figure S9: Cetuximab inhibits ligand-induced activation of downstream signaling pathways in cells expressing EGFR-RAD51.

Ba/F3 cells stably expressing EGFR-L858R and EGFR-RAD51 were serum starved for 16 hours, treated with 5 μg/mL cetuximab for 8 hours followed by 50 ng/mL EGF for 5 minutes, and subjected to western blot analysis with the indicated antibodies.

Supplementary Figure S10: cDNA sequence of *EGFR-RAD51*.

Sequence of the *EGFR-RAD51* fusion based on *EGFR* NM_005228 (ENST00000275493) and *RAD51* NM_002875 (ENST00000267868). Exons 1-24 of *EGFR* are highlighted in blue and written in lower case. Exons 4-10 of *RAD51* are highlighted in orange and written in capital letters.