Published in final edited form as:

Mol Cancer Res. 2017 January ; 15(1): 45–58. doi:10.1158/1541-7786.MCR-16-0157.

AXL Inhibition Suppresses the DNA Damage Response and Sensitizes Cells to PARP Inhibition in Multiple Cancers

Kavitha Balaji^{#1}, Smruthi Vijayaraghavan^{#1}, Lixia Diao², Pan Tong², Youhong Fan³, Jason PW Carey¹, Tuyen N. Bui¹, Steve Warner⁵, John V Heymach³, Kelly K Hunt⁴, Jing Wang², Lauren Averett Byers^{#3}, and Khandan Keyomarsi^{#1}

¹ Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, 6565 MD Anderson Blvd. Unit 1052, Houston, Texas 77030.

² Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 1410, Houston, Texas 77030.

³ Department of Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd. Unit 0432 Houston, Texas 77030.

⁴ Department of Breast Surgical Oncology, The University of Texas MD Anderson Cancer Center, 1400 Pressler Street. Unit 1434 Houston, Texas 77030.

⁵ Tolero Pharmaceuticals Inc., Lehi, Utah 84043.

[#] These authors contributed equally to this work.

Abstract

Epithelial to mesenchymal transition (EMT) is associated with a wide range of changes in cancer cells, including stemness, chemo- and radio-resistance and metastasis. The mechanistic role of upstream mediators of EMT has not yet been well characterized. Recently, we showed that non-small cell lung cancers (NSCLCs) that have undergone EMT overexpress AXL, a receptor tyrosine kinase. AXL is also overexpressed in a subset of triple-negative breast cancers (TNBCs) and head and neck squamous cell carcinomas (HNSCCs) and its overexpression has been associated with more aggressive tumor behavior and linked to resistance to chemotherapy, radiation, and targeted therapy. Since the DNA repair pathway is also altered in patient tumor specimens overexpressing AXL, it is hypothesized that modulation of AXL in cells that have undergone EMT will sensitize them to agents targeting the DNA repair pathway. Downregulation or inhibition of AXL directly reversed the EMT phenotype, led to decreased expression of DNA repair genes and diminished efficiency of homologous recombination (HR) and RAD51 foci formation. As a result, AXL inhibition caused a state of HR-deficiency in the cells, making them sensitive to inhibition of the DNA repair protein, PARP1. AXL inhibition synergized with PARP inhibition, leading to apoptotic cell death. AXL expression also associated positively with markers of DNA repair across TNBC, HNSCC and NSCLC patient cohorts.

* Corresponding author: Khandan Keyomarsi, Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77030, Phone: 713-792-4845, Fax: 713-794-5369, kkeyomar@mdanderson.org.

Conflicts of Interest: No conflicts to disclose

Keywords

AXL; PARP; Breast Cancer; Lung Cancer; Head and Neck Cancer; DNA damage response

---

Introduction

AXL belongs to the TAM (Tyro3, AXL, Mer) family of receptor tyrosine kinases that are expressed on the surface of cancer cells, endothelial cells (vasculature) and some normal tissues (1). AXL is associated with metastasis, invasion and migration in many cancers including breast cancer (2) and lung cancer (3). Inhibition of AXL strongly diminished proliferation and migration of MCF7 and DAL breast cancer cell lines (2). AXL knockdown in mouse xenografts of A549 (non-small cell lung) and MDA-MB-231 (breast) cancer cell lines led to a modest decrease in tumor growth (3). Additionally, AXL knockdown reduced lung metastasis in MDA-MB-231 cell lines (3) and attenuated migration and anchorage-independent growth in other cancer types such as pancreatic cancer (4) and hepatocellular carcinoma cell lines (5). Forced expression of AXL can transform NIH3T3 cells leading to neoplastic transformation (6).

Our group and others have previously shown that AXL is overexpressed in human tumors that have gone through epithelial to mesenchymal transition (EMT) (7-9), and it is one of the key genes from our published EMT signature developed in non-small cell lung cancer (NSCLC) (8). This 76-gene EMT signature predicted resistance of NSCLC cell lines and patient tumors to EGFR tyrosine kinase inhibitors (TKIs) (a standard of care for NSCLC), which could be partially reversed in preclinical models by co-targeting AXL (8). Recently, we built upon this prior finding in NSCLC to develop a pan-cancer EMT signature from 12 different cancer types (10). In that study, high AXL expression was observed at the protein and mRNA level in mesenchymal cell lines and pan-cancer cohorts from diverse cancer types. AXL expression correlated highly along with the core EMT markers in all the tumor types examined and corresponded to differences in drug sensitivity (10).

AXL has also been linked to resistance to chemotherapy, radiotherapy and targeted therapy in a large number of cancer types. For example, in lapatinib-resistant HER2+ breast cancer cell lines, there was an increase in the expression levels of both total AXL and phospho-AXL. Inhibition of AXL reversed lapatinib resistance in these cells (11). Furthermore, as with lung cancer, EMT and AXL can mediate resistance to EGFR inhibition in breast cancer cell lines (8, 9). AXL overexpression also protected chronic myelogenous leukemia cell lines from growth inhibitory potential of imatinib (leading to drug resistance), while knockdown of AXL in these cells led to apoptosis of the resistant cell lines (12). Furthermore, AXL expression has been associated with primary or acquired resistance to DNA damaging therapies and may prevent normal DNA damage response (DDR) in a number of cancer types. Specifically, AXL expression has been recently shown to be associated with radio-resistance in HNSCC and AXL inhibition increased their sensitivity to chemotherapy and radiation (13). In esophageal cancer, high AXL expression was associated with in vitro resistance to cisplatin-induced apoptosis and prevented DNA damage induced cellular responses (14). AXL gene-expression was also upregulated in cisplatin-resistant

ovarian cancer cells, although targeting AXL in these cell lines had minimal effect on growth (15). AXL silencing in a panel of breast and lung cancer cell lines led to sensitizing these cell lines to mitotic inhibitors, suggesting that AXL inhibition can sensitize mesenchymal tumors to agents causing DNA damage and cell cycle inhibition (16). Collectively, these studies suggest that inhibition of AXL in multiple malignancies is likely to result in re-sensitization to targeted therapy, radiation and chemotherapy, possibly through enhancing/promoting DDR.

In this study, we interrogated the role of AXL in EMT and DNA damage response across three different malignancies (NSCLC, TNBC and HNSCC). We show that AXL can directly mediate EMT using stable knockdown models of AXL. We also introduce a novel role for AXL in positively facilitating homologous recombination (HR) mediated DNA repair at the gene expression as well as functional levels. Finally, we show that AXL inhibition leads to a defect in the HR DNA repair pathway, sensitizing cells to PARP inhibition, thus providing a novel therapeutic combination spanning three different cancer systems. The association between AXL and DNA repair protein expression was further validated in TNBC, NSCLC and HNSCC patient cohorts, where higher AXL levels in patient tumors was associated with increased expression of multiple DNA repair proteins, especially those involved in homologous recombination DNA repair process.

Materials and Methods

Cell lines and culture conditions

All breast and lung cancer parental cell lines used in this study were purchased from American Type Culture Collection (Manassas, VA). Triple negative breast cancer cell lines, (MDA-MB-231, MDA-MB-436, MDA-MB-157) and human mammary epithelial cells line (MCF-10A) were cultured as previously described (17, 18). Non-small cell lung cancer cell lines (SKLU1, Calu1, H1993) were cultured in RPMI medium in the presence of 10% FBS. Head and neck squamous cell carcinoma cell lines (584, HN5, 1386-LN) were provided by Dr. Jeffrey Myers at MDACC and cultured in DMEM in the presence of 10% FBS and growth factors, as previously described (19). All cells were free of mycoplasma contamination. Cell lines were identified and authenticated by karyotype and short tandem repeat analysis using the MD Anderson's Characterized Cell Line Core Facility regularly (every six months). Cell cycle, invasion and western blot assays were performed as previously described (17, 20) and briefly summarized in the Supplemental Methods.

Proliferation Assays

Cells were seeded at 5×10^4 cells per well in a 6-well plate and treated with either AXL inhibitor TP0903 (provided by Tolero Pharmaceuticals, Lehi, Utah), R428 (BGB324) (Selleckchem, Houston, TX) and/or olaparib (Selleckchem, Houston, TX) at the indicated time intervals (see figure legends). Medium was changed on alternate days following drug treatment. Cells were counted by Trypan Blue assay, using a Biorad cell counter on days 1, 3, 5 and 7 post-plating. The number of cells at each time point was normalized to the cell number at plating.

qRT-PCR

Total RNA was isolated from cell culture with RNAeasy kit with DNase treatment according to the manufacture's protocol (Qiagen, Valencia, CA). 2 µg of the RNA samples was reverse-transcribed using cDNA synthesis kit (Applied Biosystems, Carlsbad, CA). Realtime PCR was done with aliquots of the cDNA samples mixed with SYBR Green Master Mix (Sigma, St. Louis, MO). Reactions were carried out in triplicate. The fold difference in transcripts was calculated by the $2^{-\Delta\Delta CT}$ method using GAPDH as a control. E-cadherin forward 5'-TGCCCAGAAAATGAAAAAGG, E-cadherin reverse 5'-GTGTATGTGGCAATGCGTTC Twist forward 5'-GGAGTCCGCAGTCTTACGAG, Twist reverse 5'-TCTGGAGGACCTGGTAGAGG; Slug forward 5'-GGGGAGAAGCCTTTTCTTG, Slug reverse 5'-TCCTCATGTTTGTGCAGGAG; Zeb1 forward 5'-TTACACCTTTGCATACAGAACCC, ZEB1 reverse 5'-TTTACGATTACACCCAGACTGC; N-cadherin forward: 5'-ACAGTGGCCACCTACAAGG, N-cadherin reverse: 5'-CCGAGATGGGGTTGATAATG; Vimentin forward: 5'-GAGAACTTTGCCGTTGAAGC, Vimentin Reverse 5'-GCTTCCTGTAGGTGGCAATC; GAPDH forward 5'-ACCCAGAAGACTGTGGATGG, GAPDH reverse 5'-CTGGACTGGACGGCAGATCT; RAD51 forward: 5'-CAACCCATTTACGGTTAGAGC, RAD51 reverse: 5'-TTCTTTGGCGATAGGCAACA, BRCA2 forward: 5'-CACCCACCCTTAGTTCTACTGT, BRCA2 reverse: 5'-CCAATGTGGTCTTTGCAGCTAT; RAD54L forward: 5'-TTGAGTCAGCTAACCAATCAACC, RAD54L reverse: 5'-GGAGGCTCATAACGAACCAAGG; E2F1 forward: 5'-CATCCCAGGAGGTCACCTTCTG, E2F1 reverse: 5'-GACAACAGCGGTCTTGCTC BRCA1 forward: 5'-ACCTTGGAAGTGTGAGAACTCT BRCA1 reverse: 5'-TCTTGATCTCCCACACTGCAATA The following conditions were used for qPCR: Denaturation: 95°C for 10 minutes; 40 cycles: 95°C for 30 seconds, 58°C for 10 seconds, 72°C for 30 seconds; Extension: 72°C: 10 minutes

Immunofluorescence

To measure DNA damage response by γ H2AX staining, cells were plated at 10,000 cells per well in an 8-well chamber slide. After 24 hours, cells were treated with the AXL inhibitor 25 nM TP0903 and incubated for 48 hours. Cells were then transferred to fresh drug-medium for 48 hours, following which they were fixed in 4% paraformaldehyde for 20 minutes before staining. For measurement of RAD51 foci, cells were plated at 10,000 cells per well in an 8-chamber slide. After 24 hours, cells were treated with DMSO or the AXL inhibitor (25 nM TP0903) in the presence of 0.5 µg/ml doxorubicin and incubated for 48 hours. Cells were then transferred to fresh drug medium for 48 hours. For the AXL KD immunofluorescence, 10,000 cells were plated per well in an 8-well chamber and treated with doxorubicin as described above. Following treatment, cells were fixed in 4% formaldehyde for 20 minutes before staining. For immunofluorescence staining, fixed wells were rinsed in PBS and permeabilized for 5 minutes in 0.1% triton-X-100. The cells were then blocked in 1% BSA for an hour at room temperature. Primary antibodies, prepared in 1% BSA were added to the cells according to the manufacturer's instructions, followed by a 2 hour incubation at room temperature in a moist chamber. Primary antibodies used were γ H2AX (mouse monoclonal, Millipore, Billerica, MA) and RAD51 (rabbit polyclonal, obtained as a kind gift from the laboratory of Dr. Junjie Chen, MD Anderson Cancer Center,

Houston, TX). Secondary antibody was added after washing cells thoroughly in PBS. Cells were incubated with secondary antibodies tagged with Alexa fluor dyes (goat-anti-mouse-Alexafluor-488 and goat-anti-rabbit-Alexa fluor-594, Invitrogen, Carlsbad, CA) for 1 hour at room temperature. After rinsing and washing thoroughly with PBS, slides were mounted using Vectashield mounting medium containing DAPI and sealed. Cells were visualized in Olympus 1X81 DSU confocal microscope and images were analyzed using Slidebook.

Apoptosis Assay

Harvested cells were resuspended in annexin-binding buffer (10mM HEPES, 140mM NaCl, and 2.5mM CaCl₂, pH 7.4) and brought to a final volume of 1×10^6 cells/mL allowing for 100 μ l per assay. 5 μ l of annexin V conjugate was added to each 100 μ l of cell suspension and incubated for 15 minutes at room temperature. Finally, 400 μ l of annexin-binding buffer was added to each sample and analyzed by flow cytometry at 650/660nm.

Homologous Recombination (HR Assay)

To measure HR efficiency, a two plasmid- HR assay was used as described previously (21). Briefly, cells were plated at about 0.4×10^6 cells per well in a 6-well plate. After an overnight incubation, the two plasmids: pSce-I and pDR-GFP were transfected into the cells using jetPRIME transfection reagent (Polyplus transfection system, New York, NY). 3 hours after transfection, cells were treated with the AXL inhibitor (TP0903). Cells were harvested 48 hours later by trypsinization, resuspended in PBS and run through BC Gallios flow cytometer. Percentage of GFP positive cells was read as a measure of HR efficiency.

High-Throughput Survival Assay (HTSA)

Cells were seeded at a density of 2,000 cells/well in a 96-well plate and treated with the indicated concentration of a single drug or combination after 24 hours. Drug medium was changed every 72 hours for 7 days. Cells were then released into complete drug-free medium for 4 days, with medium being changed every 48 hours. On the day of harvest, 100 μ l per well of 2.5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) was added to serum-free media and allowed to incubate at 37°C for 3-4 hours. After incubation, media was removed and 100 μ l solubilization solution (0.04M HCl, 1% SDS, in isopropyl alcohol) was added to each well. Plates were lightly rocked at room temperature for 1 hour and read on a plate reader (Epoch Microplate Spectrophotometer, Gen 5 software, BioTek, Winooski, VT) at wavelength 590 nm. Combination indices were measured using calcsyn.

Clonogenic Assay

For clonogenic/colony-formation assay, 5,000 to 10,000 cells (depending on the plating efficiency of each cell line; data not shown) were plated in each well of 6-well plates, treated for 6 days, and allowed to recover for 6 days in the absence of drug. Cells were then washed with PBS and stained with a 0.5% crystal violet solution in 25% methanol for 10 minutes. Plates were then scanned to obtain pictures. The plates were then solubilized with crystal violet solubilization solution (0.1% sodium citrate in 50% ethanol), and absorbance was measured at 570 nm using the Epoch Microplate Spectrophotometer (BioTek Instruments,

Inc, Winooski, VT). Values were normalized to those of their no treatment controls and analyzed in GraphPad Prism.

RPPA and gene expression analysis of human patient tumors

The MDACC PROSPECT cohort (Profiling of Resistance patterns and Oncogenic Signaling Pathways in Evaluation of Cancers of the Thorax) of surgically resected NSCLC tumors was collected and profiled by reverse phase protein array (RPPA) as previously described (22). RPPA data from tumor cohorts for lung adenocarcinoma and lung squamous patients from The Cancer Genome Atlas (TCGA) were profiled similarly using previously established methods (8, 23-25). mRNA expression data for triple negative breast cancer (TNBC) generated on Affymetrix U133A microarray platform was downloaded from Gene Expression Omnibus (GEO, accession number GSE20194) (26, 27). The dataset consists of 230 stage I-III breast cancers from fine needle aspiration specimens of newly diagnosed breast cancers before any therapy. AXL levels (protein or mRNA) were correlated with the expression of other protein markers (lung and head and neck cancer) or genes (mRNA, breast cancer) by Pearson correlation. For this exploratory analysis, markers correlating with AXL at a corresponding False Discovery Rate (FDR) less than 5% and with a Spearman correlation of at least ± 0.35 were shown for each tumor type. For in vitro RPPA experiments, Analysis of Variance (ANOVA) was used to assess variation in RPPA levels among different treatment conditions. Data statistical analyses were preformed using R, a publically available statistical computing tool (<https://www.r-project.org/>).

Results

Direct role of AXL in regulating EMT

To examine the ability of AXL to directly regulate cell survival and EMT, AXL was stably downregulated in NSCLC and TNBC cell lines (at least 2 cell lines per tumor type). Western blot analysis shows that while AXL was differentially expressed in each cell line, infection of shRNA to AXL resulted in >70% downregulation of the protein (**Fig. 1A and Supp. Fig. 1A, 1B**) compared to scrambled control. Downregulation of AXL in both NSCLC and TNBC cell lines increased expression of apoptotic markers, cleaved PARP and cleaved caspase-7 (**Fig. 1A and Supp. Fig. 1A**). AXL knockdown also reduced cellular proliferation (**Fig. 1B**) over a 7-day period, as seen by the increase in doubling times in TNBC (**Supp. Table 1**). The doubling time of HCC1806 increased modestly (1.3 fold) in one of the stable knockdown model, while doubling time of SKLU1 increased by 3.3 fold upon AXL downregulation (**Supp. Table 1**). The greater effect in SKLU1 cell line can potentially be explained by higher levels of AXL expression in SKLU1 accompanied by a higher mesenchymal score (8). Next, the effect of AXL on EMT status was examined using the EMT transcription factors Twist, Slug, ZEB1 and mesenchymal markers N-cadherin and vimentin. Stable knockdown of AXL suppressed all of the EMT transcriptional factors and mesenchymal markers as measured by qRT-PCR in all the cell lines examined (**Fig. 1C**). Additionally, AXL knockdown led to a reversal of the EMT phenotype, seen by an increase in E-cadherin protein levels and decrease in ZEB1 protein levels (**Fig. 1D and Supp. Fig. 1C**). Finally, invasion through Matrigel was significantly decreased upon AXL downregulation in both TNBC (HCC1806) and NSCLC (SKLU1) cell lines (**Fig. 1E and**

Supp. Fig. 1D). Collectively, these studies suggest that the receptor tyrosine kinase AXL may be a driver of EMT in TNBC and NSCLC cell lines.

Next, we tested if treatment with an AXL inhibitor, TP0903 (28), could reverse TGF β -induced EMT in an epithelial cell line, H1993. Stimulation with TGF β alone resulted in increase in vimentin and N-cadherin gene expression, an increase in ZEB1 and AXL protein levels and a decrease in E-cadherin protein levels, showing induction of EMT with TGF β (**Supp. Fig. 1D, 1E**). AXL inhibition alone led to decrease in vimentin gene expression and decrease in ZEB-1 protein levels as seen previously. Interestingly, AXL inhibition resulted in reversal of TGF β -induced EMT seen by decrease in vimentin and N-cadherin gene expression ($p < 0.005$), increase in E-cadherin protein and decrease in ZEB1 protein levels, thus suppressing TGF β -induced EMT (**Supp. Fig. 1D, 1E**).

Regulation of DDR by AXL in NSCLC, breast, and head and neck cancers

Since EMT has been linked to DNA damage response (DDR) (29, 30) and AXL to radio-resistance (13, 31), we hypothesized that AXL inhibition may directly regulate DNA repair machinery such that cells will become sensitive to DNA damaging agents. To directly test this hypothesis, we measured the accumulation of DNA damage upon AXL inhibition and stable AXL knockdown across all three-cancer systems. NSCLC (SKLU1), TNBC (HCC1806) and HNSCC (584) cell lines were treated with a specific AXL inhibitor, TP0903 (25 nM) for 96 hours and DNA damage response was measured by γ H2AX staining of cells and reverse phase protein array (RPPA) analysis (**Supp. Table 2**) of major players involved in DNA repair, including DDR, mismatch repair and base excision repair proteins using cell lysates of treated cells. Results revealed that AXL inhibition led to an accumulation of DNA damage over a period of 96 hours (**Fig. 2A**), suggesting that AXL inhibition may interfere with an inherent cellular mechanism of DNA damage and repair. For example, we observed a significant increase in the percent cells exhibiting more than 10 γ H2AX foci per cell following treatment with TP0903 in all cell lines examined. Specifically, the percentage of cells exhibiting more than 10 foci/cell increased by 7 fold in HCC1806 ($p < 0.0001$), 1.5 fold in SKLU1 cells ($p < 0.0001$) and 6-fold in 584 ($p < 0.0001$) (**Fig. 2A**). Annexin staining of these cell lines in the presence of the AXL inhibitor showed only a marginal increase in apoptosis at 24, 48 and 96 hours of treatment, indicating that the increase in γ H2AX staining can be attributed to an increase in DNA damage rather than apoptosis (**Supp. Fig. 2A**). Further, RPPA analysis revealed a cancer-system dependent decrease in the protein levels of both AXL (data not shown) and DNA repair proteins following early time points (24h and 48h) of TP0903 treatment (**Fig. 2B**). For example, as early as 24 hours post TP0903 treatment, NSCLC cell lines showed a decrease in the protein levels of RAD50 (SKLU1) and RAD51 (Calu1), a protein directly involved in homologous recombination (HR) DNA repair (**Fig. 2B**, top panel, $p < 0.0001$). In TNBCs, there was a consistent decrease in the protein levels of c-Myc ($p < 0.0001$ HCC1806 and $p < 0.005$ MDA-MB-231), which is involved in the transcription of DNA repair proteins such as RAD51 (32) (**Fig. 2B**, middle panel). In HNSCC cell lines, there was a decrease in the protein levels of ATM (**Fig. 2B**, bottom panel, $p < 0.0001$ 1386-LN and $p < 0.005$ 584). Furthermore, treatment of cells with TP0903 also resulted in cancer type dependent alteration of DNA repair proteins. For example, in TNBC, AXL inhibition leads to a decrease in pCHK1, pATR and BRCA2, while

in NSCLCs inhibition of AXL leads to down regulation of MRE11, ATR and E2F and in HNSCC, levels of BAP1, XRCC1 and CHK1 are downregulated (**Supp. Fig. 2B**). Furthermore, knockdown of AXL in MDA-MB-157 (TNBC) and SKLU1 (NSCLC) cell lines resulted in a significant increase in γ H2AX foci in both cell lines (MDA-MB-157: $p<0.0001$, SKLU1: $p<0.001$), verifying the accumulation of DNA damage upon AXL downregulation (**Supp. Fig. 3A**). Lastly, using AXL stable knockdown cells, we show that RAD51 level (as measured by RPPA analysis) was significantly downregulated in TNBC ($p=0.01$ HCC1806, $p=0.028$ MDA-MB-157), HNSCC ($p=0.008$) and NSCLC ($p=2.2E-05$) cell lines (**Fig. 2C**).

We next interrogated if DNA repair genes are altered at the mRNA level following treatment (48 hours) with the AXL inhibitor, TP0903. Results revealed that the mRNA expression of key DNA repair proteins (RAD51, BRCA2, E2F1, RAD54L and BRCA1) decreased significantly at 48 hours post-treatment with 25 nM TP0903 in TNBC (MDA-MB-157, HCC1806), NSCLC (SKLU1) and HNSCC (584) cell lines (**Fig. 3A**). For example, RAD51 levels were reduced by 2-fold in MDA-MB-157 ($p<0.005$) and SKLU1 ($p<0.05$) and 2.5 fold in HCC1806 ($p<0.005$) and 584 ($p<0.05$) cell lines. Additionally, BRCA2 levels decreased by 2-fold decrease in MDA-MB-157 ($p<0.05$), 2.5 fold in HCC1806 and 584 ($p<0.005$ and <0.05 respectively) and almost ten-fold in SKLU1 ($p<0.0001$) cell lines. Fold decrease in E2F1 and RAD54L were subtler, albeit significant in the majority of cell lines examined. Lastly, BRCA1 levels did not show any significant changes in MDA-MB-157 and HCC1806, but decreased by 3-fold and 2-fold respectively in SKLU1 ($p<0.005$) and 584 ($p<0.0001$) cell lines. To examine the specificity of TP0903 for AXL, we measured the RNA levels of RAD51, BRCA2, E2F and RAD54L in cell lines stably knocked down for AXL, which showed a decrease in expression of the DNA repair genes in all the cells lines examined (**Fig. 3B and Supp. Fig. 3B**). Specifically, RAD51 gene expression decreased by 2-fold in AXL shRNA-infected MDA-MB-157 ($p<0.01$) and HCC1806 ($p<0.005$), 2.5 fold in SKLU1 and Calu1 ($p<0.05$) and by 3-fold in 584 ($p<0.005$) and 1386-LN ($p<0.0001$) cell lines. BRCA2 showed more significant changes in gene expression such as 10 fold in SKLU1 ($p<0.005$), 3-fold in MDA-MB-157 ($p<0.05$) and 2.5-fold in HCC1806 ($p<0.05$), Calu1 ($p<0.005$), 584 ($p<0.05$) and 1386-LN ($p<0.005$) upon AXL knockdown. We also observed a subtle yet significant decrease in gene expression of E2F1 and RAD54L in MDA-MB-157 and SKLU1 cell lines stably knocked down for AXL ($p<0.05$) (**Supp. Fig. 3B**).

We next examined the functional effect of AXL on HR mediated DNA repair by immunofluorescence staining of RAD51 and γ H2AX foci, following treatment with doxorubicin to induce DNA damage in the presence and absence of the AXL inhibitor. Results from the immunofluorescence assay showed a decrease in the percentage of cells exhibiting greater than 20 RAD51 foci upon AXL inhibitor treatment (25nM TP0903, 96 hours), despite increased accumulation of DNA damage, compared to control (**Fig. 3C, 3D**). For example, MDA-MB-157 cells showed a 4-fold decrease and SKLU1 cells showed a 2-fold decrease in the percent of cells exhibiting more than 20 RAD51 foci per cell ($p<0.05$) (**Fig. 3D**). The decrease in efficacy of HR mediated DNA repair in the presence of an AXL inhibitor was substantiated using fluorescent reporter constructs that enable sensitive and

quantitative measurement of baseline HR in cell lines. Results revealed that treatment of TNBC, NSCLC and HNSCC cell lines with the AXL inhibitor (25nM TP0903, 48 hours) led to 2-fold decrease in the efficiency of the cells to carry out HR-mediated DNA repair in MDA-MB-231 ($p<0.005$) and HCC1806 ($p<0.05$) cells and 2.5 fold decrease in SKLU1 and 584 ($p<0.0001$) cell lines (**Fig. 3E**). To further validate the effect of AXL on HR mediated DNA repair, we performed immunofluorescence of RAD51 in MDA-MB-157 cells stably knocked down for AXL, in the presence of doxorubicin. Knockdown of AXL significantly diminished (3.2 fold; $p=0.05$) the ability of MDA-MB-157 cells to form RAD51 foci despite the induction of DNA damage by doxorubicin (**Fig. 3F, 3G**).

Inhibition of AXL sensitizes cells to PARP inhibition

Since AXL inhibition leads to an increase in accumulation of DNA damage by compromising homologous recombination (HR) in all the three cancer types examined, we next hypothesized that combination therapy of an AXL inhibitor with agents that target an alternate DNA repair pathway would lead to synthetic lethality. Defects in HR-mediated DNA repair have been shown to sensitize cells to PARP inhibition, which interferes with single stranded DNA repair mechanism (33). We thus reasoned that AXL inhibition might sensitize cells to PARP inhibitor treatment. To test this hypothesis we treated TNBC (MDA-MB-157), NSCLC (SKLU1) and HNSCC (584) cell lines with increasing concentrations of AXL inhibitor (TP0903), PARP inhibitor (olaparib) or a combination of the two drugs and determined if the combination was synergistic, additive, or antagonistic using isobologram kinetics. Treatment of TNBC, NSCLC and HNSCC mesenchymal cell lines with the combination of olaparib and TP0903 resulted in a significant growth inhibition (at TP0903 concentrations of 25nM or higher) compared to treatment with the single inhibitors alone (**Fig. 4A, 4C and Supp. Fig. 4A**). Combination index (CI) generated from isobologram plots indicated synergy in mesenchymal cell lines from all three cancer systems ($CI<1$ for all) (**Fig. 4A and Supp. Fig. 4A**). In contrast, the epithelial cell lines H1933 (NSCLC) and HN5 (HNSCC) showed minimal to no synergy across all of the concentrations of the AXL inhibitor in combination with olaparib ($CI>1$) (**Fig. 4B and Supp. Fig. 4B**). Similarly, MCF10A, an immortalized human breast epithelial cell line used here as a negative control, was unresponsive to olaparib as a single agent or in combination with TP0903 (**Fig 4B, 4C and Supp. Fig. 4B**). Cell counting following treatment with the AXL and PARP inhibitors as single agents or in combination for 7 days, showed a greater decrease in cell numbers with the combination compared to the individual inhibitors alone in HCC1806, MDA-MB-157, SKLU1 and 584 cell lines (**Supp. Fig. 4C**). While treatment with olaparib alone resulted in little to no difference in cell numbers compared to control, treatment with AXL inhibitor (25nM TP0903) led to a 3-5 fold decrease in cell numbers. Combining olaparib with the AXL inhibitor led to a further and significant decrease in cell numbers in all the four cell lines (**Supp. Fig. 4C**). To further confirm the synergy between AXL and PARP inhibitors, we carried out a clonogenic colony formation assay with MDA-MB-157 and SKLU1 cell lines. Results showed a significant decrease in colony formation with combined treatment of PARP inhibitor (0.5 μ M olaparib) and AXL inhibitor (25nM TP0903) ($p<0.005$ and $p<0.05$ respectively) (**Fig. 4D**). While treatment with the PARP or AXL inhibitor alone led to a 40-60% increase in colony formation, the combination led to a >80% increase in colony formation compared to control (**Fig. 4D - right**).

To further validate our finding that AXL inhibition caused synthetic lethality with PARP inhibitors, we tested the combination of the PARP inhibitor, olaparib with another highly selective AXL inhibitor, R428 (also called BGB324). Similar to TP0903, the combination of R428 and olaparib was also highly synergistic, yielding a combination index of <1 in MDA-MB-157 (TNBC) and SKLU1 (NSCLC) cell lines (**Fig. 4E**).

We next validated if the key DNA repair/damage proteins identified by RPPA from cells treated with single-agent TP0903 (Figure 2) were also modulated by the combination treatment with TP0903 and olaparib. Western blot analysis revealed that treatment of three cell lines, MDA-MB-157 (TNBC), SKLU1 (NSCLC) and 584 (HNSCC) with AXL inhibitor alone (25, 50 and 100 nM TP0903) led to a concentration-dependent decrease in protein levels of AXL, RAD51, MRE11, E2F1 and c-Myc. However, treatment with PARP inhibitor alone (1 uM olaparib) had minor effects on these proteins (**Fig. 4F and Supp. Fig. 4D**). Notably, when the AXL inhibitor was combined with olaparib, there was a cumulative increase in the levels of cleaved PARP, indicating that the combination of AXL and PARP inhibitors induce apoptosis (**Fig. 4F and Supp. Fig. 4D**). Since all pharmacologic agents can have off target effects, we examined the specificity of the synergism between AXL and PARP inhibitors by interrogating whether stable knockdown of AXL (in lieu of pharmacologic inhibition) was sufficient to sensitize cells to the PARP inhibitor. Similar to the AXL inhibitors, cell counting assay showed that stable AXL knockdown sensitized cells to the PARP inhibitor across all the three cancer systems (HCC1806, SKLU1 and 584 cell lines) ($p < 0.05$) (**Fig. 4G**). Moreover, stable knockdown of AXL in MDA-MB-157 and SKLU1 cells, and AXL KD in combination with PARP inhibitor (1uM olaparib) treatment diminished protein levels of RAD51, E2F1 and MRE11, similar to TP0903 treatment (**Supp. Fig. 4E**).

We next interrogated the biological effects of AXL (25 nM TP0903) and PARP inhibitors (0.5 uM olaparib) as single agents or in combination (TP0903+olaparib), by examining apoptosis using Annexin-V staining and cell cycle analysis. The AXL inhibitor alone resulted in 20-40% Annexin V positivity, while single agent PARP inhibitor did not cause significant apoptosis in TNBC, NSCLC and HNSCC cell lines (**Fig. 5A**). In contrast, the combination caused a much larger increase in the percentage of Annexin-V positive cells (between 30-80% with the combination, versus 10-50% with either drug alone, depending on the cell line) following a 7-day treatment interval in mesenchymal cell lines in all three cancer types (**Fig. 5A**). Examination of sub-G1 population in the treated cells also showed a significant increase in the percentage of cells at the sub-G1 stage when treated with the combination, but not with single agents alone. (**Fig. 5B, 5C and Supp. Fig 5**). Further, an increase in G2/M phase of the cell cycle was also observed with the combination treatment in the mesenchymal cell lines, HCC1806, SKLU1 and 584 (**Fig. 5C and Supp. Fig 5**). To decipher if the increase in apoptosis and changes in cell cycle mediated by the combination is specific to mesenchymal cell lines, we treated MCF-10A and H1993 cell lines with AXL and PARP inhibitors, either alone or in combination and measured apoptosis and cell cycle profile. Results revealed that neither MCF10A nor H1993 cell lines showed an increase in Annexin-V positivity, increase in percent sub-G1 cell population or change in G2/M population when treated with the combination compared to the co treatment or single

inhibitors controls, showing specificity of response to the combination treatment in the mesenchymal cell lines that express high AXL (**Fig. 5A-5C and Supp. Fig. 5C**). To validate the increase in apoptosis and cell cycle changes seen with the combination treatment, we used cells stably knocked down for AXL and treated them with olaparib. AXL knockdown alone caused a significant increase in apoptosis compared to the control cells containing a scramble shRNA (**Fig. 5D**). This was further enhanced in the presence of olaparib in MDA-MB-157 and SKLU1 cells, confirming the synergy between inhibition of AXL and PARP ($p<0.005$) (**Fig. 5D**). Further, treatment of MDA-MB-157 and SKLU1 AXL knockdown cells with olaparib led to a moderate increase in sub-G1 population and an increase in G2/M compared to Scrambled and no treatment AXL KD cells (**Fig. 5E**). Collectively, the data presented recognize AXL as a targetable mediator of DNA damage repair and demonstrate a synergistic response with AXL and PARP inhibition in mesenchymal tumors.

Association between AXL expression and DDR in patient tumors

Based on our *in vitro* findings, we then investigated the relationship between the expression of AXL and DNA repair genes in tumors from NSCLC, HNSCC, and breast cancer patients. Specifically, we analyzed gene (microarray) or protein expression (RPPA) profiles from 4 different patient cohorts NSCLC (NSCLC-MDACC PROSPECT N=140, TCGA LUSC N=112), HNSCC (protein) (TCGA HNSCC, N=200), TNBC (mRNA) (MD Anderson N=230) and the TCGA LUAD (protein) (N=181) (**Supp. Fig. 6**). Molecular profiling revealed that AXL expression associates positively with survival mechanisms linked with cellular stress such as DNA repair (RAD51, BRCA2, MSH2, MRE11, XRCC-1, ATR) and negatively with apoptosis in all patient cohorts (**Fig. 6A-6D and Supp. Fig. 6, 7A, 7B**) (FDR<5% and Spearman rho value greater than ± 0.35). In NSCLCs, AXL expression correlated positively with DNA repair proteins, including proteins such as RAD51, MRE11, XRCC1 and BRCA2 (**Fig. 6A and Supp. Fig. 7A**). In lung squamous cell carcinoma (LUSC), positive correlations were seen with BRCA2, RAD51, E2F1 and X53BP1, which are shown to facilitate DNA repair (**Fig. 6B and Supp. Fig. 7A**). With HNSCC, a consistent positive association was seen with RAD51, BRCA2, MRE11 and E2F1 (**Fig. 6C**). These findings were then validated in TNBC by correlating AXL mRNA levels with those markers identified in the lung and HNSCC cohorts. In TNBCs, AXL gene expression with was positively associated with ATM, CHEK2, BRCA2 and MRE11, substantiating the positive association of AXL with major mediators of DNA repair in cancer cells (**Fig. 6D**). This observation that high AXL expression is associated with increased expression of DNA repair genes across multiple tumor types is consistent with our *in vitro* results that indicate a direct role for AXL in regulating DNA repair.

Discussion

AXL has been established as one of the major players mediating intrinsic and acquired resistance to anti-cancer drugs. For example, AXL expression was shown to be higher in patients with drug-resistant leukemia and AXL was induced by chemotherapy drugs in leukemia cell lines (34) and by cisplatin in non-small cell lung cancer cells (35). Several studies by our group and others have shown the involvement of AXL in causing EMT-associated resistance to targeted therapy such as EGFR inhibitors and PI3K inhibitors (8)

(36). In these studies, inhibition of AXL in the mesenchymal cancer cells re-sensitized these cells to either chemotherapy or targeted therapy (8, 9, 16, 36). Further, the relationship between EMT and DNA repair pathways has been recently observed in several systems. For example, the DNA repair protein ATM has been shown to stabilize ZEB1, which in turn stabilizes CHK1 (37). In another study, radioresistant prostate cancer cells have an increased ability to form colonies, spheroids and invade, indicating that the EMT phenotype is linked to upregulation of DNA repair pathways (38).

AXL is a receptor tyrosine kinase that is enriched in mesenchymal tumors and a key marker of EMT, but its functional role in EMT has not yet been demonstrated. While AXL's role in cisplatin resistance and radio-resistance was established in esophageal, lung and head and neck cancer types, the mechanism remains unknown (13, 14). In this study, we utilized stable knockdown systems and high-throughput proteomics to demonstrate a novel relationship between AXL and DNA repair proteins that are crucial for homologous recombination (HR)-mediated DNA repair. These results establish for the first time, a link between AXL and DNA repair, and provide evidence for AXL inhibition mediated sensitization to PARP inhibitor therapy.

We first show that AXL can directly regulate EMT using a stable knockdown system, which revealed an increase in E-cadherin and decrease in mesenchymal factors with AXL downregulation. Similar assays in AXL downregulated HNSCC cell lines produced very similar results (data not shown). Thus, the effect of AXL on EMT transcription factors and EMT-mediated cellular behavior was seen to be consistent across all cells lines examined in this study belonging to the three cancer types (i.e. TNBC, NSCLC, HNSCC), implicating AXL as a mediator of EMT. Furthermore, AXL also suppressed TGF β -induced EMT, suggesting a role for AXL downstream of TGF β , and this warrants future investigation.

Functionally, we have unveiled that AXL inhibition can lead to decrease in mRNA levels of genes, which, when expressed, play a major role in HR DNA repair. As a result, inhibition of AXL can hinder the HR DNA repair process. Thus, a state of "HR deficiency" is created when AXL is inhibited or downregulated (**Supp. Fig. 7C**). Hence combination with PARP inhibitors, which are known to affect base excision repair, results in accumulation of DNA damage, leading to apoptosis (**Fig. 6E**). Interestingly, at early time points, the DNA repair genes that are downregulated seem cancer-system or cell line specific. However, over 4 days of treatment, RAD51, MRE11 and E2F1 seem commonly downregulated across all the cancer systems. It is notable that c-Myc and E2F1 are known transcription factors that promote expression of genes involved in G2/M and S-phase transitions (DNA replication and repair) such as RAD51, RAD54L, CHK1 and RPA3 (23, 32, 39).

Moreover, the link between AXL and DNA repair proteins in treatment-naive human patient tissue samples supports the physiological significance of this connection. The state of HR deficiency that is induced by AXL inhibition provides rationale to effectively combine them with drugs affecting an alternate DNA repair pathway, including PARP inhibitors. This study provides preclinical evidence that this combination of AXL and PARP inhibitors is synergistic, elicits a synthetic lethal effect and induces apoptosis specifically in the cancer cells that express high AXL. Thus, the EMT signature previously developed by our group,

which also includes AXL as a mesenchymal marker, could potentially be used as a biomarker, to identify which tumors will be the most susceptible to the combination treatment.

Collectively, this study has opened up a possibility of a novel therapeutic strategy involving inhibitors that affect two different cellular pathways to which cancer cells are addicted for survival and to mediate drug resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Institutes of Health through a Cancer Center Support Grant to MD Anderson, CA016672 and grants CA87548 and CA1522218 to K. Keyomarsi; through Susan G. Komen for the Cure grant KG100521 to K.K. Hunt; through LUNgevity Foundation, MDACC Physician Scientist Program, MDACC R. Lee Clark Fellow Award and The Sheikh Khalifa Bin Zayed Al Nahyan Institute for the Personalized Cancer Therapy's (IPCT's) Center for Professional Education and Training to LA Byers. KB was supported in part by The University of Texas SPORE in Lung Cancer, 5P50 CA070907-10. SV was supported in part by the Cancer Prevention and Research Institute of Texas (CPRIT) Training grant RP140106.

References

1. Lemke G. Biology of the TAM receptors. *Cold Spring Harb Perspect Biol.* 2013; 5:a009076. [PubMed: 24186067]
2. Zhang YX, Knyazev PG, Cheburkin YV, Sharma K, Knyazev YP, Orfi L, et al. AXL is a potential target for therapeutic intervention in breast cancer progression. *Cancer research.* 2008; 68:1905–15. [PubMed: 18339872]
3. Li Y, Ye X, Tan C, Hongo JA, Zha J, Liu J, et al. Axl as a potential therapeutic target in cancer: role of Axl in tumor growth, metastasis and angiogenesis. *Oncogene.* 2009; 28:3442–55. [PubMed: 19633687]
4. Koorstra JB, Karikari CA, Feldmann G, Bisht S, Rojas PL, Offerhaus GJ, et al. The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. *Cancer biology & therapy.* 2009; 8:618–26. [PubMed: 19252414]
5. Reichl P, Dengler M, van Zijl F, Huber H, Fuhrlinger G, Reichel C, et al. Axl activates autocrine transforming growth factor-beta signaling in hepatocellular carcinoma. *Hepatology.* 2015; 61:930–41. [PubMed: 25251599]
6. O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, et al. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. *Molecular and cellular biology.* 1991; 11:5016–31. [PubMed: 1656220]
7. Asiedu MK, Beauchamp-Perez FD, Ingle JN, Behrens MD, Radisky DC, Knutson KL. AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene.* 2014; 33:1316–24. [PubMed: 23474758]
8. Byers LA, Diao L, Wang J, Saintigny P, Girard L, Peyton M, et al. An epithelial mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2013; 19:279–90. [PubMed: 23091115]
9. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, et al. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nature genetics.* 2012; 44:852–60. [PubMed: 22751098]
10. Mak MP, Tong P, Diao L, Cardnell RJ, Gibbons DL, William WN, et al. A Patient-Derived, Pan-Cancer EMT Signature Identifies Global Molecular Alterations and Immune Target Enrichment

- Following Epithelial-to-Mesenchymal Transition. *Clin Cancer Res.* 2016; 22:609–20. [PubMed: 26420858]
11. Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. *Cancer research.* 2009; 69:6871–8. [PubMed: 19671800]
  12. Dufies M, Jacquell A, Belhacene N, Robert G, Cluzeau T, Luciano F, et al. Mechanisms of AXL overexpression and function in Imatinib-resistant chronic myeloid leukemia cells. *Oncotarget.* 2011; 2:874–85. [PubMed: 22141136]
  13. Brand TM, Iida M, Stein AP, Corrigan KL, Braverman CM, Coan JP, et al. AXL Is a Logical Molecular Target in Head and Neck Squamous Cell Carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2015; 21:2601–12. [PubMed: 25767293]
  14. Hong J, Peng D, Chen Z, Sehdev V, Belkhir A. ABL regulation by AXL promotes cisplatin resistance in esophageal cancer. *Cancer research.* 2013; 73:331–40. [PubMed: 23117882]
  15. Macleod K, Mullen P, Sewell J, Rabiasz G, Lawrie S, Miller E, et al. Altered ErbB receptor signaling and gene expression in cisplatin-resistant ovarian cancer. *Cancer research.* 2005; 65:6789–800. [PubMed: 16061661]
  16. Wilson C, Ye X, Pham T, Lin E, Chan S, McNamara E, et al. AXL Inhibition Sensitizes Mesenchymal Cancer Cells to Antimitotic Drugs. *Cancer research.* 2014; 74:5878–90. [PubMed: 25125659]
  17. Nanos-Webb A, Jabbour NA, Multani AS, Wingate H, Oumata N, Galons H, et al. Targeting low molecular weight cyclin E (LMW-E) in breast cancer. *Breast cancer research and treatment.* 2012; 132:575–88. [PubMed: 21695458]
  18. Jabbour-Leung NA, Chen X, Bui T, Jiang Y, Yang D, Vijayaraghavan S, et al. Sequential Combination Therapy of CDK Inhibition and Doxorubicin Is Synthetically Lethal in p53-Mutant Triple-Negative Breast Cancer. *Mol Cancer Ther.* 2016; 15:593–607. [PubMed: 26826118]
  19. Zhao M, Sano D, Pickering CR, Jasser SA, Henderson YC, Clayman GL, et al. Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2011; 17:7248–64. [PubMed: 21868764]
  20. Duong MT, Akli S, Wei C, Wingate HF, Liu W, Lu Y, et al. LMW-E/CDK2 deregulates acinar morphogenesis, induces tumorigenesis, and associates with the activated b-Raf-ERK1/2-mTOR pathway in breast cancer patients. *PLoS genetics.* 2012; 8:e1002538. [PubMed: 22479189]
  21. Nakanishi K, Cavallo F, Brunet E, Jasin M. Homologous recombination assay for interstrand cross-link repair. *Methods in molecular biology.* 2011; 745:283–91. [PubMed: 21660700]
  22. Cardnell RJ, Behrens C, Diao L, Fan Y, Tang X, Tong P, et al. An Integrated Molecular Analysis of Lung Adenocarcinomas Identifies Potential Therapeutic Targets among TTF1-Negative Tumors, Including DNA Repair Proteins and Nrf2. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2015; 21:3480–91. [PubMed: 25878335]
  23. Cancer Genome Atlas Research N. Comprehensive molecular profiling of lung adenocarcinoma. *Nature.* 2014; 511:543–50. [PubMed: 25079552]
  24. Hu J, He X, Baggerly KA, Coombes KR, Hennessy BT, Mills GB. Non-parametric quantification of protein lysate arrays. *Bioinformatics.* 2007; 23:1986–94. [PubMed: 17599930]
  25. Neeley ES, Baggerly KA, Kornblau SM. Surface Adjustment of Reverse Phase Protein Arrays using Positive Control Spots. *Cancer informatics.* 2012; 11:77–86. [PubMed: 22550399]
  26. Shi L, Campbell G, Jones WD, Campagne F, Wen Z, Walker SJ, et al. The MicroArray Quality Control (MAQC)-II study of common practices for the development and validation of microarray-based predictive models. *Nature biotechnology.* 2010; 28:827–38.
  27. Popovici V, Chen W, Gallas BG, Hatzis C, Shi W, Samuelson FW, et al. Effect of training-sample size and classification difficulty on the accuracy of genomic predictors. *Breast cancer research : BCR.* 2010; 12:R5. [PubMed: 20064235]
  28. Feneyrolles C, Spenlinhauer A, Guet L, Fauvel B, Dayde-Cazals B, Warnault P, et al. Axl kinase as a key target for oncology: focus on small molecule inhibitors. *Molecular cancer therapeutics.* 2014; 13:2141–8. [PubMed: 25139999]

29. Zhang P, Wei Y, Wang L, Debeb BG, Yuan Y, Zhang J, et al. ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. *Nat Cell Biol.* 2014; 16:864–75. [PubMed: 25086746]
30. Sayan AE, Griffiths TR, Pal R, Browne GJ, Ruddick A, Yagci T, et al. SIP1 protein protects cells from DNA damage-induced apoptosis and has independent prognostic value in bladder cancer. *Proceedings of the National Academy of Sciences of the United States of America.* 2009; 106:14884–9. [PubMed: 19706487]
31. Cheng P, Phillips E, Kim SH, Taylor D, Hielscher T, Puccio L, et al. Kinome-wide shRNA screen identifies the receptor tyrosine kinase AXL as a key regulator for mesenchymal glioblastoma stem-like cells. *Stem cell reports.* 2015; 4:899–913. [PubMed: 25921812]
32. Luoto KR, Meng AX, Wasylishen AR, Zhao H, Coackley CL, Penn LZ, et al. Tumor cell kill by c-MYC depletion: role of MYC-regulated genes that control DNA double-strand break repair. *Cancer research.* 2010; 70:8748–59. [PubMed: 20940401]
33. Dedes KJ, Wilkerson PM, Wetterskog D, Weigelt B, Ashworth A, Reis-Filho JS. Synthetic lethality of PARP inhibition in cancers lacking BRCA1 and BRCA2 mutations. *Cell cycle.* 2011; 10:1192–9. [PubMed: 21487248]
34. Hong CC, Lay JD, Huang JS, Cheng AL, Tang JL, Lin MT, et al. Receptor tyrosine kinase AXL is induced by chemotherapy drugs and overexpression of AXL confers drug resistance in acute myeloid leukemia. *Cancer letters.* 2008; 268:314–24. [PubMed: 18502572]
35. Kurokawa M, Ise N, Omi K, Goishi K, Higashiyama S. Cisplatin influences acquisition of resistance to molecular-targeted agents through epithelial-mesenchymal transition-like changes. *Cancer science.* 2013; 104:904–11. [PubMed: 23566288]
36. Scaltriti M, Elkabets M, Baselga J. Molecular Pathways: AXL, a Membrane Receptor Mediator of Resistance to Therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2016; 22:1313–7. [PubMed: 26763248]
37. Yang Y, Ahn YH, Chen Y, Tan X, Guo L, Gibbons DL, et al. ZEB1 sensitizes lung adenocarcinoma to metastasis suppression by PI3K antagonism. *J Clin Invest.* 2014; 124:2696–708. [PubMed: 24762440]
38. Chang L, Graham PH, Hao J, Ni J, Bucci J, Cozzi PJ, et al. Acquisition of epithelial mesenchymal transition and cancer stem cell phenotypes is associated with activation of the PI3K/Akt/mTOR pathway in prostate cancer radioresistance. *Cell death & disease.* 2013; 4:e875. [PubMed: 24157869]
39. Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA, et al. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes & development.* 2002; 16:245–56. [PubMed: 11799067]

Implications

The novel role for AXL in DNA repair, linking it to EMT suggests that AXL can be an effective therapeutic target in combination with targeted therapy such as PARP inhibitors in several different malignancies.

Figure 1. AXL is a direct mediator of EMT

A) TNBC and NSCLC cell lines infected with scramble shRNA (Scr) or AXL-specific shRNA (sh#1 and sh#2) and subjected to immunoblot analysis of AXL and apoptosis markers, cleaved PARP and cleaved caspase-7. Actin was used as a loading control. **B)** HCC1806 (TNBC) and SKLU1 (NSCLC) cell lines infected with scramble shRNA or AXL shRNA and counted (with trypan blue for viability) over the indicated time points. Data represent average of cell numbers from three independent experiments. Doubling times of the cells were calculated and presented in Supp. Table 1. **C)** TNBC (MDA-MB-157 and HCC1806) and NSCLC cell lines (Calu1 and SKLU1) infected with scramble shRNA (Scr) or AXL-specific shRNA (AXL KD) and subjected to qRT-PCR analysis for EMT

transcription factors, Slug, Twist, ZEB1 and mesenchymal markers, vimentin and N-cadherin. GAPDH was used as loading control for normalization. Data is representative of three independent experiments, each performed in triplicate. **D)** MDA-MB-157 (TNBC) cells infected with scramble shRNA (Scr) or AXL-specific shRNA (sh#1 and sh#2) and immunoblotted for AXL and EMT markers, E-cadherin and ZEB-1. Actin was used as a loading control. **E)** HCC1806 (TNBC) and SKLU1 (NSCLC) cell lines infected with scramble or AXL shRNA and plated in Matrigel coated Boyden chambers. Invasion was measured by crystal violet staining over a 24 hour time point as described in the Methods section and quantified.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. AXL inhibition causes DNA damage and reduces levels of DNA repair markers

A (Left): HCC1806 (TNBC), SKLU1 (NSCLC) and 584 (HNSCC) cell lines were treated with 25 nM of the AXL inhibitor (TP0903) for 96 hours, following which they were fixed and stained for γ H2AX. Data represents percentage cells exhibiting >10 γ H2AX foci measured using Slidebook. **(Right):** Images corresponding to γ H2AX staining in DMSO or 25 nM TP0903 treated cells. Blue: DAPI, Green: γ H2AX. **B)** Reverse phase protein array (RPPA) analysis corresponding to the indicated DNA repair markers in TNBC, NSCLC and HNSCC cell lines, which were treated with vehicle (DMSO) or 25 nM TP0903 for 24 or 48

hours. $n=3$, $FDR<0.05$. **C)** Reverse phase protein array (RPPA) analysis of RAD51 expression in scramble-shRNA or AXL-shRNA infected cell lines. Data represent average from three independent biological replicates; $p<0.05$, $FDR<0.05$, $n=3$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. AXL inhibition diminishes homologous recombination (HR)

A) TNBC (MDA-MB-157 and HCC1806), NSCLC (SKLU1) and HNSCC (584) cell lines were treated with vehicle or 25 nM TP0903 for 48 hours and subjected to qRT-PCR analysis of the indicated HR genes. Data is representative of three independent experiments, each performed in triplicate. **B)** TNBC (MDA-MB-157 and HCC1806), NSCLC (SKLU1 and Calu1) and HNSCC (584 and 1386-LN) cell lines infected with scramble or AXL shRNA and subjected to qRT-PCR analysis to measure RNA levels of the HR genes, RAD51 and BRCA2. Data is representative of three independent experiments each performed in

triplicate **C, D**) Images (C) corresponding to formation of RAD51 and γ H2AX foci in TNBC (MDA-MB-157) and NSCLC (SKLU1) following treatment with doxorubicin and DMSO or 25 nM TP0903 for 96 hours (red: RAD51, blue: DAPI, green: γ H2AX) and its quantification (percentage of cells containing >20 RAD51 foci) (D). Data is representative of three independent experiments. **E**) TNBC (MDA-MB-157 and HCC1806), NSCLC (SKLU1) and HNSCC (584) cell lines, transfected with the two-plasmid system (DR-GFP and pSceI) were treated with vehicle or 25 nM TP0903 for 48 hours. HR efficiency assay was performed on the cell lines as described in the Methods section. Data represents fold-decrease in GFP+ cells in 25 nM TP0903 treated cells compared to DMSO treated cells from three independent experiments. **F, G**) Images (F) corresponding to formation of RAD51 foci in TNBC (MDA-MB-157) and NSCLC (SKLU1) following infection with Scrambled or AXL shRNA and treatment with doxorubicin for 48 hours (red: RAD51, blue: DAPI); G) Quantification of RAD51 foci (percentage of cells containing >5 foci calculated from 3 fields with min of 75 cells per field).

Figure 4. AXL inhibition is synergistic with PARP inhibition

A) Combination index calculated using CalcuSyn for the AXL and PARP inhibitors (TP0903 and olaparib) in the indicated mesenchymal cell lines. Data is representative of three independent experiments. **B)** Combination index calculated in epithelial cell lines using CalcuSyn for the AXL and PARP inhibitors (TP0903 and olaparib). Data is representative of three independent experiments. **C)** High throughput survival assay (HTSA - Methods) showing concentration dependent proliferation curves in the TNBC (MDA-MB-157), NSCLC (SKLU1) and HNSCC (584) cell lines. MCF10A cells were used as a normal

mammary epithelial cell control. Cells were treated with single agent (TP0903 or olaparib) or indicated concentration of the drug combinations. Data represents average of three independent experiments. **D).** Clonogenic assay in MDA-MB-157 (157) and SKLU1 cells treated with TP0903, olaparib or combination of TP0903 and olaparib for 7 days and recovery in drug free media for 5 days. (Right) Quantification of the clonogenic assay in the indicated cell lines. **E)** Combination index calculated from HTSA performed in TNBC (MDA-MB-157) and NSCLC (SKLU1) cell lines, following treatment with the AXL inhibitor, R428 alone, 1 μ M olaparib or the combination of R428 and olaparib. **F)** TNBC (MDA-MB-157 and HCC1806), NSCLC (SKLU1) and HNSCC (584) cell lines were treated with vehicle, 25 nM TP0903, 1 μ M olaparib or the combination of both TP0903 and olaparib for 7 days. Cells were lysed and immunoblot analysis was performed for markers of DNA repair (RAD51, MRE11, E2F1, c-Myc) and apoptosis (cleaved PARP). Actin was used as a loading control. **G.** Scramble or AXL shRNA infected TNBC (MDA-MB-157), NSCLC (SKLU1) and HNSCC (584) cell lines were treated with DMSO or 1 μ M olaparib and cell number was counted on day 7. Data is representative of normalized mean cell number from three independent experiments.

Figure 5. AXL inhibition in combination with PARP inhibition causes apoptosis

A) AnnexinV +ve PI+ve population measured in the indicated cell lines treated with DMSO, 25 nM TP0903, 0.5 μ M olaparib or 25 nM TP0903+0.5 μ M olaparib. Data is representative of three independent experiments. **B)** Sub-G0 population measured by propidium iodide staining in the indicated cell lines treated with DMSO, 25 nM TP0903, 0.5 μ M olaparib or 25 nM TP0903+0.5 μ M olaparib. Data is representative of three independent experiments. **C)** Cell cycle analysis from three independent experiments measured by flow cytometry in the indicated cell lines treated with DMSO, 25 nM TP0903, 0.5 μ M olaparib or 25 nM TP0903+0.5 μ M olaparib. **D)** Control (Scr) and AXL-shRNA (AXL KD) infected MDA-MB-157 and SKLU1 cells were treated with DMSO or 0.5 μ M olaparib and Annexin-V+ve / PI+ve population was measured. Data is representative of three independent experiments. **E)** Cell cycle analysis from three independent experiments measured by flow cytometry in

MDA-MB-157 and SKLU1 cells infected with control (Scr) or AXL-shRNA (AXL KD) and treated with DMSO or 0.5 μ M olaparib. Data is representative of three independent experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. AXL expression corresponds to expression of DNA repair proteins and EMT markers in patient cohorts

A-C) Correlation analysis of AXL protein with other protein markers in NSCLC PROSPECT cohort (A), the TCGA LUSC cohort (B) and the TCGA HNSCC cohorts (C). Heat maps show the top significant markers that correlate with AXL expression (FDR<5%, Spearman Rho value > +/- 0.35). **D)** Correlation analysis of AXL gene expression with other genes in the breast cancer patient cohort, with heat map showing the top significant markers that correlate with AXL expression. **E)** Model depicting how AXL inhibition causes HR deficiency and synthetic lethality with PARP inhibitor. In mesenchymal cell lines and

tissues, AXL expression leads to higher expression of HR DNA repair proteins, facilitating higher HR-DNA repair efficiency, which is inhibited in the presence of an AXL inhibitor (TP0903). This makes the cells sensitive to inhibition of an alternate DNA repair pathway that utilizes PARP (base excision repair). Thus, a synergy occurs when AXL and PARP are inhibited simultaneously, leading to accumulation of DNA damage and apoptotic cell death.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript