Development of an inducible Cas9 for temporally-controlled CRISPR gene editing

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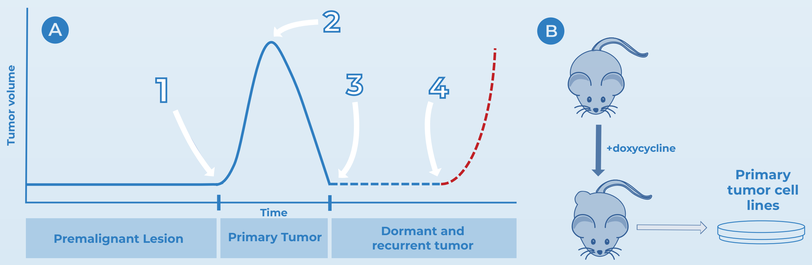
Abstract

Breast cancer is a leading cause of cancer-related death among women in the United States. Of these fatalities, a majority are due to recurrence of disease years after primary treatment. While the field of cancer biology has made significant progress in explaining the formation and behavior of these instances of primary disease, the epigenomic etiology of regression and recurrence that often follows is far less understood. To better address these issues, we optimized a CRISPR-Cas9 endonuclease system with inducible editing capabilities to conduct knockout screens in a HER2-expressing mammary adenocarcinoma. Once it has been refined for use *in vivo*, this system may provide an unprecedented window into late cancer behavior and disease resurfacing.

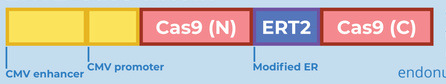
Tumor Progression



Timing of tumorigenesis, dormancy, and recurrence. Progression of adenocarcinoma is marked by four discrete phases, as indicated in **figure A**. At each of the four timepoints, the knockout of target oncogenes by guided Cas9 endonuclease activity offer insight as to the role of genomic features in facilitating tumor formation, survival, quiescence, and eventual recurrence. As shown in **figure B**, primary adenocarcinomas are propagated in a MMTV-rt-TA; Tet-Her2 (MTB/TAN) murine model through administration of doxycycline. Tissue samples are extracted at the inception of primary and recurrent tumor growth and cultured for in-vitro experimentation.

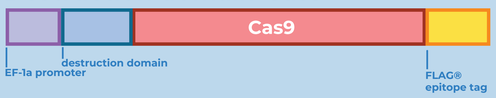
Mechanisms of Cas9 Inducibility

ERT2-Cas9 intein



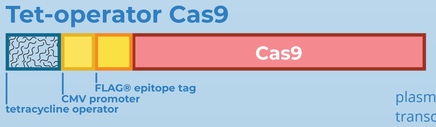
Catalytic Inhibition. Once the protein is translated, the structural orientation of an ERT2 intein domain obstructs the RuvC+ catalytic site of Cas9, restricting endonuclease activity. Binding of 4-hydroxytamoxifen to ERT2 triggers intramolecular cleavage of the receptor, thereby rescuing Cas9 activity.

Destruction domain-Cas9



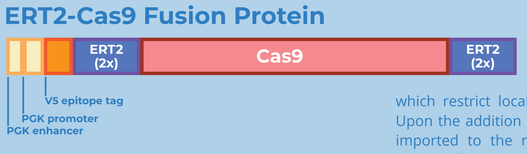
Transcript Destabilization. A destruction domain upstream of the Cas9 transgene destabilizes Cas9 transcripts in the absence of shield1, a small-molecule ligand capable of stabilizing the complex.

Tet-operator Cas9



Transcriptional Enhancement. When introduced into the genome of a tet-controlled transcriptional activator-expressing (tTA+) cell, this recombinant plasmid allows for modulation of Cas9 transgene transcription by administration of tetracycline derivatives. Due to the dependence of the MTB/TAN model on a doxycycline, data from *in vitro* inducible Tet-o Cas9 optimization was not included in this presentation.

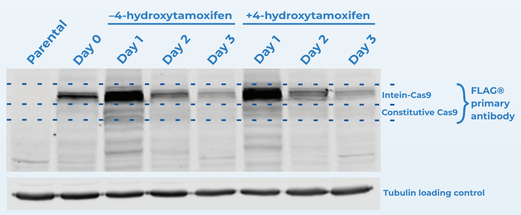
ERT2-Cas9 Fusion Protein

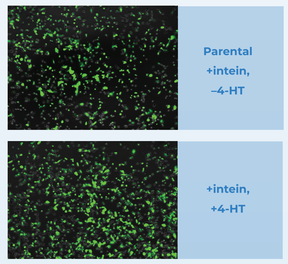


Spatiotemporal Localization. Cas9 is translated with two ERT2 estrogen receptors affixed to its N and C terminii, which restrict localization of the complex to the nucleus. Upon the addition of an estrogen analogue, the complex is imported to the nucleus, where it begins surveying the genome for target sequence(s).

Mechanisms in vitro

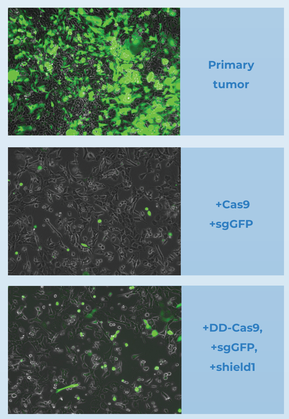
ERT2-Cas9 intein





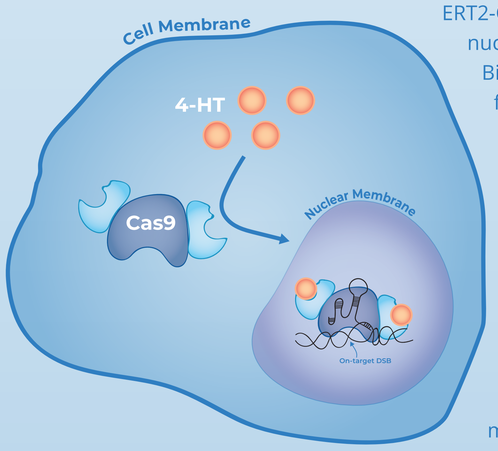
Primary HER2-positive tumor cells were transduced with a lentiviral ERT2-Cas9 intein vector and assayed in a western blot to assess functional Cas9 transcript translation efficiency. In the presence of 4-HT, formation of active Cas9 complexes was not observed, suggestig that ligand binding was interrupted at the post-translational level.

Destruction domain-Cas9



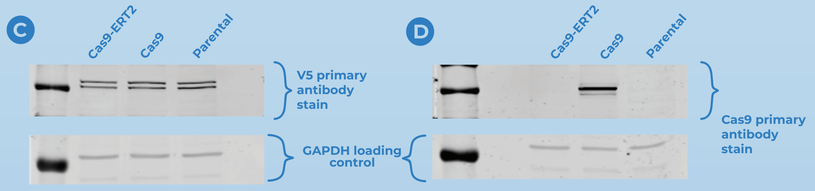
Primary HER2+ cells were transduced with a lentiviral DD-Cas9 vector. Compared to the +shield1 condition, active Cas9 expression in cells deprived of shield1 was expected to decrease and produce higher levels of EGFP expression. The presence of a band in this lane, along with the image to the right, suggests that Cas9-mediated knockdown may be acheived in cells to which no FKBP12-binding ligand had been administered.

ERT2-Cas9 Fusion Protein



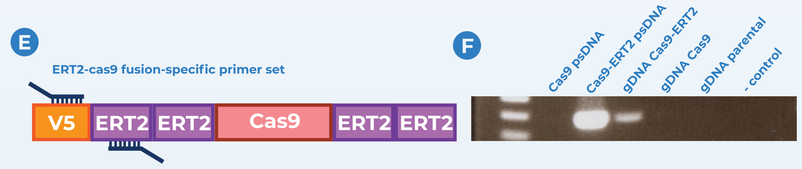
In the absence of 4-hydroxytamoxifen, ERT2-Cas9 is excluded from the nucleus of the mammalian cell. Binding tamoxifen allows the fusion protein to localize to the nucleus, bind with guide RNAs, and cleave target sequence(s) of genomic DNA.

The lack of interference with key signaling pathways of the MTB/TAN model for in vivo studies of tumor dynamics makes this system a highly compatible choice for mediating inducible gene edits.



Western blot of CRISPR-associated transgenic cell lines. Following transduction of the primary HER2-on tumor cells with Cas9 and ERT2-Cas9 lentiviral vectors, the cells were lysed with RIPA buffer and assayed on an 8% polyacrylamide gel. Figure C depicts primary antibody staining for the V5 epitope tag specific to the ERT2-Cas9 fusion protein. Although no cas9-specific band was observed in the ERT2-Cas9 lane of figure D, genomic DNA from three populations were assayed to select for an ERT2-cas9 amplicon (figures E & F).

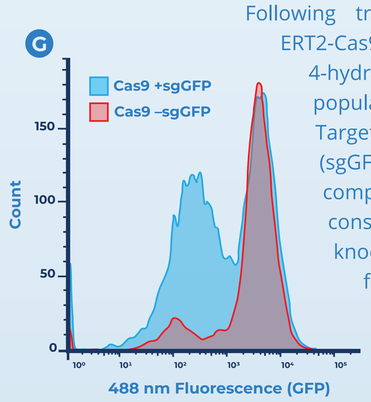
Primer Design and Validation

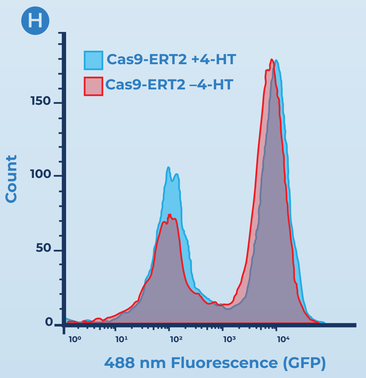


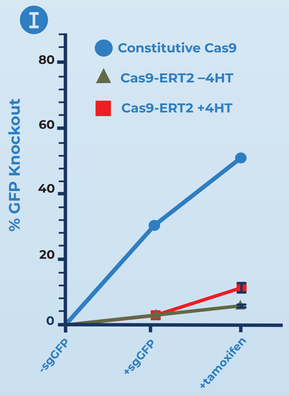
Probing pre-transcriptional expression. To assess transduction efficiency of the ERT2-Cas9 vector in our cancer cell lines, sequence-specific primers were designed using NCBI Primer BLAST as shown in **figure E**. The amplicon visualized in **figure F** is nearly 290 basepairs in length, suggesting that the 292 basepair target sequence was present in transduced cells.

Functional Assay

Following transduction, one population of primary, ERT2-Cas9-expressing cells were cultured in 10 uM 4-hydroxytamoxifen media, along with one population cultured in tamoxifen free media. Target knockout directed by small guide GFP (sgGFP) was analyzed using flow cytometry in comparison to parental cells transduced with constitutively-active Cas9. **Figures G** and **H** depict knockout of GFP in the range of 488 nm fluorescence. **Figure I** offer a comparison of on-target knockout efficiency between Cas9 and ERT2-Cas9 populations.







Implications/Future Work

HER2 positive primary tumor cells were successfully transduced with a ERT2-Cas9 lentiviral vector and cultured at high confluency. In the presence of 4-hydroxytamoxifen, the ERT2-Cas9 mechanism mediated knockout of target DNA sequences of genomic DNA. To better characterize the efficacy of this inducible-Cas9 mechanism in the context of the HER2 positive cell model, future work will aim to collect flow cytometry data at successive timepoints following 4-HT administration. Ultimately, this inducible Cas9 system may be implemented orthotopically in the MTB/TAN mouse model to provide temporal control over *in vivo* gene editing.

Aknowledgements

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