AXL-mediated activation of ABL and SFK induces YAP signaling to confer bypass resistance to erlotinib in lung adenocarcinoma

This manuscript was automatically generated on July 21, 2021.

## Authors

* **Marc Creixell** ORCID [0000-0003-3981-934X](https://orcid.org/0000-0003-3981-934X) · Github [mcreixell](https://github.com/mcreixell) · twitter [mcreixell\_](https://twitter.com/mcreixell_) Department of Bioengineering, University of California, Los Angeles
* **Eric B. Haura** Department of Bioengineering, University of California, Los Angeles
* **Forest M. White** Department of Bioengineering, University of California, Los Angeles
* **Aaron S. Meyer** ORCID [0000-0003-4513-1840](https://orcid.org/0000-0003-4513-1840) · Github [aarmey](https://github.com/aarmey) · twitter [aarmey](https://twitter.com/aarmey) Department of Bioengineering, University of California, Los Angeles; Department of Bioinformatics, University of California, Los Angeles; Jonsson Comprehensive Cancer Center, University of California, Los Angeles; Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles

## Abstract

Abstract.

## Summary points

* A
* B
* C
* D

## Introduction

Receptor tyrosine kinases (RTKs) play a central role regulating cell response to environmental cues during development and homeostasis ([*1*](#ref-14U8Zsben)). RTK dysregulation contributes to a variety of diseases including cancer ([*2*](#ref-LMqTuh6n)). RTK-targeted therapies are approved for a subset of lung cancer patients and successfully extend and improve patients’ lives. However, the effectiveness of these therapies is always limited by resistance. How resistance occurs varies widely, including through mutation of the drug target to block the effect of therapy, amplification of the drug target to overcome inhibition, pharmacokinetic barriers that block trafficking of the drug to tumor cells, and “bypass” switching to alternative pathways not targeted by therapy ([*3*](#ref-7S9uEWa), [*4*](#ref-eBhtfcG)). In the case of RTK-targeted therapies, many non-targeted RTKs may become activated to provide bypass resistance ([*5*](#ref-1A6zM7LPZ)). Two well-studied combinations are the ability of HER3 to provide resistance to HER2-targeted therapy in breast carcinoma, and the ability of Met to provide resistance to EGFR-targeted therapies in lung carcinoma ([*6*](#ref-uu3baD5W)–[*10*](#ref-16VTNu6s8)). In each case, the resistance-conferring receptors may contribute to intrinsic or acquired resistance; can become activated by multiple means including ligand-mediated autocrine or paracrine induction, amplification, or mutation; and can effectively be blocked by combination therapy ([*8*](#ref-QzzeTuTF), [*11*](#ref-chIpSlW9), [*12*](#ref-qmZcVWkr)).

## Results

### The ability of AXL to confer erlotinib resistance varies among PC9 AXL mutants

Figure 1: **Phenotypic consequences of AXL YF mutations.** (A-B) After knocking AXL out from PC9 cells using CRISP/Cas9, lentiviral vectors containing the AXL phosphosite mutations and a EGFP separated by an IRES were transduced into the PC9 AXL KO cells. After puromycin selection, the AXL mutant cell lines were sorted for GFP expressing cells. Phenotypic assays were formed wherein cells were treated with either 1µM erlotinib only or and erlotinib and 300nM AXL-activating antibody AF154 to quantify cell viability and death (YOYO), cell migration, and an island effect. C) Cell migration was observed using Incucyte via a scratch wound assay. D) PC9 WT cells form “islands” induced by erlotinib which disolve through concomitant activation of AXL. The island effect was quantified by taking the cell-to-cell distance and modeled using Ripley’s K function. (E-F) PCA analysis of AXL mutant phenotypes across conditions. In addition to cell migration and island effect, cell viability and cell death were monitored using Incucyte via measuring confluency levels and counting YOYO+ cells, respectively, over time. (G-H) Cell viability time lapse of WT-like Y698F and AXL KO-like Y821F. (I-J) Cell death time lapse of WT-like Y821F and KO-like Y750F. (K-L) Cell migration time lapse of WT-like Y726F and KO-like Y821F. (M-N) Cell island effect quantified by Ripley’s K function across radii of WT-like Y726F and KO-like Y750F.

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### Our model predicts several upstream components of the YAP patwhay as drivers of AXL-mediated malignant phenotypes

Figure 2: **Reconstruction of signaling networks driving AXL-mediated malignant phenotypes.** A) Hierarchical clustering of the phosphoproteomic state of AXL mutants treated with erlotinib for 4h and subsequently with erlotinib and AF154 for 10min. B) Actual vs predictive pearson’s correlation coefficients fitting PLSR models with different clustering strategies. The best performing number of components were picked for each model. (C-D) PSLR scores and loadings of a PLSR model fit to DDMC cluster centers. (E-H) Centers and (I-L) PSSM motifs of clusters 1, 3, 4 and 5. (M) Upstream kinase predictions calculated using the Frobenius distance between PSPL profiles and cluster PSSMs.

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### AXL activates YAP to promote cell growth in the presence of erlotinib

Figure 3: **YAP promotes cell growth in an AXL-dependent manner.** (A-H) Cell viability time course of PC9 WT (A-D) and AXL KO cells (E-H) in the presence of erlotinib, erlotinib and R428, and erlotinib and AF154 in addition to a varying concentration of dasatinib. (I) Dasatinib dose response inhibition of YAP. (J) AXL-mediated activation of YAP.

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## Discussion

Discuss…

## Methods

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| AXL Genotype | Known Intracellular interactors | ScanSite | NetworKIN | NetPhorest | PDB | Known Phenotype |
| Wild-type | PI3K, AKT, GRB2, p85, PLCγ, SRC, LCK | N/A | N/A | N/A | N/A | Cell survival, proliferation, migration, and immunosuppression |
| Knock-Out | N/A | N/A | N/A | N/A | N/A | Inverse phenotyes of WT |
| Transduced Wild-type | N/A | N/A | N/A | N/A | N/A | N/A |
| K567R | N/A | N/A | N/A | N/A | N/A | Kinase dead, typically recapitulates KO phenotypes |
| Y634F | - | SHC | - | CRKL, SRC, PTP R3 | - | - |
| Y643F | - | p85 | - | - | - | - |
| Y698F | - | - | INPPL1, BCAR3 | FLT3, CSF1R, KIT, PDGFR, PTP R3 | - | - |
| Y726F | - | - | CBL, SYK | PTP R3 | - | - |
| Y750F | - | - | - | SH2D | - | - |
| Y821F | PI3K, AKT, GRB2, p85, PLCγ, SRC, LCK | p85, FGR, GRB2 | GRB2, PI3K, GRAP, MIST SHP2 | PIK3R, GRB2, SRC, PTP R3 | - | - |

#### Search Parameters:

Scansite: Stringency = “High” for Y821, and “medium” for the rest. NetworKIN: Minimum score = 1.11 for Y812 ; 0.89 for the rest. NetPhorest: Minimum score = 0.20 for Y634, Y643, and Y821 ; = 0.10 for Y726 and Y750

### Reagents and Cell Culture

Erlotinib was obtained from LC Laboratories. The AXL activating antibody AF154 was obtained from R&D Systems. PC9 (Sigma Aldrich) cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. HEK293T cells were grown in DMEM supplemented with 10% FBS and 1% GlutaMAX (Thermo Fisher Scientific).

### Generation of Mutant Cell Lines

The PC9 AXL KO cell line was generated by transfecting cells with a CRISPR/Cas9 and GFP vector containing a gRNA targeting the AXL kinase domain. gRNA sequence, as well as cell culturing and sorting methods, have been previously described ([*13*](#ref-a2AgZtV5)). Plasmids containing the AXL phosphosite mutations were generated from an AXL-IRES-Puro vector (Addgene #65627) using a site directed mutagenesis kit (New England Biosciences).

Each mutant was copied from its respective plasmid, digested, and inserted into a lentiviral vector containing a puromycin resistance gene (Addgene #17448). For viral packaging, HEK 293T cells were seeded 4.5 x 106 per 10 cm dish. After 24 hours, the lentiviral AXL expression vector, VSV-G envelope vector, and packaging vector (Addgene #12259 and #12260 respectively) were combined in a 10:1:10 mass ratio and diluted in Opti-MEM (Thermo Fisher Scientific). TransIT-LT1 (Mirus Bio) was added dropwise and the solution was mixed gently by swirling and incubated at room temperature for 20 minutes. The solution was then added dropwise to the cells. After 18 hours, transfection media was replaced by media supplemented with 1% BSA fraction V (Thermo Fisher Scientific). Cells were incubated for 24 hours, after which the virus-containing media was removed and stored at 4℃. The media was replaced and the cells incubated a further 24 hours to generate a second batch of viral media. The harvested batches were then pooled, filtered through a .45μm PVDF membrane to remove packaging cells, and flash frozen followed by storage at -80ºC until use.

PC9 AXL KO cells were seeded with antibiotic-free media in a 6-well plate at a density of 1.5 x 105 cells per well and incubated for 24 hours. Cells were then infected with viral particles in antibiotic-free media supplemented with polybrene (MilliporeSigma). After 18 hours, the media was replaced with fresh antibiotic-free media. Cells were observed for a GFP positive population and passaged into a 10cm plate until confluent. The virally transduced cell lines were then sorted for GFP expressing cells using a BD FACSAria cell sorter. The mutant cell populations were then subcultured for later experiments.

### Preparation of Cell Lysates

Cell lines were grown to confluence in 10 cm dishes over the course of 72-96 hours, washed, and treated by addition of media containing 1 μM erlotinib. Cells were incubated for 4 hours at 37ºC and then additionally treated with media containing 1 μM erlotinib and 300 ng/mL AXL activating antibody for 10 minutes. The cells were then placed immediately on ice, washed with ice-cold phosphate-buffered saline, and lysed with cold 8 M urea containing Phosphatase Inhibitor Cocktail I and Protease Inhibitor Cocktail I (Boston BioProducts). The lysates were then centrifuged at 20,000xg, 4℃ to pellet cell debris, and the supernatants removed and stored at -80℃.

### Cell Viability Assay

Cells were seeded in a 96-well plate at a density of 1.05 x 103 cells per well. After 24 hours, treatments were added in media containing 300nm YOYO-3 (Thermo Fisher Scientific). Cells were cultured and imaged every 3 hours using an IncuCyte S3 (Essen Bioscience) at 20x magnification with 9 images per well. The phase, green, and red channels were manually thresholded and then analyzed by IncuCyte S3 software (Essen Bioscience) to determine cell counts and fraction of area covered.

### Cell Migration Assay

96-well IncuCyte ImageLock plates (Essen Bioscience) were coated with a Collagen-I solution (Thermo Fisher Scientific), washed twice, and then seeded with 4 x 104 cells per well. After a 4 hour incubation, cells were wounded using the IncuCyte WoundMaker, washed twice to remove detached cells, and then treated with respective conditions. Images of the center of the wound were taken every 2 hours at a magnification of 10x, one image per well. The phase and green channels were thresholded and analyzed as above to determine migration measurements.

All analysis was implemented in Python, and can be found at <https://github.com/meyer-lab/resistance-MS>.

## Acknowledgements

This work was supported by NIH U01-CA215709 to A.S.M. and in part by the UCLA Jonsson Comprehensive Cancer Center (JCCC) grant NIH P30-CA016042.

## Author contributions statement

A.S.M., E.B.H., F.M.W. conceived the project.

## Supplementary Figures

### Cell viability and death of PC9 AXL mutants

Figure S1: Cell viability and death complete time courses of PC9 AXL mutants. A) Fold-change confluency levels to erlotinib only-treated cells after 96h. (B-K) Cell viability measurements of PC9 AXL mutants throughout 96h normalized to initial time point 24h and erlotinib only-treated cells. (L) YOYO+ cell counts normalized to erlotinib only-treated cells after 96h. (M-V) Cell death measurements of all cell lines throughout 96h normalized to initial time point 24h.

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### Cell migration and island effect of PC9 AXL mutants

Figure S2: Cell migration and island effect of AXL mutants. A) Fold-change relative wound density (RWD) percentage of PC9 cells to erlotinib only-treated cells after 14h. (B-K) Cell migration measurements throughout 24h. L) Fold-change K estimate of Ripley’s K function to erlotinib only-treated cells. (M-V) K estiamtes of PC9 AXL mutant cells across different radii.

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### Signaling supplements

Figure S3: PCA loadings of phosphoproteomic data and signal of specific peptides. A) PCA loadings scores B) Phosphorylation signal of AXL, EGFR, and ERK1/2.

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### DDMC cluster centers and PSSM motifs

Figure S4: DDMC AXL model outputs. DDMC cluster centers (A-E) and PSSM motifs (F-J).

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