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TITLE: Nuclear ErbB3 Predicts Sensitivity to Androgen Receptor Inhibitors in Castration-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Maitreyee Jathal, Ph.D.

CONTRACTING ORGANIZATION: The Regents of the University California (Davis)

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14. ABSTRACT
This research focuses on ErbB3, a member of the epidermal growth factor receptor (EGFR) family in castration-resistant prostate cancer (CRPC), and the role that its localization may play by altering efficacy of AR inhibitors (AR-I) in prostate cancer (CaP). In Aim 1 we will investigate whether subcellular localization of ErbB3 renders CaP cells resistant or responsive to AR-I. In Aim 2 we will investigate whether secondary markers of ErbB3 localization can predict response or resistance to AR-I. We have thus demonstrated that some metastatic, hormone-sensitive prostate tumors show increased efficacy towards AR-I when ErbB3 is silenced. Our overall goal is understanding whether ErbB3 can predict which patients will benefit most from treatment with AR-I.

15. SUBJECT TERMS
ErbB3, subcellular localization, prostate cancer, androgen receptor inhibitors, abiraterone acetate

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1. INTRODUCTION:

The subject of this research was the role of ErbB3, a member of the epidermal growth factor receptor (EGFR) family, in castration-resistant prostate cancer (CRPC) and how its localization may mediate prostate cancer (CaP) responsiveness or resistance to AR inhibitors (AR-I). The purpose of the research was to study the biochemical and functional roles of nuclear ErbB3 in a 2D cell culture model of human prostate tumor progression as well as in normal-like immortalized human prostate cells and patient serum samples. The scope of this research is to based on the hypothesis that CaP with nuclear ErbB3 will be sensitive to AR-I. The proportion of patients with AR-independent tumors will substantially increase over time, owing to earlier treatments with AR inhibitors, and eventual resistance to these treatments. If prostate tumor cells remain androgen-dependent - or responsive to AR inhibitors for as long as possible – then overall survival is greatly improved when tumors take longer to become castration-resistant.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

ErbB3, subcellular localization, prostate cancer, androgen receptor inhibitors, abiraterone acetate, nucleocytoplasmic transport

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The first goal was to create abiraterone-resistant cell lines and examine nuclear and cytoplasmic ErbB3 expression levels and physiological effects with or without AR inhibitors (AR-I) in CRPC cells in vitro. This was to be performed during months 1-6 but owing to the delay in generation of the drug-resistance cell lines is still ongoing. It is therefore 85% complete. The second goal was to identify transport mechanisms for ErbB3 in CRPC cells also during months 1-6. It is therefore 100% complete, using pharmacological inhibitors for the cell lines LNCaP, C4, C4-2 and C4-2B. The third goal was to create ErbB3 NLS and NES mutants and determine physiological effects on CRPC cell lines with or without AR-I in vitro, during months 6-12. The plasmids have been generated but not transfected or tested therefore this goal is only 25% complete. The fourth goal was to statistically analyse the results from the preceeding 3 goals. This is ongoing and is best described as 50% complete, pending results from Goal 3 and additional results from Goal 1 using the drug-resistant cell lines.

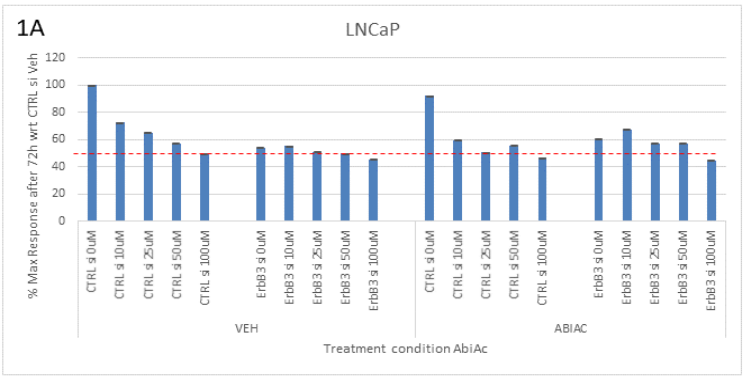
What was accomplished under these goals?

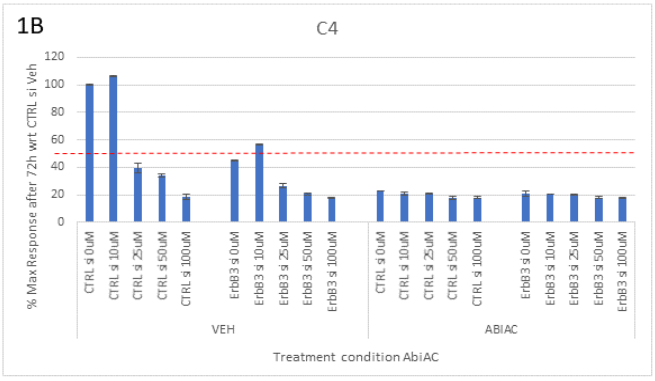
The major activities for this reporting period were the generation, characterization and testing of abiraterone acetate (Abi) LNCaP and C4 cell lines (along with comparable vehicle-treated cell lines), identifying transport mechanism for ErbB3 in the tumor progression model cell lines LNCaP, C4, C4-2 and C4-2B and generation, characterization and testing of ErbB3 localization mutants in normal-like, immortalized prostate epithelial cell lines RWPE-1 and RWPE-2. Cell culture methods, siRNA transfections, viability assays, subcellular fractionation, flow cytometry and immunofluorescence were performed as described in my previous publication full (Jathal MK, Steele TM, Siddiqi S, Mooso BA, Dabronzo LS, Drake CM, Whang YE, Ghosh PM, Br J Canc, 121, 237-48, 2019).

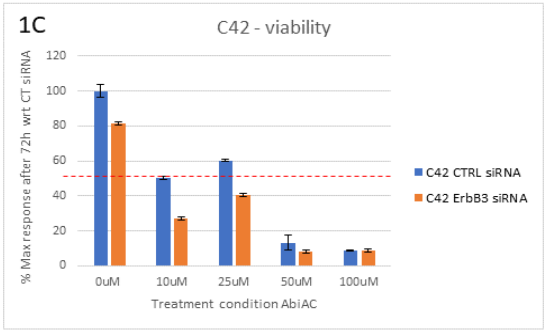
The drug-resistant cell lines were generated by continuous culture either in complete serum-containing media supplemented with 5uM or 10uM Abi (for LNCaP and C4 respectively) or an equal volume of 200 proof ethanol (vehicle in which Abi was dissolved). Drug-resistant LNCaP and C4 cells are hereby referred to as ‘LN-Abi’ or ‘C4-Abi’ respectively. Their vehicle-treated control lines are described as ‘LN-Veh’ and ‘C4-Veh’ respectively. Cells were then subjected to subcellular fractionation to determine nuclear and cytoplasmic ErbB3 protein levels as well as downstream targets. Cells were also plated for viability assays and characterized via cell cycle and cell death analyses (flow cytometry). To determine the conditions under which ErbB3 localization could be altered in response to heregulin-1β (HRG) all cell lines were subjected to additional experiments wherein alteration of ErbB3 localization was observed at varying time points using 50ng/ul of

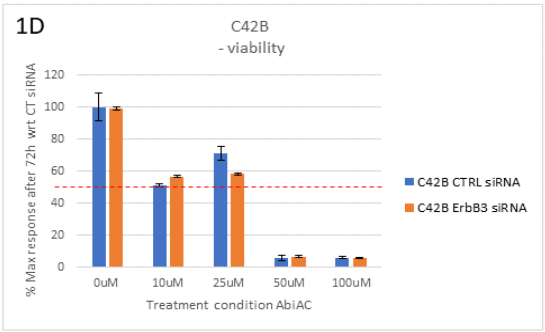
HRG. When these conditions were determined cell lines were then treated with the appropriate inhibitors of nucleocytoplasmic transport to observe which pathways were affected. All experiments were conducted in the presence or absence of 10uM of the appropriate androgen receptor inhibitors (AR-I) and in the presence of absence of commercially-purchased ErbB3 siRNA. ErbB3 knockdown was verified by immunofluorescent microscopy as well as Western blot using multiple antibodies to its N- and C-terminii as and where appropriate.

While LN-Veh and C4-Veh cells showed a dose-dependent decrease in viability with abiraterone acetate (AbiAC) treatment, LN-Abi and C4-Abi did not (1A right bars, 1B right bars). C4-2 and C4-2B cells were resistant to 25uM AbiAc (1C, 1D) but showed decreased viability above 50uM AbiAc. The dotted red lines in each bar graph indicate the 50% viability level. ErbB3 siRNA significantly enhanced the effect of AbiAc only in LN-Veh and C4-Veh cells but not in LN-Abi (1A) or C4-Abi (1B) cells or C4-2 (1C) or C4-2B (1D) cells.

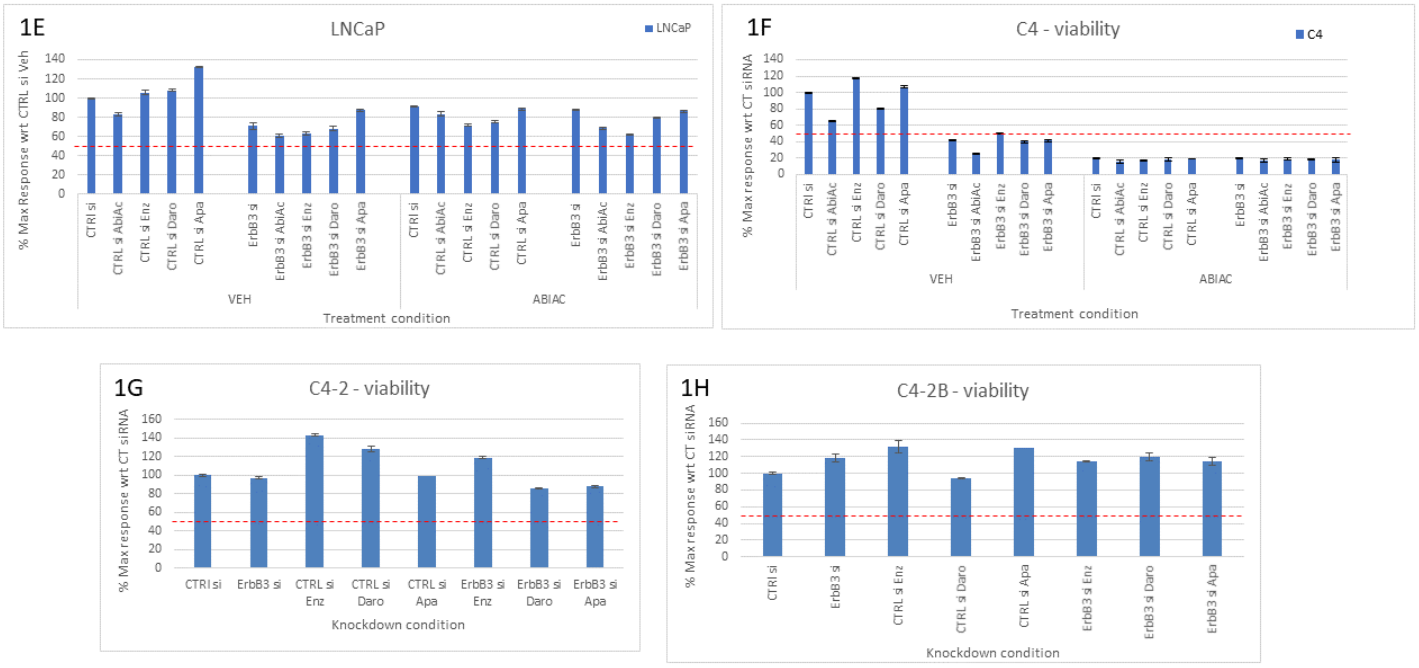




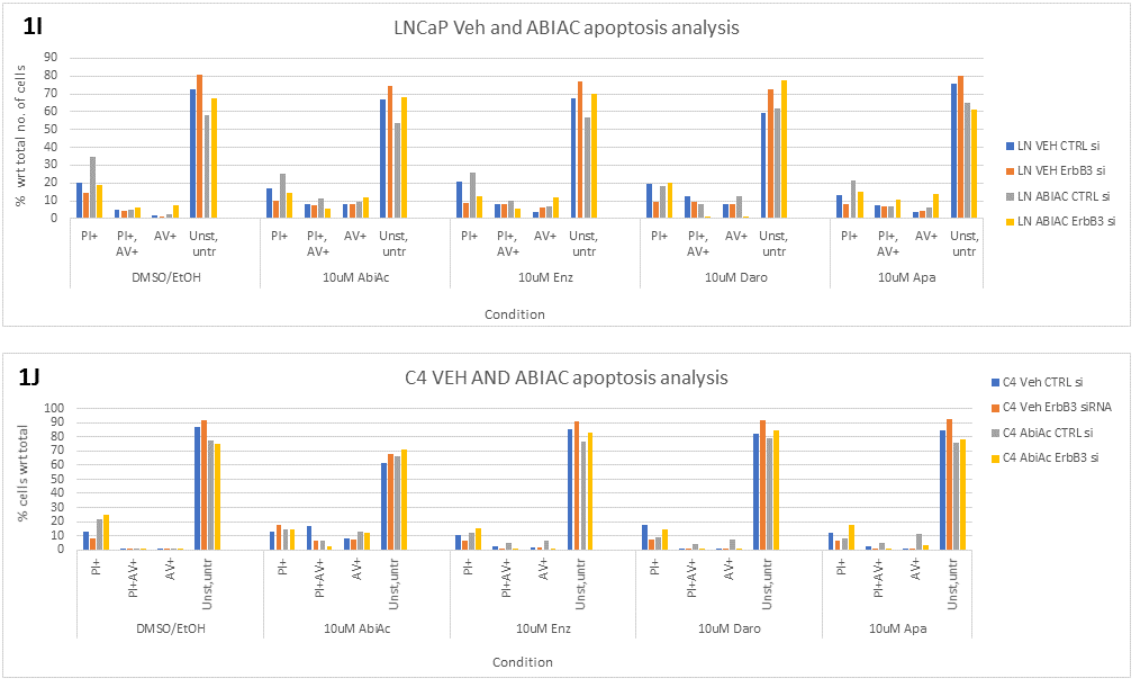




Similar responses were also seen when these cell lines were assayed in a head-to-head comparison with 10uM of the FDA-approved AR-inhibitors (AR-I), enzalutamide, darolutamide and apalutamide (1E-1H). Again, ErbB3 siRNA did not further decrease viability but strangely enough seemed to slightly increase viability. Based on unpublished observations from my doctoral work, this may suggest that inhibition of ErbB3 activates the AR (we know that the reverse is true, ie inhibition of AR increases ErbB3 expression (Chen L, Siddiqi S, Bose S et al, Canc Res, 70(14), 2010)), rendering the used concentrations of the AR-I ineffective.

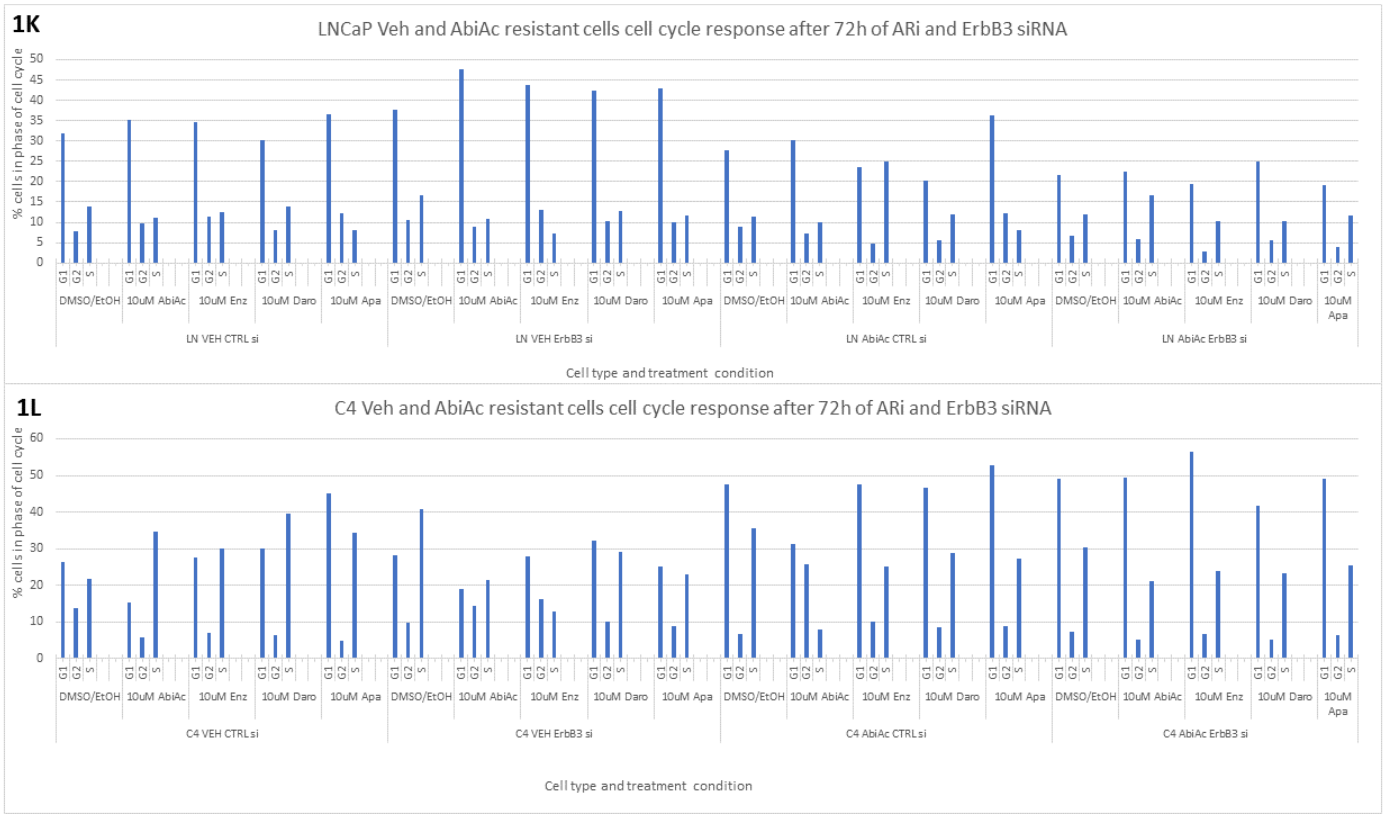


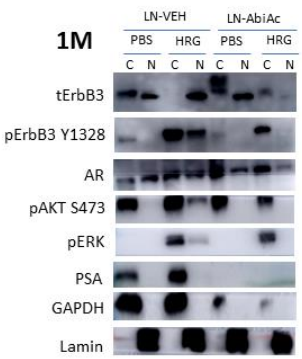
Consistent responses were again observed in cell death (1I, 1J) experiments in both LN and C4 Veh and Abi cell lines, where each resistant cell line showed increased cell death (blue vs grey-coloured bars) in response to AR-I. LN-Abi however did not show increased cell cycle arrest (1K, G1 bars) over LN-Veh in response to LN-Abi unlike C4-Abi with respect to C4-Veh (1K, G1 bars). In all cases, ErbB3 siRNA enhanced cell death via necrosis (PI+ bars, orange vs. yellow) in LN-Abi vs LN-Veh (1I) as well as C4-Abi vs C4-Veh (1J).

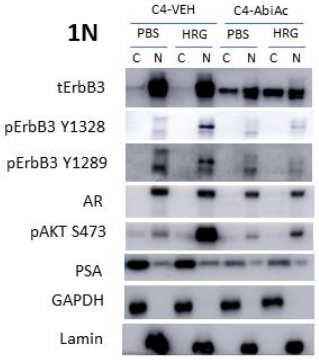


LN-Abi vs LN-Veh however showed decreased cell cycle arrest in the presence of AR-I and this was further

reduced when combined with ErbB3 siRNA (1K, G1 bars). C4-Abi vs C4-Veh however showed increased cell cycle arrest (1L, G1 bars) and this effect was enhanced when ErbB3 siRNA was applied to the cells.

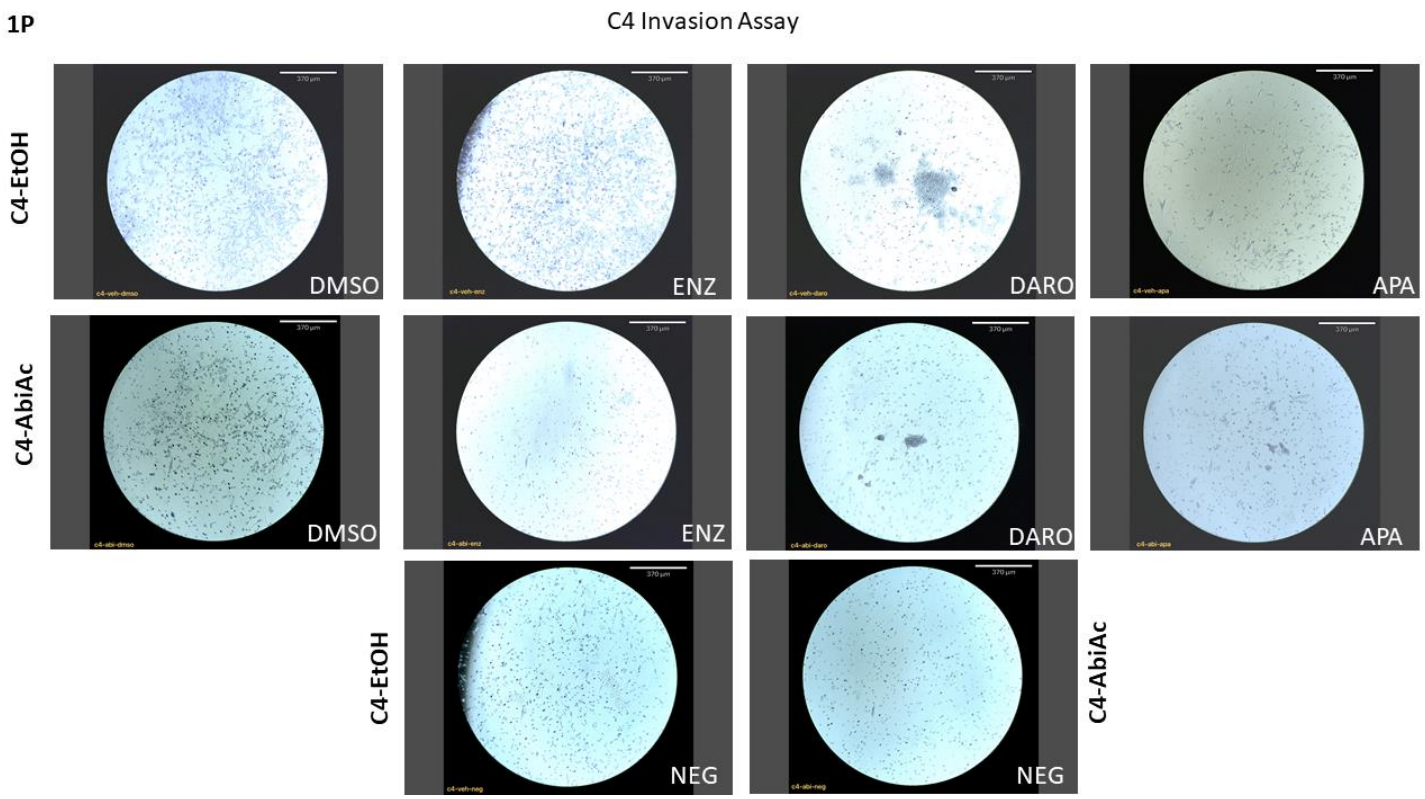


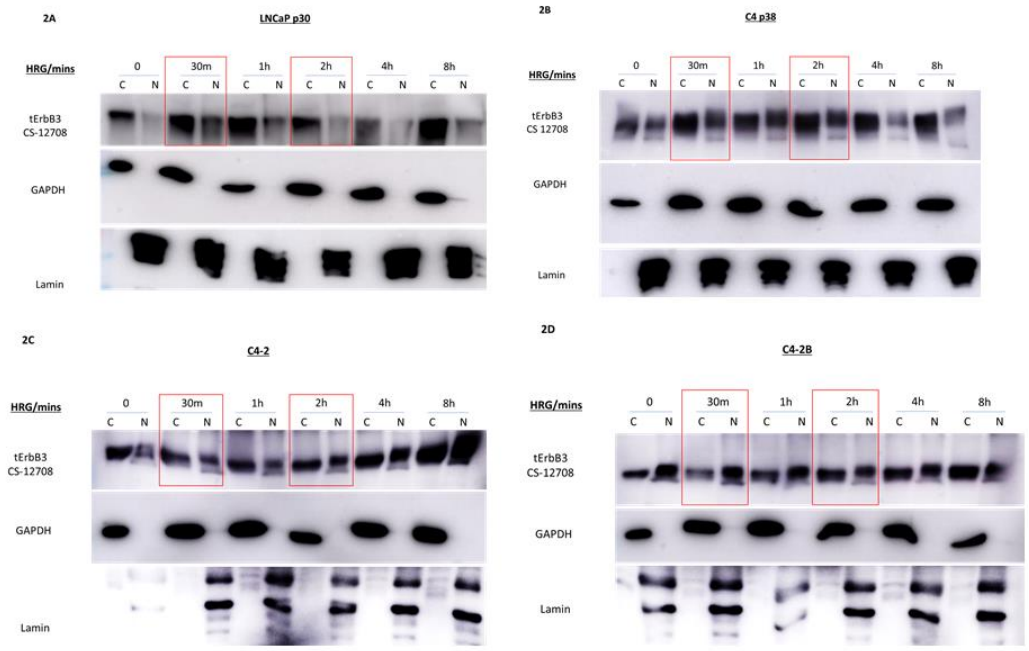




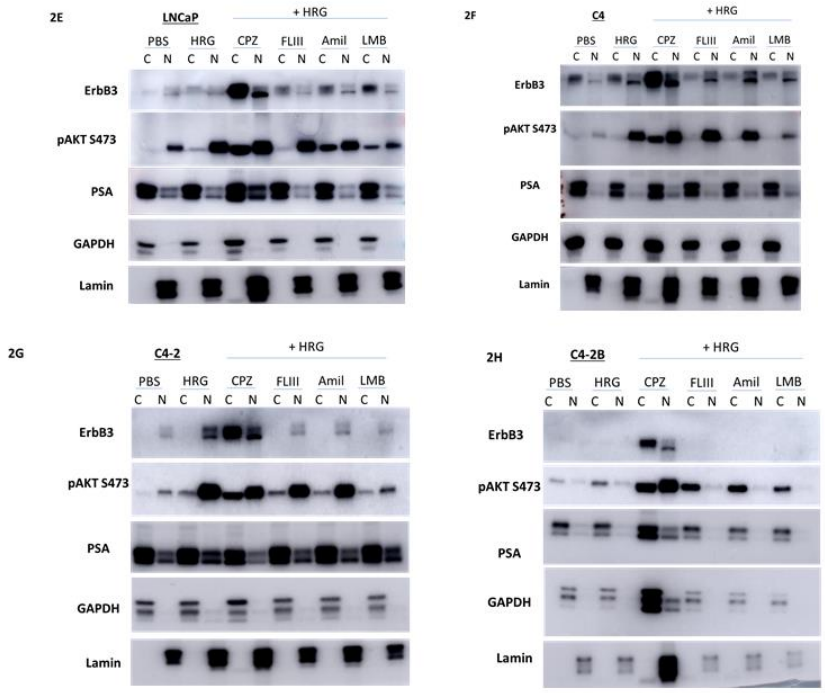
Upon examination of the protein levels of ErbB3 in fractionated cells, it was observed that LN-Abi cells showed increased cytoplasmic ErbB3 compared to LN-Veh (1M) whereas C4-Abi showed persistent nuclear ErbB3 when compared to C4-Veh (1N), lending support to the central hypothesis that nuclear ErbB3 may render cells sensitive to AR-I. The efficacy of ErbB3 siRNA is shown in 1O.

Migration of C4-Abi cells was also significantly decreased in the presence of AR-I (1P) as visualized in the decreased intensity of crystal violet staining. Experiments in LN-Veh/Abi as well as C4-2 and C4-2B are currently underway, as well as in the presence and absence of ErbB3 siRNA. Additional data depicting raw flow cytometry data and immunofluorescent verification of ErbB3 siRNA can be found in the Appendix, Supplementary Figures S1-S7.

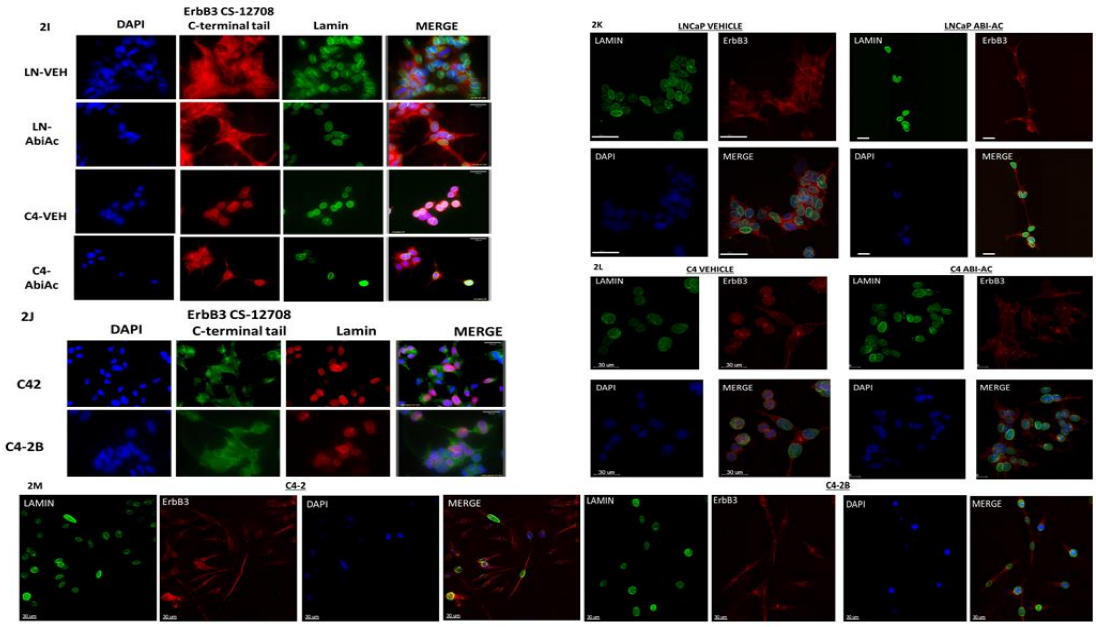




The next goal was to determine which endocytic and nuclear import-export pathways might be responsible for the differences in ErbB3 localization. HRG-1β is an ErbB3-specific ligand that is known to cause movement of ErbB3 to and from the plasma membrane. Cell lines constituting the tumor progression model were first treated with 50ng/ul of HRG-1β and analysed by fractionation at various time points to determine at what time ErbB3 localization was altered ie what time it appeared in the nuclear fraction. In all cell lines tested, ErbB3 appeared in the nuclear fraction around 30 mins to 1h after stimulation with HRG-1β (2A-2D). Therefore this time point was chosen as the starting point for subsequent experiments with the various pharmacological inhibitors of ErbB3 transport.

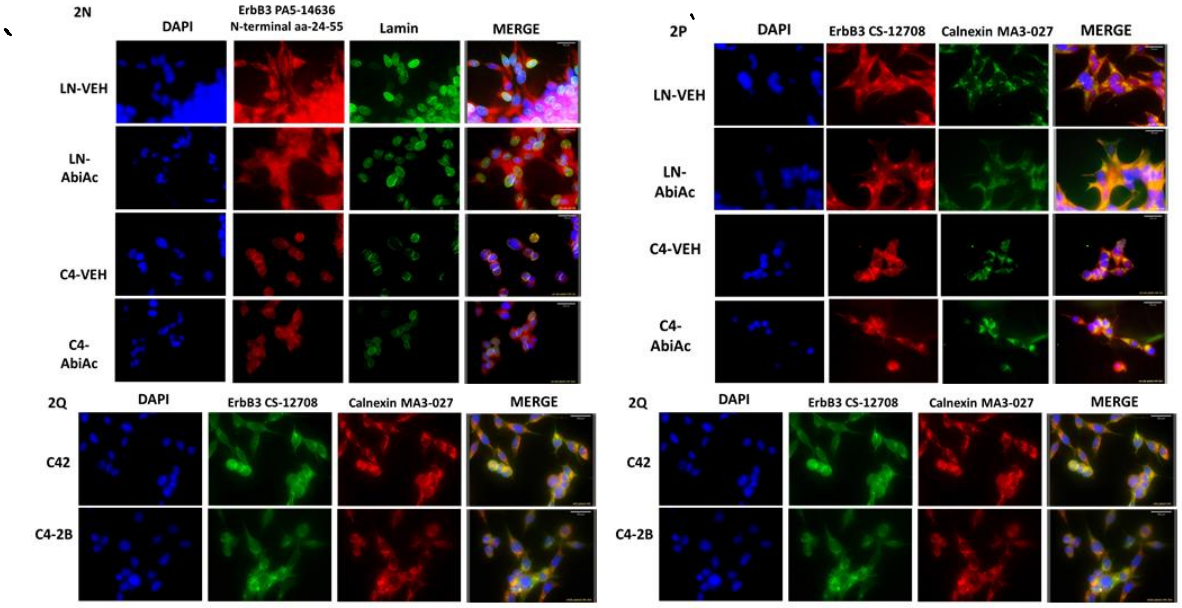


1mM chlorpromazine (CPZ, inhibitor of clathrin-dependent endocytosis), 1ug/ml filipin III (FLIII, inhibitor of clathrin-independent endocytosis), 1mM amiloride hydrochloride (Amil, inhibitor of macropinocytosis) and 2nM leptomycin B (LMB, inhibitor of nuclear import-export) were applied to all the cell lines for 1h in the presence or absence of HRG-1β. Concentrations of the various inhibitors were chosen after conducting an extensive literature search and using the most commonly-applied doses that were non-toxic to the cells. CPZ showed the largest increase in cytoplasmic ErbB3 in the presence of HRG in all cell lines tested (2E-2H) indicating that clathrin-dependent endocytosis might be the preferred internalization mechanism for ErbB3. FLIII as well as Amil showed modest increases in cytoplasmic ErbB3 in LNCaP cells only, suggesting the activity but not predominance of these internalization routes. LMB caused an accumulation of cytoplasmic ErbB3 only in hormone-sensitive LNCaP cells (2E) suggesting that the nuclear import import pathway was active only in this cell type, at the concentration tested. None of the endocytosis inhibitors were tested at higher concentrations to avoid toxicity.

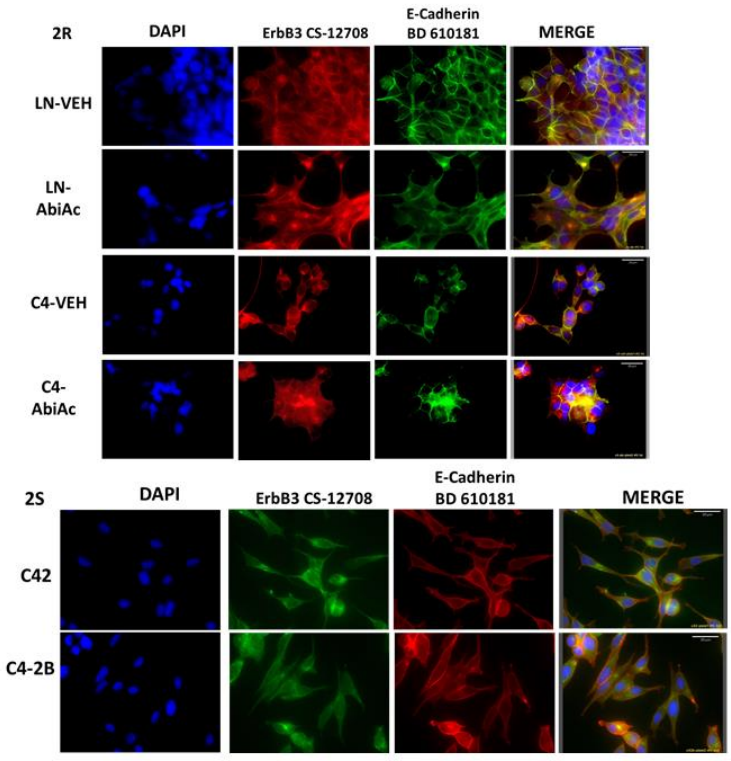


Immunofluorescent microscopy on the drug resistant cells showed that C-terminal ErbB3 localized prominently with Lamin only in C4 Veh cells (2I , lower boxes) and this localization was not disrupted by continuous culture in AbiAc (2I, lower). No such phenomenon was observed in LNCaP veh and Abi cells (2I, upper boxes) or in C4-2 and C4-2B cells (2J). Confocal microscopy performed at the UC Davis Advanced Imaging Facility on a Leica SP8 (keeping laser power, detector gain, size of z-stack acquired, pixel size and resolution constant across all cell lines imaged) revealed that ErbB3 levels decreased and lamin levels increased in Abi vs Veh cells for both LNCaP (2K) and C4 cells (2L). C4-2 cells (2M, left) and C4-2B cells (2M, right) did not display increased lamin or alterations in ErbB3 protein. Given that lamin is an outer nuclear membrane protein which is also known to play a role in transcription, an exciting future avenue for this research would be to explore the interactions between lamin and ErbB3 in drug-resistant prostate cancer cells,

and whether these interactions might be responsible for altering the transcriptional profile in treatment-resistant vs. treatment responsive cells. Multiple immunofluorescent panels were also created to ascertain the localization of ErbB3 with various subcellular markers depicting the nuclear membrane (lamin), cytoplasm (calnexin) and membrane compartments (E-Cadherin). ErbB3 colocalized significantly with Lamin only in C4-Abi cells (2N, lower) and this was seen with antibodies targeted towards the N- (2N, lower) and C-terminii of ErbB3 indicating the full-length receptor.



C-terminal ErbB3 prominently colocalized with cytoplasmic calnexin in all cell lines tested (2P, 2Q) and with membrane E-Cadherin in all but the C4-2B cell line (2S, lower).



The third goal was to design, create and test ErbB3 constructs with one or both putative nuclear localization sequences (NLS) mutated. This goal is currently underway, with the ErbB3 construct being cloned into the high copy, mammalian cloning and expression vector pcDNA 3.1(+), with 3 copies of the haemagglutinin (HA) tag being placed at the N-terminal end of the ErbB3 sequence, inframe with its coding sequence and open reading frame. The 5' cloning site is KpnI and the 3' cloning site is BamIII. This vector does not contain a prostate-specific promoter. A kanamycin selection gene has also been inserted at the N-terminal end, just proximal to the 3 x HA tag. Each putative ErbB3 NLS will be converted to AAAA (sequence of 4 alanines) and the resulting constructs transfected into low-ErbB3 expressing RWPE-1 cells and tested for subcellular localization and ability to alter physiological responses of cell to AR-I. Data acquisition is currently ongoing for this goal.

What opportunities for training and professional development has the project provided?

I was awarded a full tuition scholarship to attend a full-time, 5 day, virtual, comprehensive flow cytometry course conducted annually by the UC Davis Shared Resource Flow Cytometry Core (SRFCC). A total of 5 such scholarships are awarded by the SRFCC based on a competitive application process which is open to everyone (ie not restricted to UC Davis applicants) . This course covered flow cytometry basics, cytometer optics, fluidics and electronics, experimental design and troubleshooting, to name a few topics covered. Sessions were conducted by Dr. Bridget Mclaughlin, the Technical Director of the SRFCC, who has over 20 years of experience in flow cytometry and Mr. Jonathan van Dyke, senior Research Technician with over 10 years of experience in flow cytometry. This course was instrumental to me as I conducted my flow cytometry experiments pertaining to this project.
I was also awarded a scholarship to participate in a 9 month course on leadership, entrepreneurship and innovation called 'Leaders for the Future' and offered by the UC Davis Mike and Renee Child Institute for innovation and Entrepreneurship. This too was via an open, competitive application process which included a written application as well as individual and group interviews prior to selection. This program has helped me explore options and learn the skills that I will need to excel in diverse careers such as industry, government and NGOs, should I decide to purse a non-academic route.
As part of my training and service to the research community, I have also served as Chair of a virtual poster session at the 2021 Annual Meeting of the American Society for Biochemistry and Molecular Biology (ASBMB). At the intramural level, I served as a judge for the virtual poster session conducted by the Graduate Group in Integrative Pathobiology at UC Davis at their 2021 Annual Research Symposium, which was also the group that I conducted my PhD research in during my doctoral studies at UC-Davis.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

During the next period (Y2) I will continue to characterize and study the ErbB3 nuclear localization sequence (NLS) mutants generated in Y1 to determine what effects they may on response to androgen receptor signalling inhibitors (ARSI). I will also conduct RNA-seq and ChIP-seq experiments to learn what transcripts and signalling pathways may be involved in drug-resistant cells, as well cells overexpressing ErbB3 mutants. I will also attempt to obtain patient serum samples to detect the presence of full-length and soluble ErbB3 and learn whether levels of these proteins showed any differences depending upon the patient's response to ARSI. I will also use my findings to apply for federal, state, foundation and institutional grants to continue studying unexplored roles of ErbB3 and its targets or effectors in prostate cancer thereby embarking upon a pathway to complete research independence.

What was the impact on the development of the principal discipline(s) of the project?

The findings generated thus far will add to the knowledge of ErbB3 in prostate cancer, particularly with emphasis on the subcellular localization of this protein in and its role in mediating response or resistance to androgen inhibitory therapy. While knowledge about ErbB3 exists for other cancers such as breast, lung and ovarian (to name a few) less is known about the role of this receptor in prostate tumors. The cell lines generated will add to the existing cell culture models for the study of drug-resistant prostate cancer and eventually may also be made available upon request for use by the prostate cancer community for further investigation.

What was the impact on other disciplines?

It is possible that findings generated thus far may also be applicable to other tumors where ErbB3 is expressed (see above box) and where the data may not currently exist.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

The biggest experimental challenge faced was the significant delay in generating the drug-resistant LNCaP and C4 cell lines with their comparable vehicle-treated controls as well creating sufficient amounts of their cryopreserved stocks. LNCaP were particularly slow growing. While it was initially expected that this process would take 2 months (April-June) by the time sufficient material was available to begin generating data nearly 9 months had elapsed. This was overcome in three major ways (1) by attempting to use the smallest number of cells, smallest format of labware ie 35mm dish instead of 6cm dish etc (ie 'downscaling') for the experiment without compromising accuracy, scientific rigor or reproducibility, (2) by utilizing the time to engage virtually in learning and training activities eg online courses, seminars, workshops etc and finally (3) by conducting experiments that did not involve these cell lines (those pertaining to endocytic mechanisms of ErbB3 which utilized the tumor progression model), exploring the literature and conducting a few simple experiments other novel hypotheses pertaining to the nuclear localization of ErbB3, focusing on one compound called amiloride (which was also used in the experiments pertaining to this project). Basic findings were presented as virtual poster at the Annual Meeting of the Society for Basic Urologic Research (SBUR) in October 2021.

Another surprising outcome involved the lack of efficacy of the ErbB3 siRNA used. This will be overcome by designing targeted siRNA, failing which CRISPR knockout lines of ErbB3 will be created. To the best of my knowledge there exists no such ErbB3 knockout prostate cancer cell line, therefore this research would likely be the first to generate such a product. I also acknowledge that while ErbB3 gene knockouts are not typically found in prostate cancer (questioning the physiological relevance of this model) it will serve as an indicator of the function of ErbB3 in drug-resistant prostate cancer cells.

The effect of supply chain issues and staffing restructuring as a result of the COVID-19 pandemic also contributed to delays in ordering, processing and delivery times of research-related reagents and materials. Obtaining reagents and materials from vendors that were not available on the university marketplace required additional paperwork and procedures, adding to the delays.

Changes that had a significant impact on expenditures

Favourable significant changes in expenditure included the kind gifts of all cell lines used in this proposal by a senior Principal Investigator and long-time collaborator (Dr. Paramita M Ghosh) of my Primary Mentor (Dr. Maria M Mudryj). I will take advantage of this by now conducting advanced flow cytometry experiments to determine the ErbB3 status of drug-resistant cells that do or do not undergo early or late apoptosis or necrosis in response to AR-I.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals, biohazards and/or select agents

Nothing to report.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

1. "Nuclear/perinuclear localization of ErbB3 in prostate cancer and its role in regulating ligand-dependent activation of ErbB2/ErbB3 heterodimers and its downstream targets", Jathal MK, Mudryj MM, Chen HW, Drake CM, Ghosh PM, Annual Meeting of the Society for Basic Urologic Research, October 2021, virtual poster.

- **Website(s) or other Internet site(s)**

Nothing to report.

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- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

LNCaP and C4 cell lines that are resistant to 5uM and 10uM abiraterone acetate respectively (along with their appropriate vehicle-treated control cell lines) have been generated by this project. Also generated will bee ErbB3 constructs with one or both nuclear localization sequences (NLS) mutated in order to ascertain the function of ErbB3 and its role in mediating response or resistance to AR-I when its subcellular localization is impaired.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Maitreyee Jathal
Project Role: Postdoctoral Principal Investigator
Researcher Identifier: ORCID number
Nearest person month worked: 12
Contribution to Project: Dr. Jathal has performed all aspects of experimental design, execution, data collection, analysis, literature search, troubleshooting, preparing data for presentation and publication.
Funding Support: CDMRP PC20063 Award Number# W81XWH2110073

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

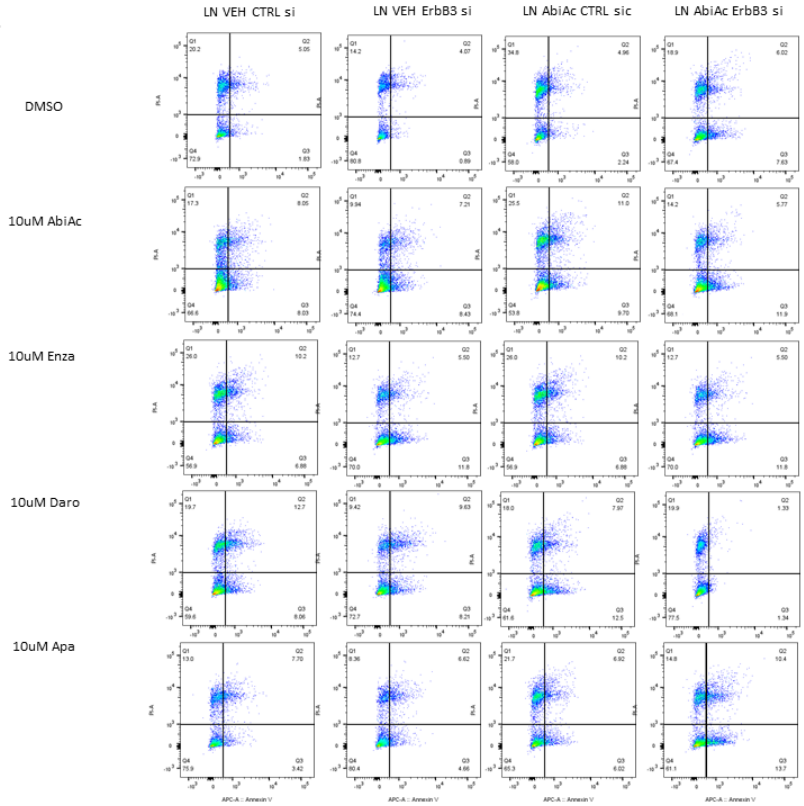
COLLABORATIVE AWARDS

QUAD CHARTS

Nothing to report.

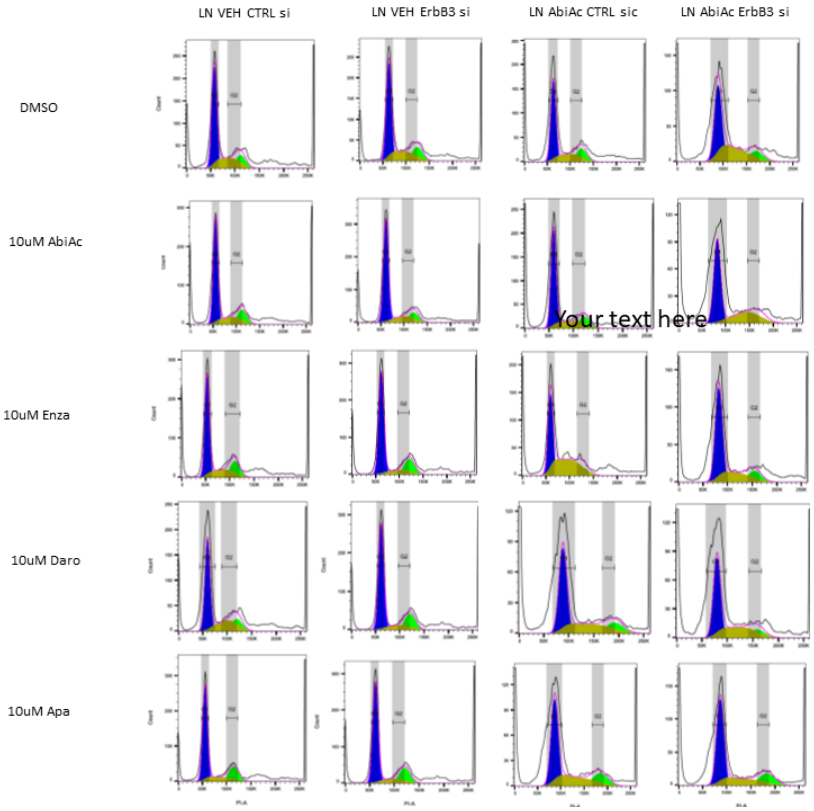
9. APPENDICES

S1



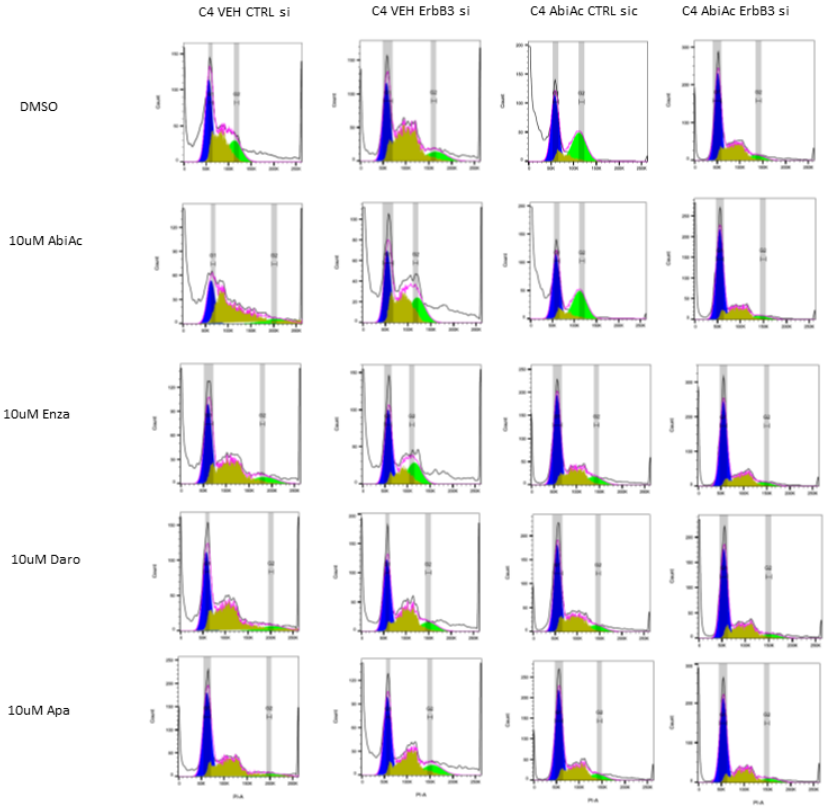
Cell death in LN-Veh and LN-Abi cells in response to AR-I and ErbB3 siRNA

S3

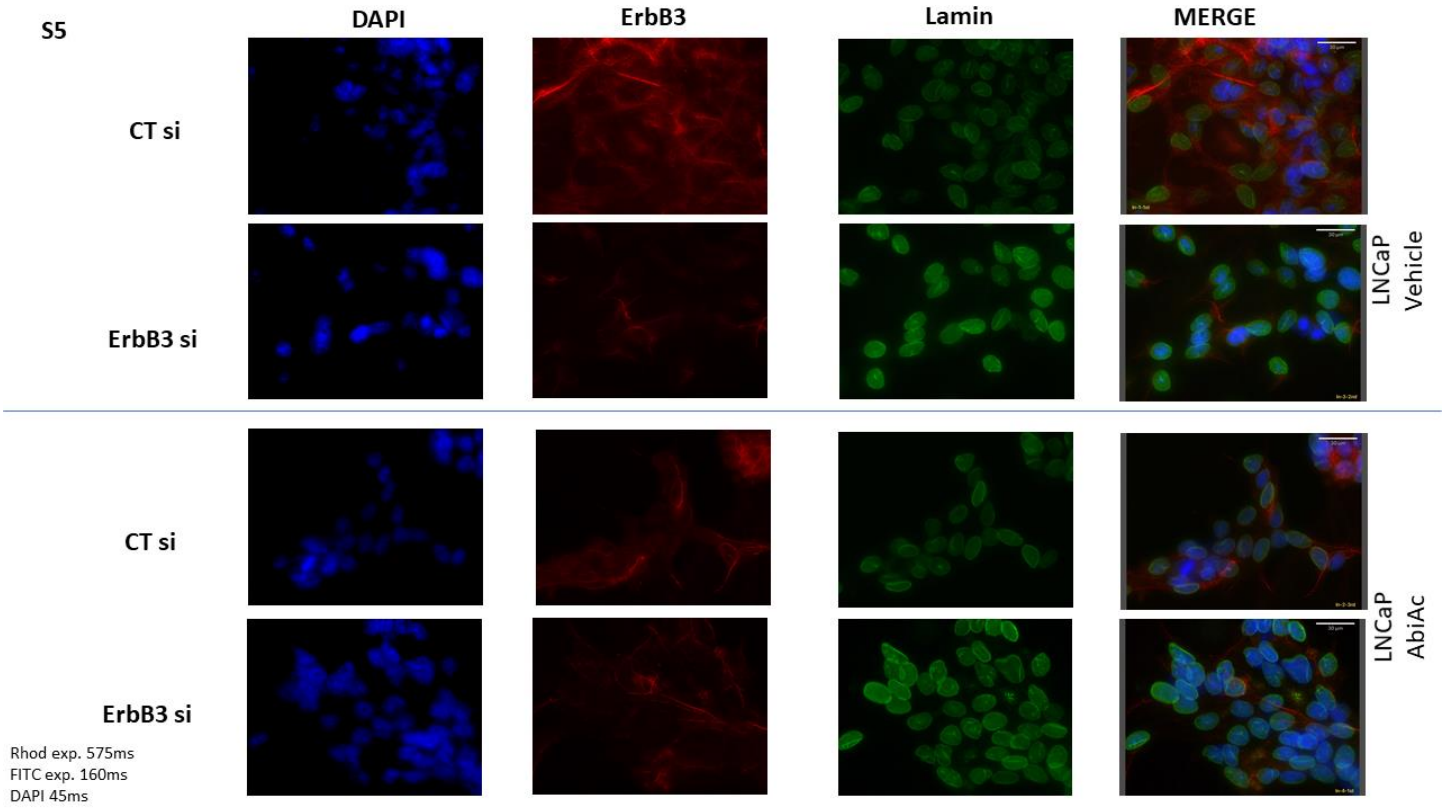


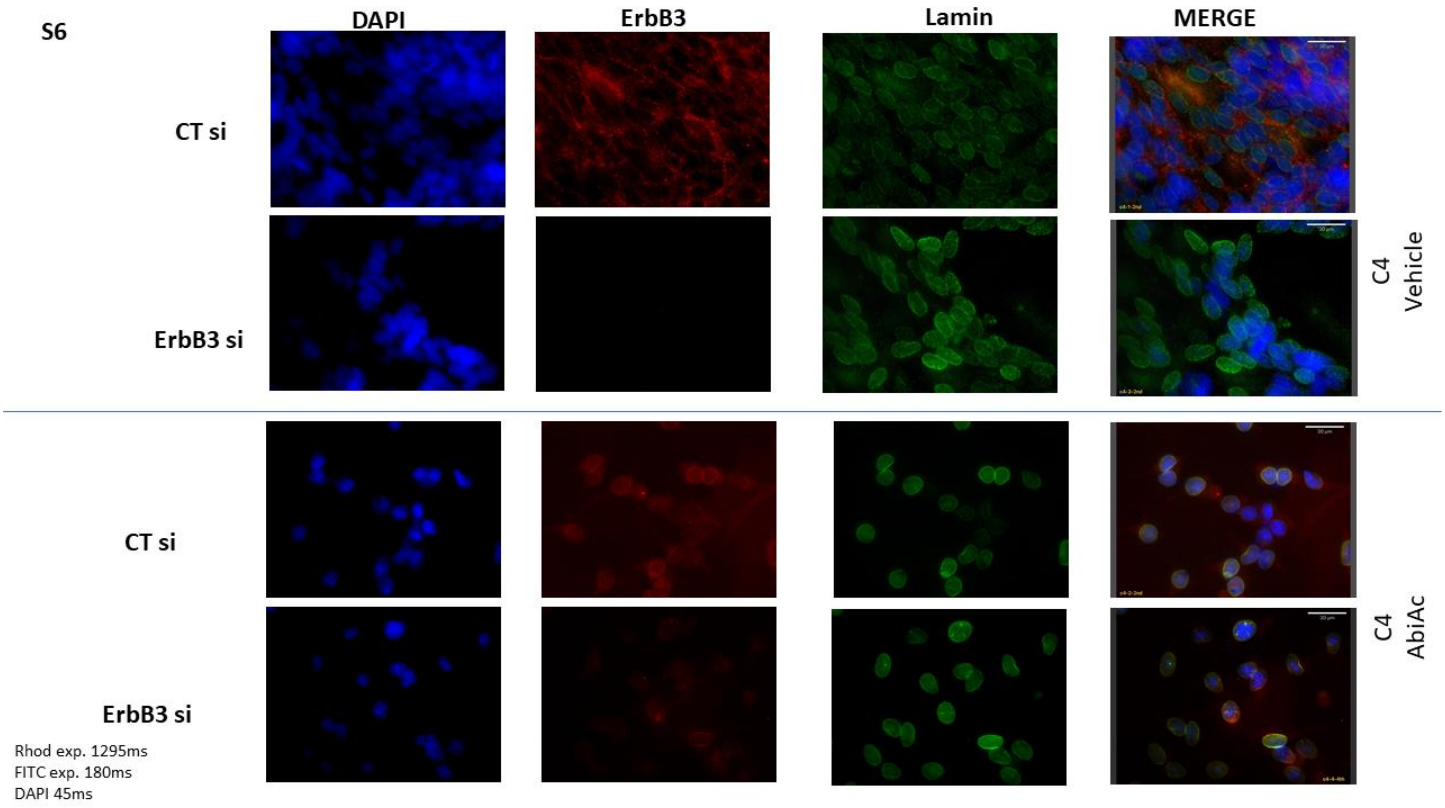
Cell cycle analysis in LN-Veh and LN-Abi cells in response to AR-I and ErbB3 siRNA

S4

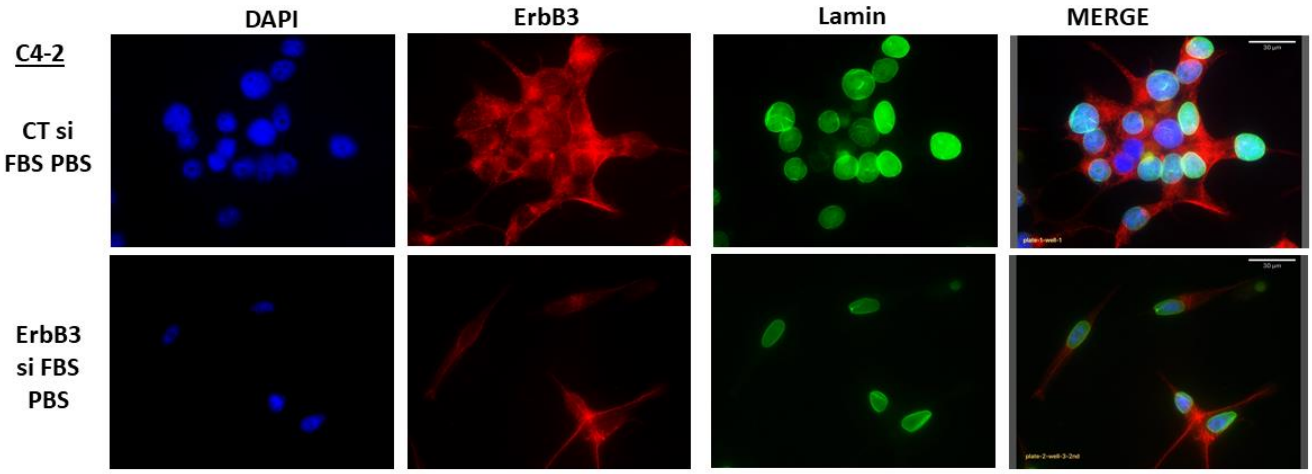


Cell cycle analysis in C4-Veh and C4-Abi cells in response to AR-I and ErbB3 siRNA


S5
DAPI
ErbB3
Lamin
MERGE
CT si
ErbB3 si
LNCaP Vehicle
CT si
ErbB3 si
LNCaP AbiAc
Rhod exp. 575ms
FITC exp. 160ms
DAPI 45ms

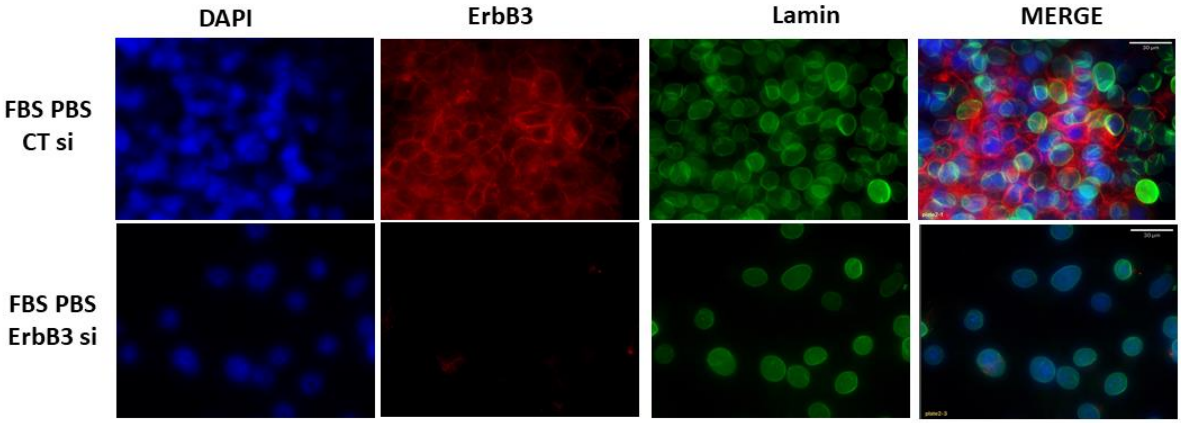

S6
DAPI
ErbB3
Lamin
MERGE
CT si
ErbB3 si
C4 Vehicle
CT si
ErbB3 si
C4 AbiAc
Rhod exp. 1295ms
FITC exp. 180ms
DAPI 45ms

S7



Rhod exp. 635ms
FITC exp. 160ms
DAPI 20ms

S8



Rhod exp. 305ms
FITC exp. 45ms
DAPI 10ms