

Summary

PURPOSE: Treatment of triple negative breast cancer (TNBC) cells with tyrosine kinase inhibitors (TKIs) targeting the epidermal growth factor receptor (EGFR-TKIs) but not chemotherapeutic drugs has been shown to reactivate the expression of the tumor suppressor AnxA6 in AnxA6-low TNBC cells. Here we sought to investigate the mechanisms by which AnxA6 is upregulated in TNBC cells in response to these otherwise poorly effective drugs.

METHODS/RESULTS: Treatment of TNBC cells expressing low levels of AnxA6 with EGFR-TKIs, calcium channel blockers, histone deacetylase (HDAC) inhibitors, or DNA methyl transferase inhibitors resulted in AnxA6 upregulation that correlated with cytotoxic effects of these compounds. We also found that AnxA6 upregulation was more conspicuous in cells with markedly reduced calcium influx e.g. following EGFR-TKI treatment. However, this effect was cell type-specific and may be attributed to the greatly variable expression of multiple calcium channels. Although treatment of TNBC cells with HDAC inhibitors potentially involves acetylation of histone H3 and triggers epithelial to mesenchymal transition (EMT) in MDA-MB-468 cells, RNAi mediated blockage of AnxA6 upregulation revealed that the EGFR-TKI induced reactivation of AnxA6 expression does not affect the epithelial to mesenchymal transition (EMT) process. Lastly, we show that drug induced AnxA6 upregulation in TNBC cells is associated with upregulation of the ER chaperone GRP78 but not HSP90 or GADD54.

DISCUSSION/CONCLUSION: These data suggest that treatment of TNBC cells with therapeutic compounds that affect calcium influx into these cells, leads to unfolded protein response or ER stress, AnxA6 upregulation and decreased cell proliferation, as part of the mechanism for the development of resistance to these drugs.

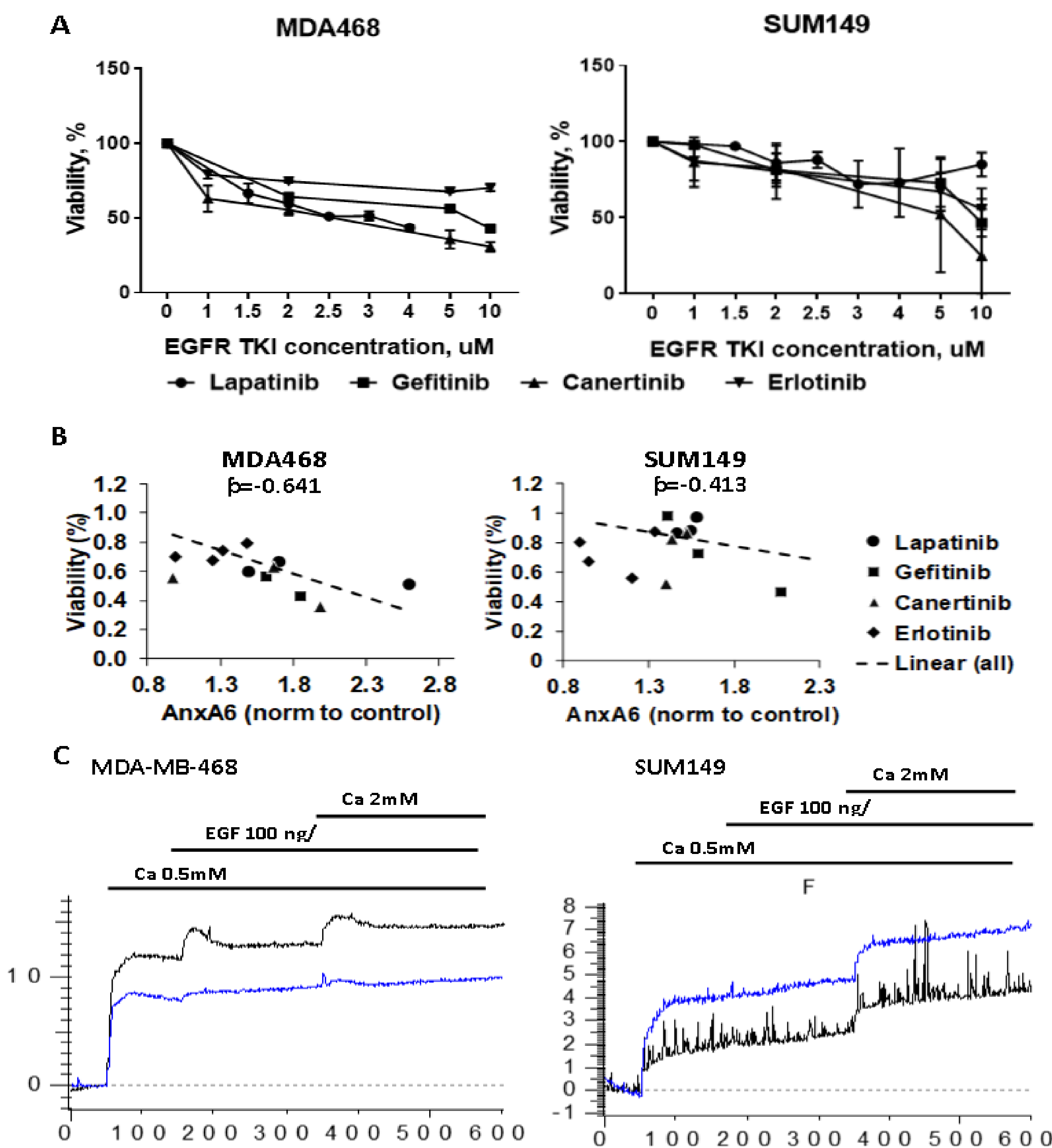


Figure 1. Upregulation of AnxA6 expression is associated with cytotoxicity but not calcium influx caused by EGFR TKIs in AnxA6-low TNBC cells. MDA468 and SUM149 cells were treated for 72 hours with various concentrations of EGFR TKIs. (A) Cell viability was assessed using the PrestoBlue assay reagent. (B) Association of EGFR-TKI induced AnxA6 expression with the viability of TNBC cells following treatment with EGFR TKIs or DMSO control. The linear trend line shows a cell type specific negative correlation of AnxA6 expression and TNBC viability and Pearson correlation were calculated for all concentrations of EGFR TKIs combined. (C) Effect of lapatinib treatment on intracellular calcium dynamics in TNBC cells. Lapatinib treated (blue) and control (black) cells.

Acknowledgements

This research is funded by NIH/NIGMS 1SC1 CA211030 and Career Enhancement Award from the Vanderbilt Breast SPORE NIH/NCI PPP50CA098131-16S1.

Methods and Results

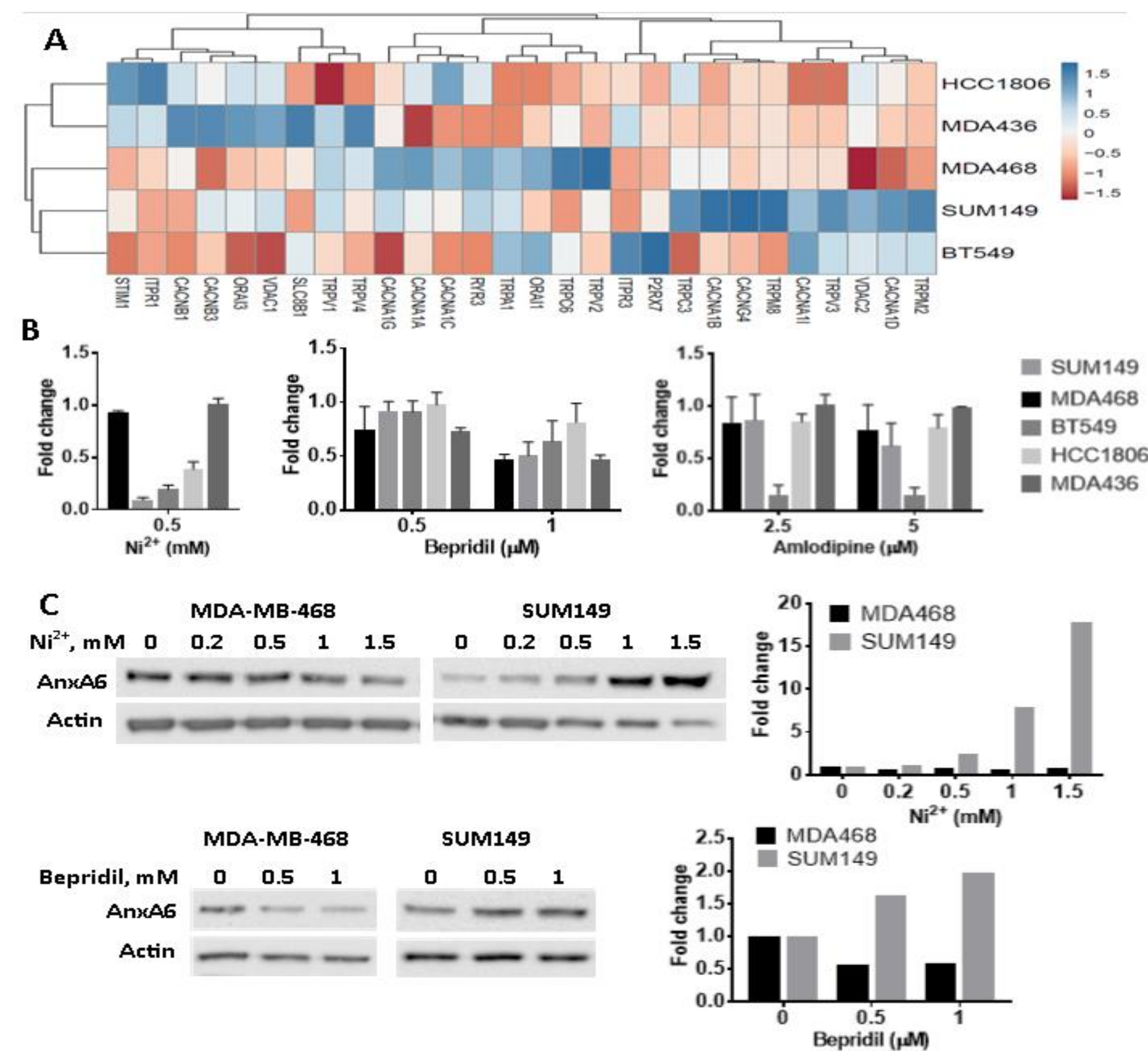


Figure 2. Heterogeneity of calcium influx channels and response to calcium influx blockage in TNBC cell lines. (A) Heat map of TaqMan Gene Expression Assay with the selected calcium channels in five TNBC cell lines was created using the Clustvis web tool. Columns were centered; unit variance scaling was applied to columns. Both rows and columns were clustered using correlation distance and average linkage. (B) Viability of TNBC cell lines after 72 hours treatment with selected calcium channels blockers was assessed by using the PrestoBlue assay. The diagrams show fold change in the viability of treated cells versus vehicle treated control. (C) Representative Western blots of the effects of different concentrations of Ni²⁺ and bepridil on AnxA6 expression in MDA-MB-468 and SUM149 cell lines. The relative intensity of the protein bands was analyzed using the NIH Image J software and quantified relative to control cells.

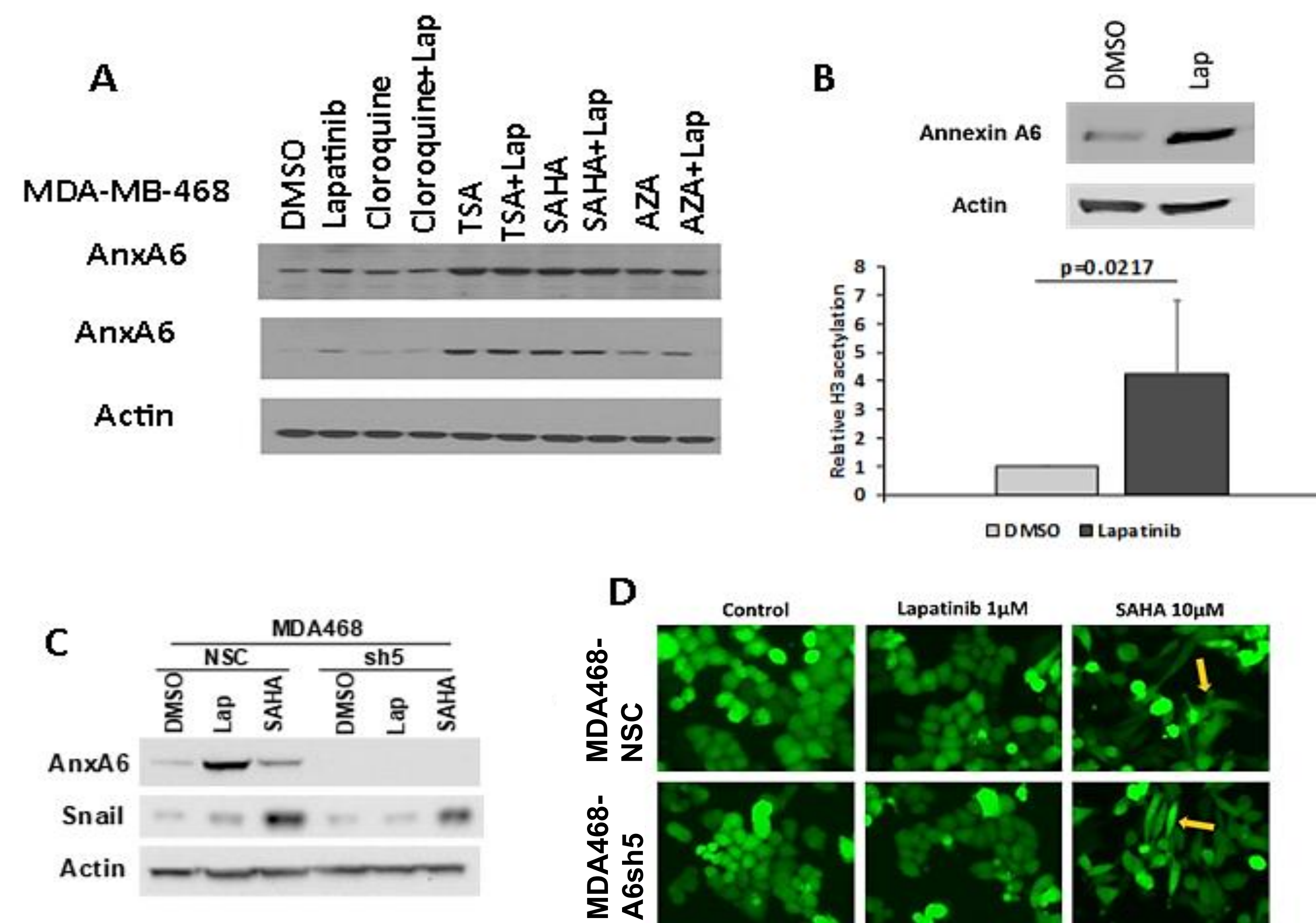


Figure 3. Drug induced expression of AnxA6 is epigenetically regulated, but AnxA6 upregulation is not required for EMT. (A) Representative Western blot of the effects of inhibitors of lysosome function (cloroquine), histone deacetylases (SAHA, TSA) and DNA methyltransferases (AZA), with or without lapatinib, on the expression of AnxA6. (B) Total Histone H3 acetylation in lapatinib treated cells compared to control cells measured by Chromatin IP. The Western blot image confirms upregulation of AnxA6 in lapatinib treated cells. (C) Expression of AnxA6 and the EMT marker Snail in lapatinib or SAHA treated MDA-MB-468 cells. (D) The morphology of AnxA6 expressing or AnxA6 depleted MDA-MB-468 cells following treatment with lapatinib or SAHA.

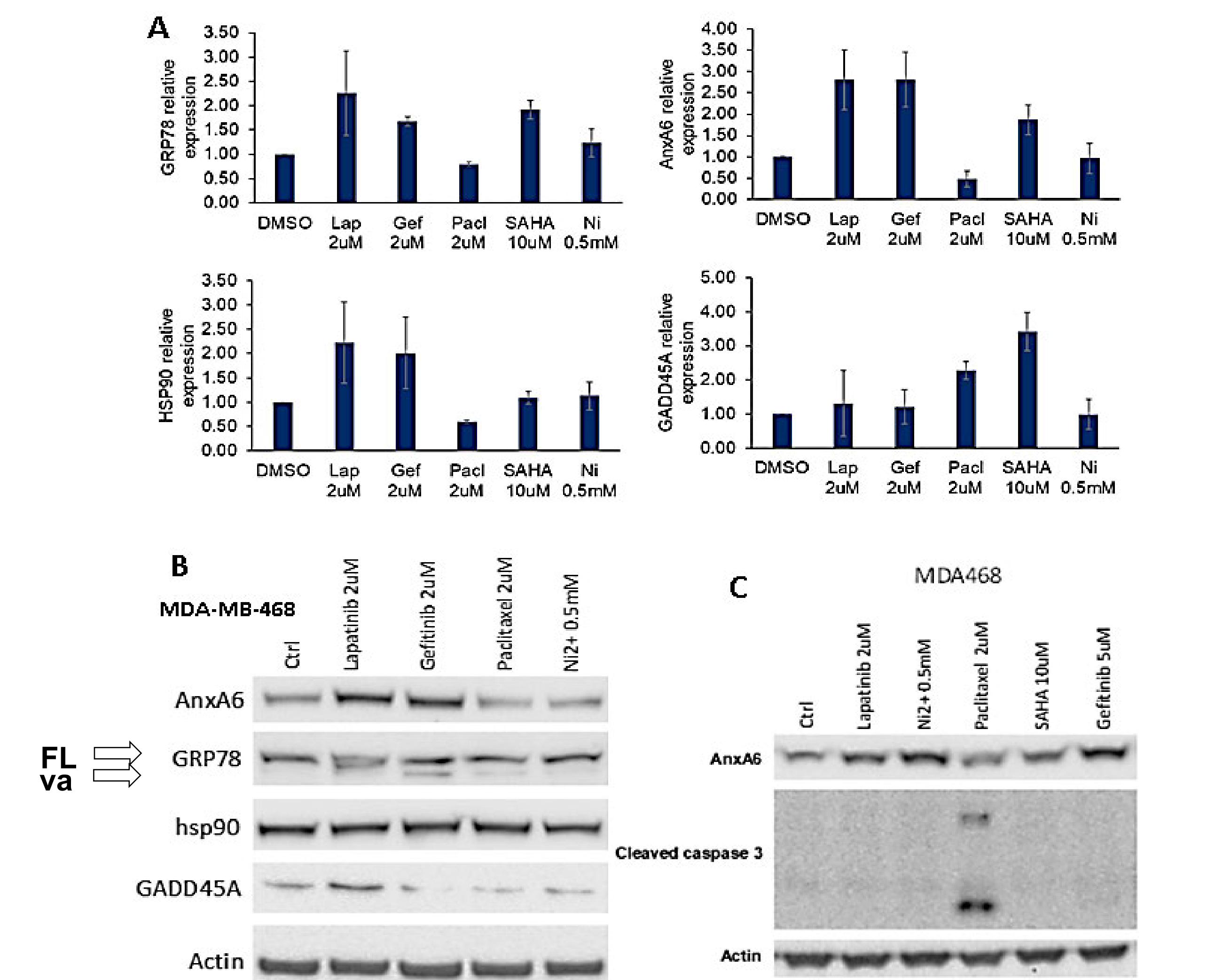


Figure 4. AnxA6 upregulation is associated with ER but not heat shock or oxidative stress. (A) Relative mRNA expression of AnxA6 and different types of cellular stress markers assayed by real time RT-PCR relative to DMSO control treated cells and 18s RNA expression as housekeeping control. (B) Representative Western blot of the effect of indicated compounds (72 hours treatment) on AnxA6 and cellular stress marker expression in MDA-MB-468 cells. (C) Expression of cleaved caspase 3 (indicative of cell apoptosis) in MDA-MB-468 cells after the treatment with indicated compounds.

GRP78: FL – full length; **va** – functional truncated form lacking ER targeting fragment thus cytosolic.

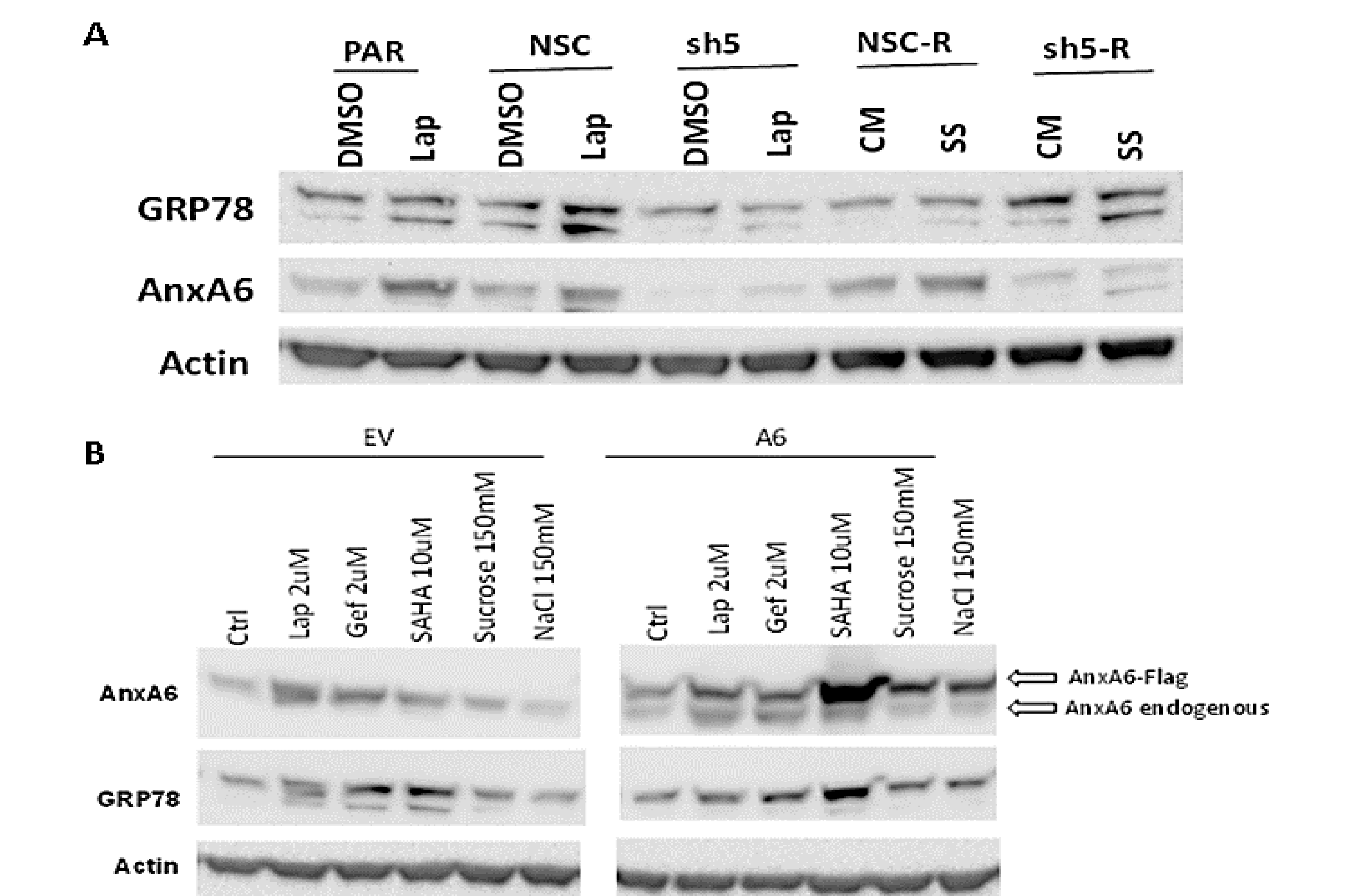


Figure 5. AnxA6 expression protects cells from cytosolic ER stress in MDA-MB-468 cells. (A) Representative Western blot of AnxA6 and GRP78 expression in MDA-MB-468 parental cells and lapatinib resistant NSC and sh5 cells with (SS) or without (CM) serum starvation overnight. (B) AnxA6 and GRP78 expression in MDA-MB-468 cells transfected with empty vector (EV) and AnxA6-Flag overexpressing cells (A6).

Conclusions

- AnxA6 expression in TNBC cells is increased by EGFR TKIs and certain calcium channel blockers at concentrations that appear to be cytotoxic for cells.
- The ability of compounds to upregulate AnxA6 may be related to their calcium influx modulating/blocking properties.
- Expression of AnxA6 does not influence the EMT mechanism in TNBC cells.
- Upregulation of AnxA6 is associated with ER stress in TNBC cells.

For questions

Please email okorolkova@mmc.edu