

AXL Regulates Neuregulin1 Expression Leading to Cetuximab Resistance in Head and Neck Cancer

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Abstract

Background

The receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR) is overexpressed and an important therapeutic target in Head and Neck cancer (HNC). Cetuximab is currently the only EGFR-targeting agent approved by the FDA for treatment of HNC; however, intrinsic and acquired resistance to cetuximab is a major problem in the clinic. Our lab previously reported that AXL leads to cetuximab resistance via activation of HER3.

Methods

In this study, we investigate the connection between AXL, HER3, and neuregulin1 (NRG1) gene expression with a focus on understanding how their interdependent signaling promotes resistance to cetuximab in head and neck cancer. Plasmid or siRNA transfections, cell proliferation assays, and clonogenic assays were conducted to test cetuximab sensitivity. Quantitative PCR and immunoblot analysis were used to analyze gene expression levels. Seven HNC patient-derived xenografts (PDXs) were evaluated for protein expression levels.

Results

We found that HER3 expression was necessary but not sufficient for cetuximab resistance without AXL expression. Our results demonstrated that addition of the HER3 ligand NRG1 to cetuximab-sensitive HNC cells leads to cetuximab resistance. Further, AXL-overexpressing cells regulate NRG1 at the level of transcription, thereby promoting cetuximab resistance. Immunoblot analysis revealed that NRG1 expression was relatively high in cetuximab-resistant HNC PDXs compared to cetuximab-sensitive HNC PDXs. Finally, genetic inhibition of NRG1 resensitized AXL-overexpressing cells to cetuximab.

Conclusions

The results of this study indicate that AXL may signal through HER3 via NRG1 to promote cetuximab resistance and that targeting of NRG1 could have significant clinical implications for HNC therapeutic approaches.

Background

Head and neck squamous cell carcinomas (HNSCC) develop from the mucosal lining of the aerodigestive tract. It is estimated that in 2020 there will be over 53,000 new cases of HNSCC in the United States and 11,000 deaths from this disease [1]. The therapy regimen used to treat this cancer typically involves surgery, radiotherapy, chemotherapy, targeted therapy, and/or immunotherapy [2]. One targeted therapy

that is commonly used to treat HNSCC is a monoclonal antibody called cetuximab. Cetuximab targets the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK) that is overexpressed in HNSCC [2, 3]. RTKs like EGFR transmit extracellular signals to activate intracellular signaling cascades that stimulate growth, proliferation, and survival of cells [3]. Targeting of EGFR with cetuximab has improved overall survival of HNSCC patients when added to radiotherapy or chemotherapy regimens [4, 5]. Despite the clinical benefit of cetuximab treatment, resistance to cetuximab can develop in patients. Thus, investigation of resistant mechanisms can provide new insights to improving treatment strategies in HNSCC. Previous studies by our lab have shown that other RTKs like AXL or HER3 can play important roles in mediating resistance to cetuximab and that targeting of AXL or HER3 can resensitize to cetuximab treatment [6, 7].

Based on our previous studies of AXL and HER3 in the context of cetuximab resistance, our lab investigated the connection between these two receptors with a focus on understanding their interdependent signaling to promote resistance to cetuximab. A model of AXL overexpression revealed that AXL leads to cetuximab resistance by activation of HER3, but genetic overexpression of HER3 is insufficient for cetuximab resistance. However, exogenous expression of the HER3 ligand neuregulin1 (NRG1) lead to cetuximab resistance. Further experimentation revealed that AXL can regulate NRG1, at the level of mRNA, to promote cetuximab resistance. Other cetuximab resistant models have relatively high AXL and NRG1 expression and targeting of NRG1 in these models is sufficient to overcome cetuximab resistance. Collectively, this data indicates that AXL can signal through HER3 via NRG1 to promote cetuximab resistance and that targeting of NRG1 could have significant clinical implications for HNSCC therapeutic approaches.

Materials And Methods

Reagents

Cetuximab (IMC-225, Erbitux) was purchased from the University of Wisconsin Pharmacy. Gas6 and NRG1 were obtained from R&D systems (Minneapolis, MN). DMSO (MilliporeSigma, St. Louis, MO) was used as the vehicle control *in vitro*. Human IgG (MilliporeSigma, St. Louis, MO) was the control for cetuximab.

Cell lines and HNSCC PDXs

UMSCC1, UMSCC6 and HNSCC PDXs were obtained from SPORE resources. The HN30 and PCI37A cell lines were a gift from Dr. Ravi Salgia, City of Hope, Duarte, CA and Dr. Jennifer Grandis, UCSF, CA, respectively. All cell lines were cultured in DMEM with 4.5g/dL glucose, 10% FBS, penicillin (100units/mL), and streptomycin (100mg/mL). Cell line identity was confirmed using short tandem repeat analysis and publicly available databases by the TRIP lab at the University of Wisconsin-Madison. STR reference of PCI37A was not available in public, however the genomic integrity remains similar more than 2 years. Mycoplasma testing was completed through the WiCell Core Service at the University of Wisconsin-Madison.

Plasmids and transfection

Plasmids were prepared and selected as previously described [8]. Transfection was performed using Lipofectamine3000 and Opti-MEMI (Life Technology, Carlsbad, CA) according to the manufacturer's instructions [7] [8]. Blasticidin (3ug/mL) was used as the selective antibiotic when maintaining cells.

siRNA transfection

Non-targeting control pool siRNA (Cat#D-001810), SMARTpool siRNA targeting HER3 (Cat#L-003127), AXL (Cat#L-003104) and NRG1 (Cat#L-004608) were purchased from Dharmacon, Inc (Lafayette, CO) and utilized for transfection with Lipofectamine RNAiMAX (Life Technologies) [7].

Cell proliferation assay and clonogenic assay

Cell proliferation and clonogenic assays using crystal violet or Cell Counting Kit-8 (CCK8, Dojindo Molecular Technologies, MD) were performed as described previously [9, 10]. All treatments were performed in duplicate or triplicate.

Immunoblot analysis

Whole-cell protein lysis, and immunoblot analysis were performed as previously described [8, 9]. Antibodies were used according to the manufacturer's instructions: AXL (Cell Signaling Technologies, MA (CST) #8661), pAXL/pMerTK/pTyro3 (CST #44463), HER3 (CST #12708), pHER3 Y1197 (CST #4561), AKT (CST #2920), pAKT (CST #4060), NRG1 (CST #2573), GAPDH (CST #2118), α -tubulin (MilliporeSigma #CP06).

RNA isolation, cDNA synthesis and qPCR

RNA isolation, cDNA synthesis and qPCR were performed as previously described [7]. Eukaryotic 18S rRNA (4333760, Life Technologies) and human ACTB (4333762, Life Technologies) were used as the endogenous controls for the normalization of initial RNA levels.

Statistical analysis

Statistical analyses were performed using Prism 8 software (GraphPad Software, Inc.). Differences between multiple groups were evaluated using a repeated measures ANOVA with a Bonferroni post-hoc test [11]. Differences were considered significant when $P < 0.05$.

Results

AXL leads to cetuximab resistance and increased HER3 activity

Previously, we reported that many cetuximab-resistant HNSCC cell lines exhibited increased expression and activation of AXL relative to cetuximab-sensitive cell lines. In addition, we showed that HNSCC cell lines that expressed AXL were dependent on this receptor for proliferation by using small interfering RNA (siRNA) analysis [6, 9]. Since siAXL impaired the proliferation of cetuximab-resistant HNSCC cells, we first

sought to identify if ligand-induced activation of AXL may mediate cetuximab resistance. We stimulated cetuximab-sensitive HN30 cells with Gas6, an AXL ligand, to determine if this would lead to increased resistance to cetuximab. Stimulation of AXL by Gas6 in HN30 cells did result in resistance to cetuximab treatment (Figure 1A). Immunoblot analysis indicated that HN30 cells stimulated with Gas6 had robust expression and phosphorylation of AXL and HER3 receptors, even in the presence of cetuximab. AKT was also highly phosphorylated by Gas6 stimulation. This result suggests that activation of AXL and HER3 by Gas6 can stimulate the cell proliferation and survival pathway, thereby escaping the inhibitory effects of cetuximab.

To further evaluate whether overexpression of AXL increased HER3 phosphorylation in HNSCC cells, we overexpressed AXL in the cetuximab-sensitive cell lines HN30 (HN30-AXL) and PCI37A (PCI37A-AXL) via stable transfection. Endogenous level of AXL and HER3 in HN30 and PCI37A cell lines were evaluated via immunoblot (**Supplemental Figure 1**). Proliferation assays demonstrated that AXL overexpressing cell lines were significantly resistant to increasing doses of cetuximab than vector cells (Figure 1B). Immunoblot analysis confirmed that total AXL expression was increased in the cells, and it also indicated that phosphorylation of HER3 was increased in HN30-AXL and PCI37A-AXL cells. Collectively, these results suggest that overexpression of AXL leads to cetuximab resistance and increased HER3 activity.

HER3 is necessary for AXL to mediate cetuximab resistance

To determine if HER3 is important in cetuximab-resistant cells whose resistance is caused by AXL overexpression, cell proliferation assays were performed using cetuximab treatment and siRNAs targeting HER3 (Figure 2A). Cell proliferation of HN30-AXL, PCI37A-AXLC1, and PCI37A-AXLC2 cells was significantly inhibited when treated with the combination of siHER3 and cetuximab compared to either treatment alone. Immunoblot analysis showed that the combination of siHER3 and cetuximab treatment decreased phosphorylation of AKT.

Because siHER3 inhibited the proliferation of HN30-AXL and PCI37A-AXL cells, we next investigated if targeting AXL in cell lines that are *intrinsically* resistant to cetuximab (UMSCC6, UMSCC1) would enhance cetuximab sensitivity and decrease phosphorylation of HER3. Cell proliferation analysis was performed after treatment of UMSCC6 and UMSCC1 HNSCC cells with siNontarget (siNT), cetuximab, siAXL, or the combination of siAXL and cetuximab. The results of these experiments demonstrated that the combination of siAXL and cetuximab had a significant anti-proliferative effect on these cells compared to either treatment alone (Figure 2B). Immunoblot analysis indicated that phosphorylation of HER3 was inhibited by the combination treatment. These results indicate that HER3 signaling collaborates with AXL to regulate cellular proliferation and response to cetuximab. To further investigate whether the expression of HER3 alone is sufficient to mediate resistance to cetuximab, two HNSCC cell lines that are sensitive to cetuximab and express little or no endogenous HER3 were manipulated to overexpress HER3 and treated with cetuximab for cell proliferation analysis (Figure 3). Results of this experiment indicated that both HN30 and PCI37A cells remained sensitive to cetuximab treatment even with overexpression of HER3.

Collectively, these results demonstrate that HER3 overexpression without high AXL expression is insufficient for cetuximab resistance.

Exogenous expression of NRG1 leads to cetuximab resistance

We have previously reported that ligand-mediated activation of HER family receptors, especially HER3, could mediate resistance to cetuximab [7, 12]. In this study, however, HER3 expression was necessary but not sufficient for cetuximab resistance without AXL expression. Therefore, we hypothesized that another pathway downstream of AXL must influence expression of HER3 and the HER3 ligand, NRG1. To test this hypothesis, we first stimulated the HN30 and PCI37A cells with NRG1 to assess if this would lead to increased resistance to cetuximab. Addition of NRG1 to HN30 or PCI37A cells did result in resistance to cetuximab (Figure 4A). Immunoblot analysis indicated that phosphorylation levels of HER3 and AKT were increased in both cell lines after NRG1 stimulation and not inhibited by cetuximab in the presence of NRG1. This result suggested that presence of NRG1 is sufficient to stimulate HER3, leading to regulation of cell proliferation and survival pathways, thus bypassing the inhibitory effects of cetuximab.

To determine the importance of HER3 activation in cetuximab resistance, we treated the cetuximab-sensitive cell lines HN30 and PCI37A with siHER3 and cetuximab for 72 hours and subsequently stimulated them with exogenous NRG1. Analysis of cell proliferation indicated that both HN30 and PCI37A cells were sensitive to cetuximab after HER3 knockdown and NRG1 stimulation (Figure 4B). This result further confirms that HER3 activation is necessary for cetuximab resistance.

On the basis of these results, we next evaluated whether AXL stimulates NRG1 to regulate cell proliferation in cetuximab-resistant cell lines. Quantitative PCR (qPCR) and immunoblot analysis were used to analyze NRG1 expression levels in HN30-AXL or PCI37A-AXL cells compared to the HN30-vector or PCI37A-vector control, respectively (Figure 5A, **Supplemental Figure 2**). The abundance of NRG1 was increased at both the mRNA and protein levels in HN30-AXL and PCI37A-AXL cells.

To expand these findings, HNSCC patient-derived xenografts (PDXs) were evaluated for NRG1 expression levels (**Figure 5B**). Seven HNSCC PDXs were previously characterized and evaluated for cetuximab response [11]. PDX samples were harvested from early-passage tumors and evaluated for NRG1 expression by immunoblot analysis. There were three cetuximab-sensitive PDXs (UWSCC-22, UWSCC-34 and UWSCC-36) and four cetuximab-resistant PDXs (UWSCC-1, UWSCC-17, UWSCC-25 and UWSCC-64). Immunoblot analysis showed that the four cetuximab-resistant PDXs on average expressed ~6-fold more NRG1 than cetuximab-sensitive PDXs. Collectively, these data demonstrate that NRG1 is overexpressed in cetuximab-resistant HNSCC.

AXL regulates NRG1 to lead to cetuximab resistance

Our data indicated that AXL increased HER3 activation, leading to cetuximab resistance. Furthermore, overexpression of AXL increased NRG1 expression levels in cetuximab-resistant cells, and cetuximab-resistant PDXs have more NRG1 expression than cetuximab-sensitive PDXs (**Figure 1, 5**). Thus, we

hypothesized that NRG1 may be critical for AXL to mediate cetuximab resistance. To test this, we targeted NRG1 by siRNA in PCI37A-AXL cells and treated with cetuximab for 72 hours. Cell proliferation assays indicated that loss of NRG1 expression re-sensitized cells to cetuximab (**Figure 6A**). In PCI37A-AXL cells treated with siNRG1 and cetuximab, there was a substantial decrease in phosphorylation of HER3 and AKT. Collectively, these results demonstrated that AXL is signaling through NRG1 to promote cetuximab resistance.

Discussion

The current report presents data suggesting that AXL can signal through HER3 via NRG1 to promote cetuximab resistance in HNC. Notably, our models demonstrated that NRG1 is necessary and sufficient for cetuximab resistance. Further investigation revealed that AXL can regulate the expression of NRG1, thereby promoting resistance to cetuximab. Together these findings suggest that AXL and NRG1 expression could predict patient responses to cetuximab therapy and strengthen the rationale for the use of AXL or NRG1 targeted therapies in HNC treatment strategies.

Previous research by our laboratory and other investigators has demonstrated an increase in expression levels of several receptor tyrosine kinases, including AXL and HER3, after development of resistance to EGFR-targeting [13–15]. Altered expression of AXL in cancer has been studied as a mechanism of acquired resistance to cetuximab [6, 16] and targeting of AXL has been shown to resensitize cells and tumors to EGFR-targeted therapy [8, 17]. Increased expression of EGFR family member HER3 has also been observed in cetuximab-resistant models leading to development of several EGFR-HER3 co-targeting therapeutic strategies [7, 18–21]. In this study, we found that overexpression of AXL leads to cetuximab resistance and increased HER3 activity (Figure 1), and that HER3 was necessary for AXL to mediate cetuximab resistance (Figure 2). We also found that HER3 overexpression alone was insufficient for cetuximab resistance in HNSCC cells. These results suggested that overexpression of both AXL and HER3 may be necessary for cetuximab resistance. Despite wide pre-clinical success of HER3 targeting to overcome cetuximab resistance [22–24], the combination of a HER3 inhibitor with cetuximab did not demonstrate clinical success over cetuximab therapy alone [25–27]. Thus, our lab has continued to investigate the signaling role of HER3 discovering that perhaps AXL and NRG1 are of more importance in the cetuximab resistance pathway.

In the current study, we found that the addition of NRG1 to the cetuximab-sensitive HN30 and PCI37A cell line rendered these cells resistant to cetuximab (Figure 4A). In line with this data, we previously reported that NRG1 autocrine signaling is a major driver of acquired resistance to cetuximab [7]. We also found that cetuximab-resistant HN30-AXL and PCI37A-AXLC1 cells relatively expressed more NRG1 than the vector control (Figure 5A) and that cetuximab-resistant PDX tumors have more NRG1 than cetuximab-sensitive PDXs (Figure 5B). These findings suggested that AXL could regulate NRG1 in cetuximab-resistant HNSCC cells. Interestingly, PCI37A-AXLC2 cells did not express more NRG1 compared to vector control despite AXL being overexpressed (Figure 5A), and cetuximab-resistant PDX tumor (UW-SCC25) did not express NRG1 even though they had AXL expression (Figure 5B). Many groups have begun

investigating NRG1 expression in cancer and how this correlates with therapeutic response with varied results. Meetze *et al.* found a significant correlation between NRG1 expression and tumor growth inhibition by the HER3 inhibitory antibody AV-203 [28], and another study demonstrated that HER3 inhibition could be quite effective in NRG1-rearranged cancers [29]. Baro *et al.* also identified upregulation of autocrine NRG1 signaling as a mechanism of cetuximab resistance in HNSCC tumors. Using the HER3 antibody CDX-3379, they were able to overcome cetuximab resistance and enhance tumor growth delay and radiosensitivity [30]. In contrast, one study found that expression of HER3 did not indicate sensitivity to a HER3 antibody or cetuximab. They demonstrated that NRG1 expression along with other EGFR-activation biomarkers correlated with better anti-HER3 response [31]. In this study, knockdown of NRG1 expression by siRNA combined with cetuximab treatment led to diminished cell proliferation by impairing AKT survival signaling in cetuximab-resistant PCI37A-AXL cells (Figure 6A). Because of the varying results in different cancer models, more investigation of NRG1 expression and cetuximab response must be completed. Additional testing of NRG1 inhibition and re-sensitization to cetuximab could also be investigated using two high-affinity monoclonal antibodies to NRG1 [32] or an anti-NRG1 antibody that has shown inhibition of tumor growth in preclinical models of pancreatic cancer [33]. Collectively, the data presented within explores a signaling connection between AXL, NRG1, and HER3 in the context of cetuximab resistance in HNC (Figure 6B).

Conclusions

We have shown that NRG1 expression, more than HER3 expression, is necessary and sufficient for resistance to cetuximab in our models and that the RTK AXL can regulate expression of NRG1. This data corroborates findings that suggest AXL regulates NRG1 expression [34] and that a combination of HER3 and AXL therapy might be more effective than targeting either alone [35]. Further exploration must be done to identify if the co-expression of AXL and NRG1 could be as used as biomarkers to predict resistance to cetuximab or if a targeting strategy for AXL or NRG1 would provide a therapeutic advantage in the setting of resistance.

Abbreviations

CCK8: Cell Counting Kit-8

Ctx: cetuximab

EGFR: epidermal growth factor receptor

HNC: Head and Neck cancer

HNSCC: Head and neck squamous cell carcinomas

NRG1: neuregulin1

PDXs: patient-derived xenografts

qPCR: quantitative PCR

RTK: receptor tyrosine kinase

siRNA: small interfering RNA

siNT: siNontarget

Declarations

Ethics approval and consent to participate

The generation of patient derived xenografts from deidentified tissues has been deemed to be not-human subjects research and exempt from University of Wisconsin-Madison IRB review. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

All authors have approved the final article and consent to publication.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no potential conflicts of interest.

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Authors' contributions

M.I., N.M., and D.L. designed the study. M.I., N.M., N.W. and C.K. carried out data acquisition and analysis. M.I., N.M. K.K. and D.L. wrote the manuscript. M.I., N.M., K.K., N.W., C.K., C.L. contributed to preparing and

making figures. C.L., J.B., S.H., and R.S. contributed to reviewing and editing the manuscript. All authors have read and approved the final manuscript.

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Figures

Figure 1

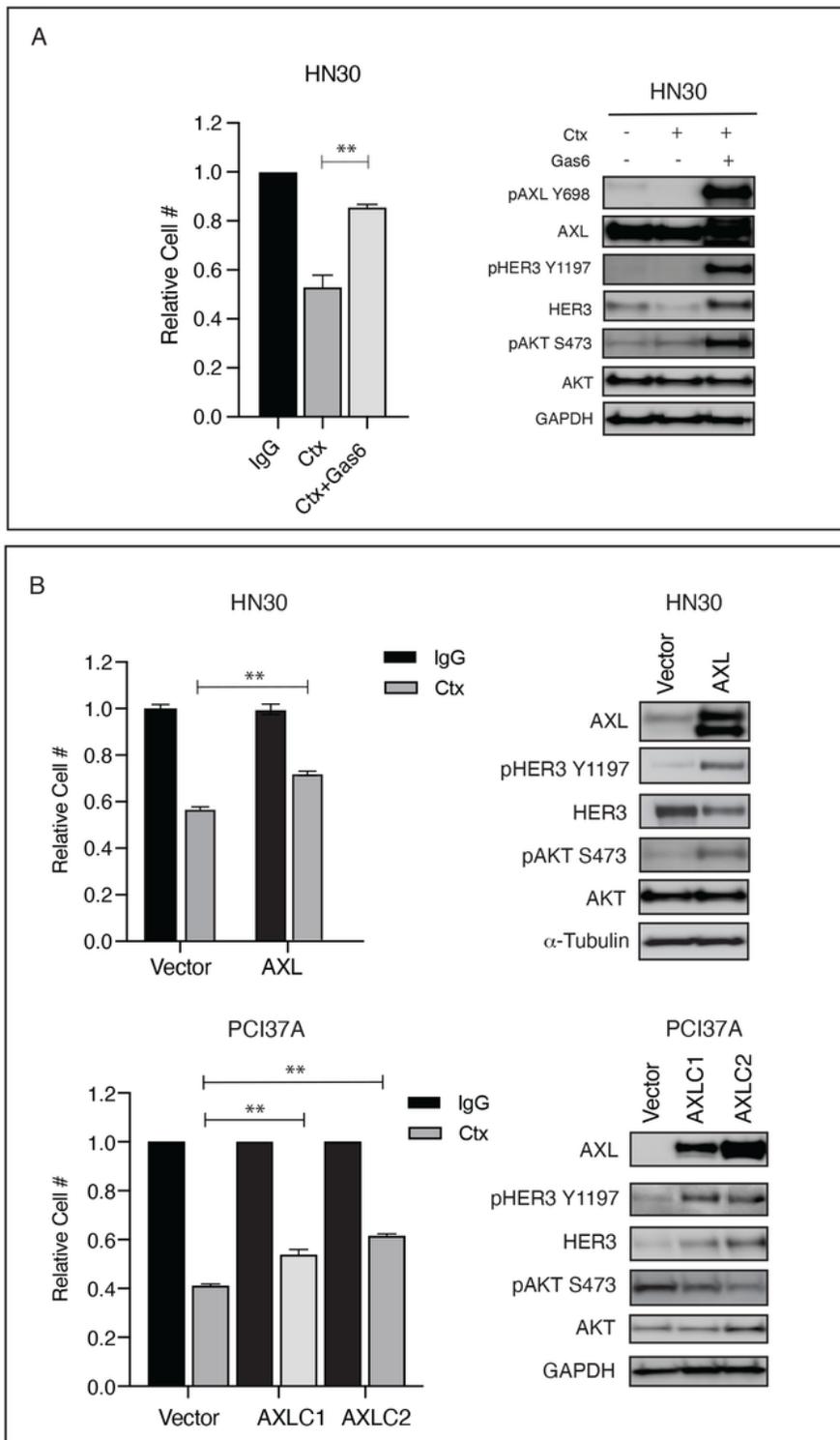


Figure 1

AXL leads to cetuximab resistance and increased HER3 activity. A: HN30 cells were treated with 100nM of IgG, 100nM of cetuximab (Ctx), or combination of cetuximab and Gas6 (200ng/uL) for 72 hours and relative cell numbers were determined by CCK8 assay. Mean values, SEs, and statistical analyses are representative of two independent experiments. N=3, **P<0.01. Whole cell lysates were harvested and fractionated via SDS-PAGE, followed by immunoblotting for the indicated proteins. GAPDH was used as a

loading control. B: Cell proliferation in AXL overexpressed cells was measured via CCK8 assay after 72 hours of treatment with cetuximab and relative values were determined. Mean values, SEs, and statistical analyses are representative of three independent experiments. N=3, **P<0.01. Whole cell lysates were harvested and fractionated via SDS-PAGE, followed by immunoblotting for the indicated proteins. α -Tubulin and GAPDH were used as loading controls.

Figure 2

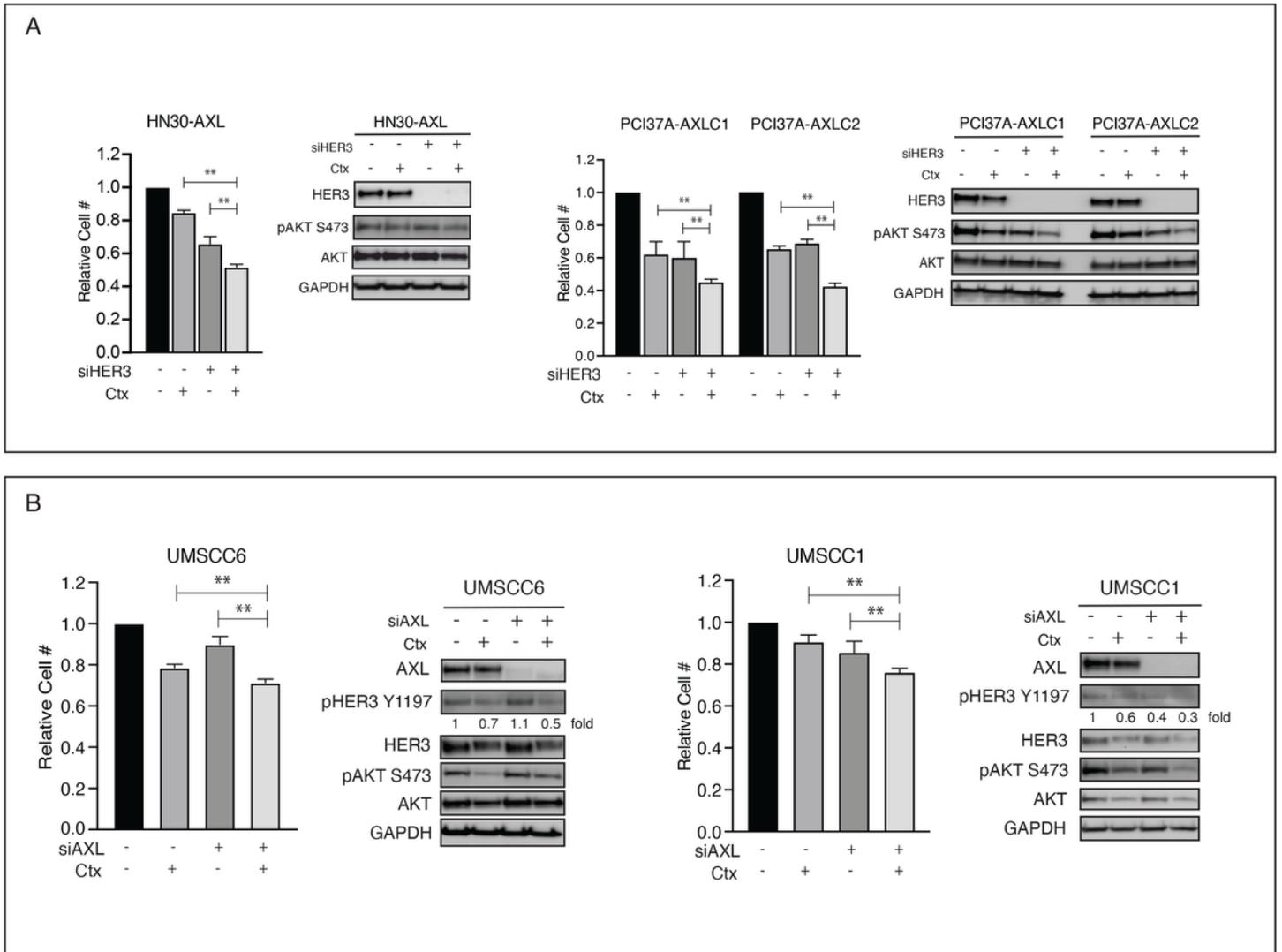


Figure 2

HER3 is necessary for AXL to mediate cetuximab resistance. A: HN30-AXL and PCI37A-AXL cells were plated and treated with 30nM of HER3 siRNA (siHER3) or 30nM non-target siRNA (siNT). The next day, cells were treated with 100nM of IgG or 100nM cetuximab for 72 hours. Growth was measured after drug treatment by CCK8 assay. Mean values, SEs, and statistical analyses are representative of three independent experiments. N=5-10, **P<0.01. Whole cell lysates were collected 24 hours after treatment, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. GAPDH was used as a loading control. B: UMSSC1 and UMSSC6 HNSCC cells were plated and treated with 30nM of siAXL or 30nM non-target siRNA. The next day, cells were treated with 100nM of IgG or 100nM cetuximab for 72 hours. Cell

proliferation was measured after drug treatment by CCK8 assay. Mean values, SEs, and statistical analyses are representative of three independent experiments. N=3, **P<0.01. Whole cell lysates were collected at 24 hours after treatment, fractionated by SDS-PAGE, and immunoblotted for the indicated proteins. GAPDH was used as a loading control.

Figure 3

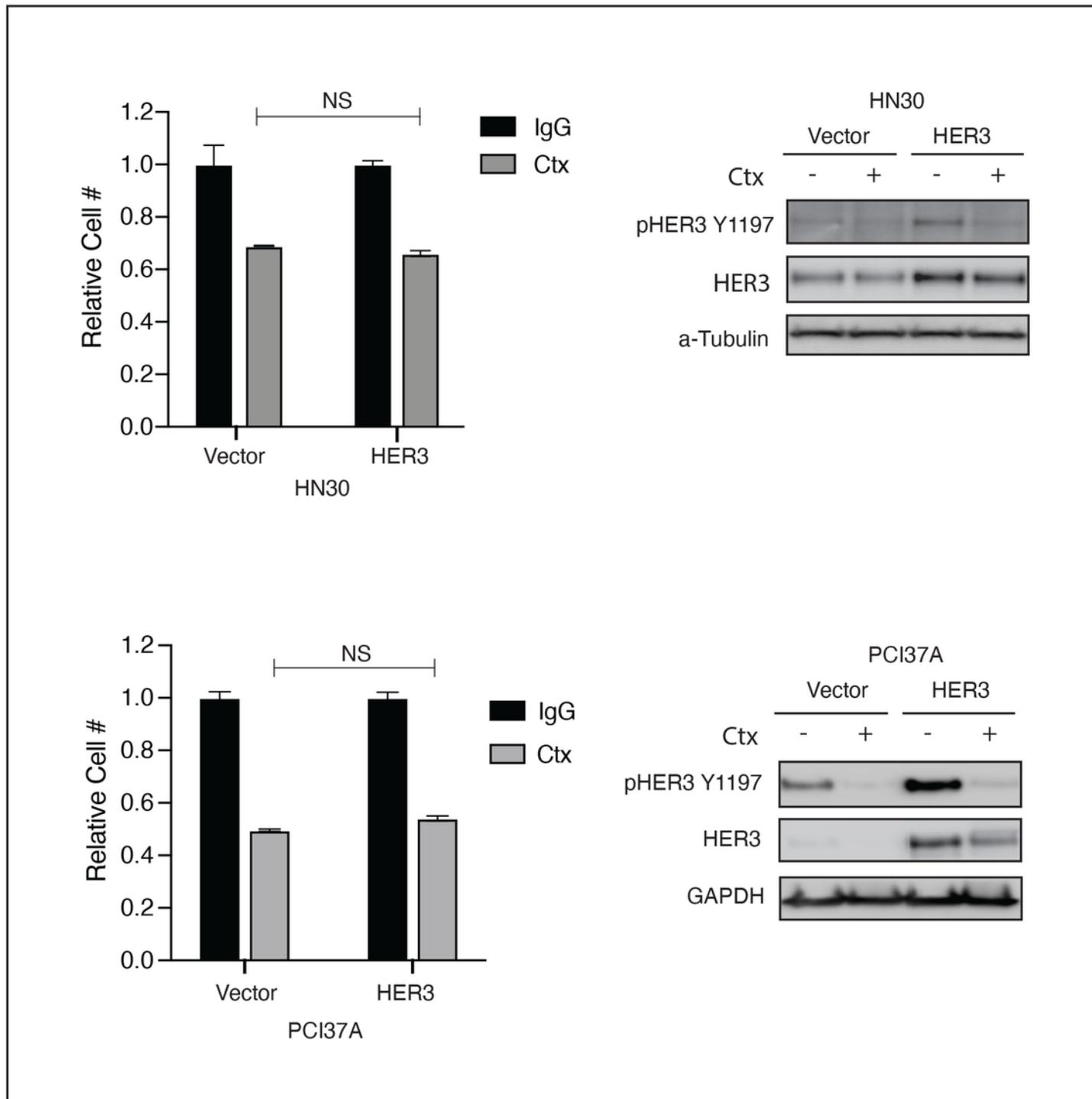


Figure 3

HER3 overexpression alone is insufficient for cetuximab resistance. A: HN30 and PCI37A cells stably overexpressing HER3 or the pcDNA6.0 vector were treated with 100nM of cetuximab for 72 hours before performing crystal violet or CCK8 proliferation assays. Whole cell lysate was harvested at 24 hours after treatment and subjected to immunoblot analysis following fractionation via SDS-PAGE. GAPDH or α -Tubulin was used as a loading control. Mean values, SEs, and statistical analyses are representative of two or three independent experiments. N=3-6. NS: not significant.

Figure 4

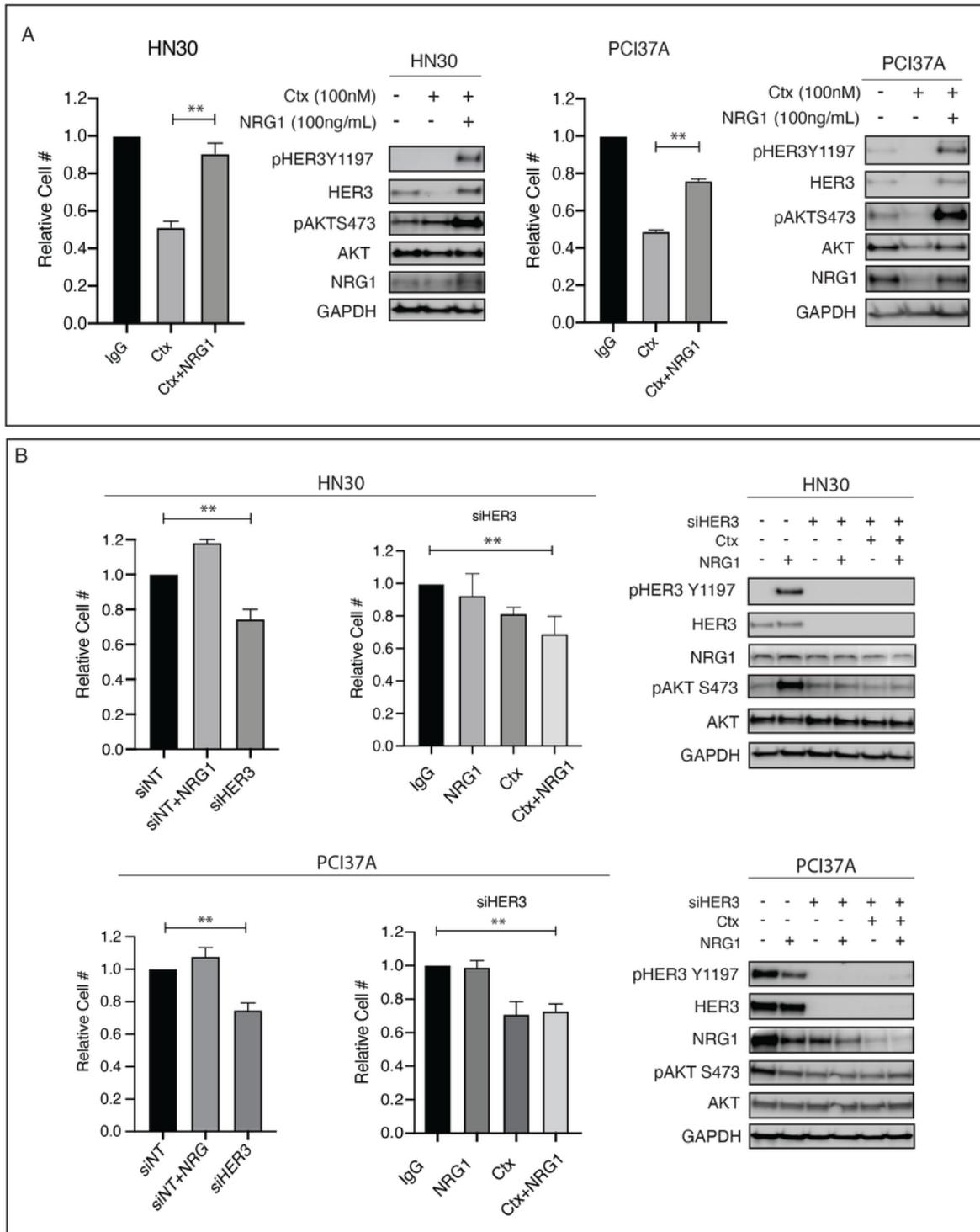


Figure 4

Exogenous expression of NRG1 leads to cetuximab resistance. A: HN30 and PCI37A cells were plated and treated with 100nM of cetuximab, 100ng/mL of NRG1, or the combination of cetuximab and NRG1 for 72 hours. Relative cell numbers were determined by crystal violet or CCK8 assay. Mean values, SEs, and statistical analyses are representative of two independent experiments. N=6, **P<0.01. Whole cell lysates were harvested at 24 hours after treatment and fractionated via SDS-PAGE, followed by immunoblotting for the indicated proteins. GAPDH was used as a loading control. B: HN30 and PCI37A cells were transfected with 30 nM siHER3 or 30nM siNT for 24 hours before treatment with cetuximab (100nM) or NRG1 (100 ng/ml) for an additional 72 hours. Relative cell numbers were determined by CCK8 assay. Mean values, SEs, and statistical analyses are representative of two or three independent experiments. N=3-10, **P<0.01. Whole cell lysate was harvested at 24 hours after treatment and subjected to immunoblot analysis following fractionation via SDS-PAGE. GAPDH was used as loading.

Figure 5

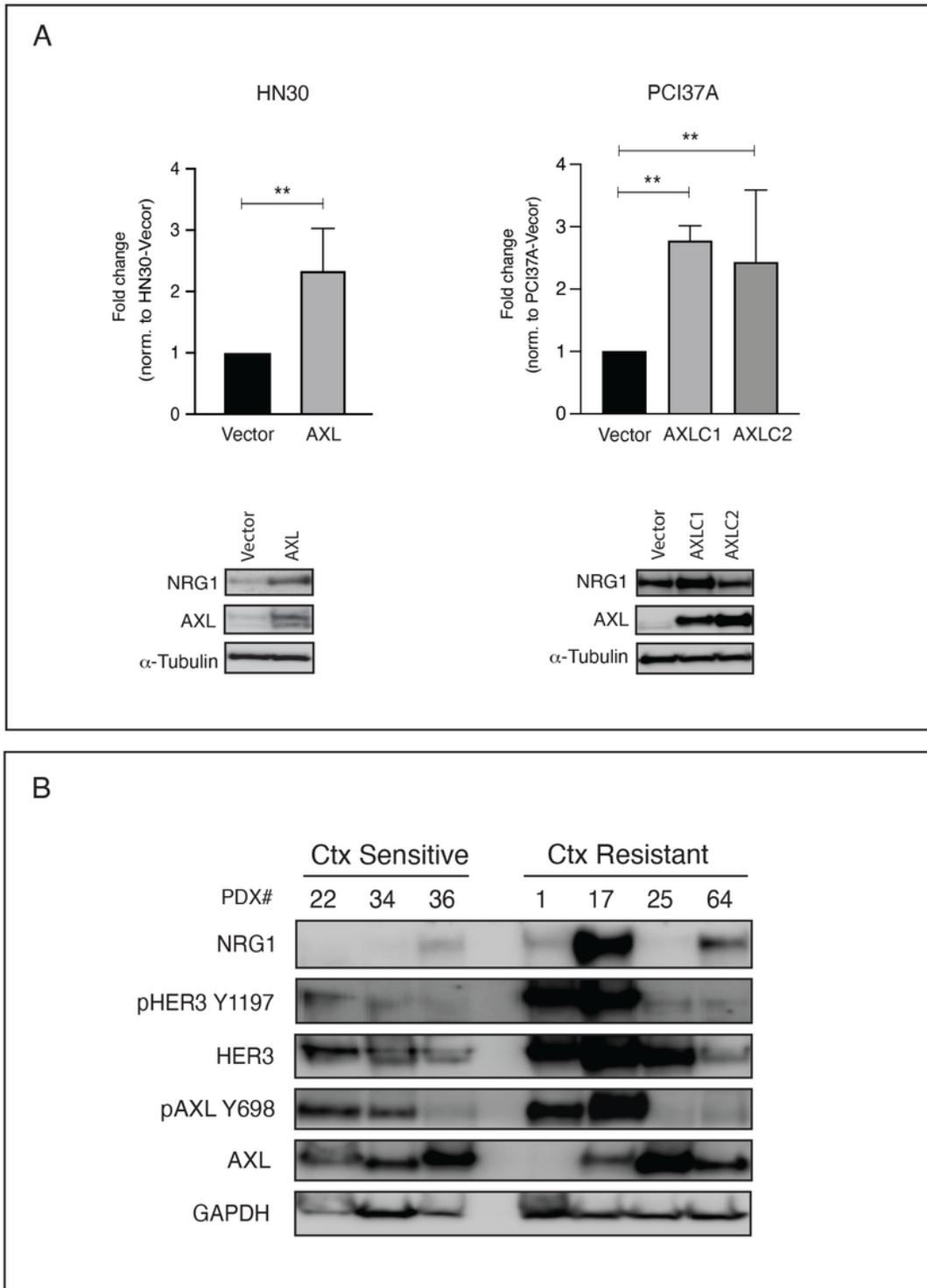


Figure 5

AXL regulates NRG1. A: The expression levels of NRG1 in HN30-Vector, HN30-AXL PCI37A-Vector and PCI37A-AXL cells were determined by qPCR and immunoblot analysis. α -Tubulin was used as a loading control. Mean values, SEs, and statistical analyses are representative of three independent experiments. N=2-4. B: Whole cell lysates were harvested from HNSCC PDX tumors and fractionated via SDS-PAGE, followed by an immunoblot for NRG1, HER3, and AXL. GAPDH was used as a loading control.

Figure 6

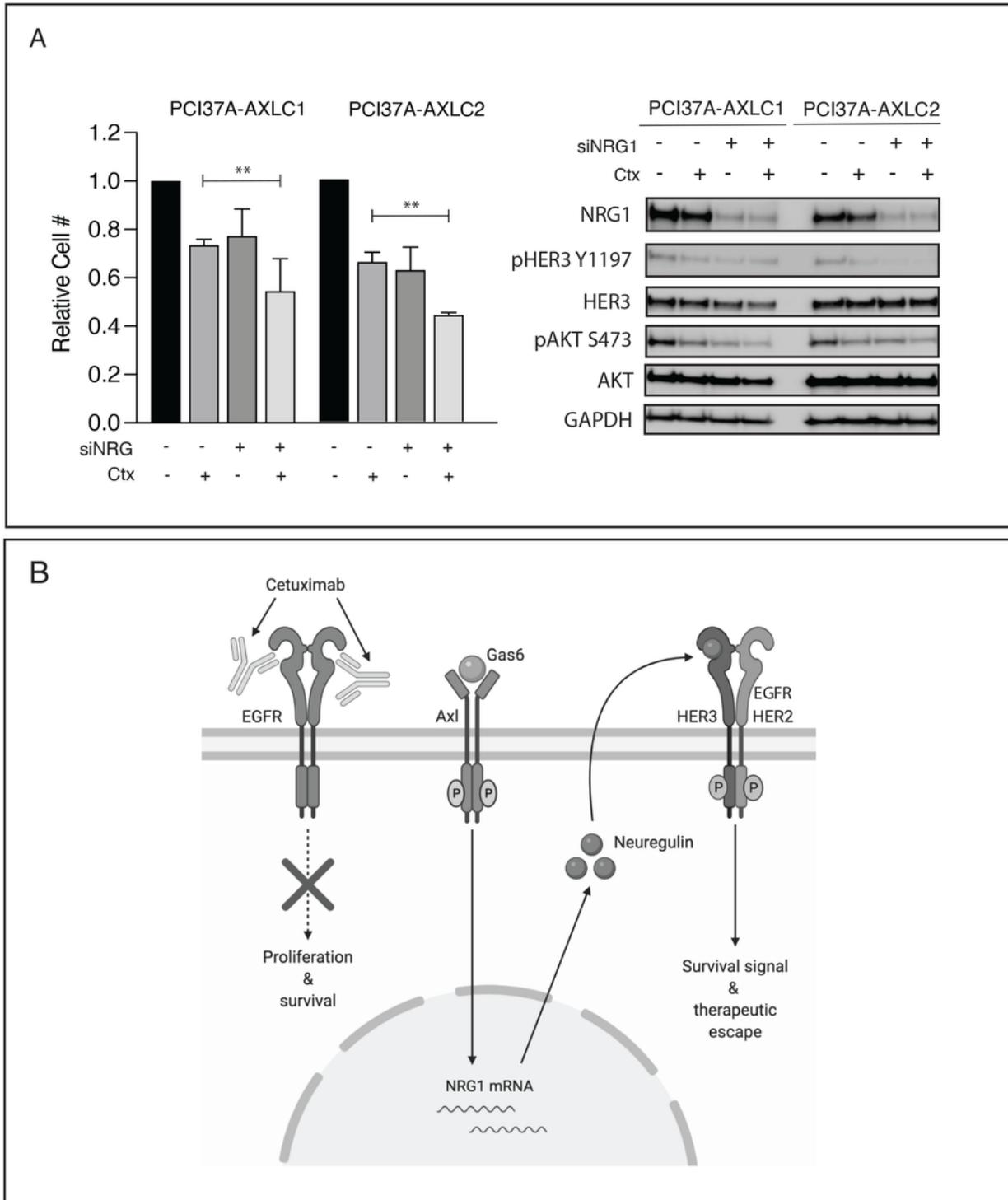


Figure 6

AXL regulates NRG1 to lead to cetuximab resistance. A: PCI37A-AXL cells were plated and treated with 30nM of NRG1 siRNA or 30nM non-targeting siRNA. The next day, cells were treated with 100nM of IgG or 100nM cetuximab for 72 hours. Growth was measured after drug treatment using the CCK8 assay. Mean values, SEs, and statistical analyses are representative of seven independent experiments. N=6-10, **P<0.01. Whole cell lysates were collected at 24 hours after treatment, fractioned

by SDS-PAGE, and immunoblotted for the indicated proteins. GAPDH was used as a loading control. B: Proposed model for AXL regulation of NRG1 leading to cetuximab resistance.

Supplementary Files

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- [SupplementalFigures.pdf](#)