

6-month Progress Report for APA Young Investigator in Pancreatology Award:

The bulk of progress made on the proposed work during this time has centered on the second aim that seeks to uncouple mechanisms driving local asparagine production and release by PDA cells. The premise of this project stems from our observation that multiple metabolic subtypes of cancer cells co-exist within pancreatic tumors. We had previously shown that constitutive ATF4 activation, a key mediator of the integrated stress response (ISR) in clonal populations of pancreatic cancer cells results in upregulation of asparagine synthetase (ASNS). ASNS is the enzyme responsible for biosynthesis of asparagine from aspartate. In the stressed (ISR-HI) cells, asparagine is produced in excess, released from the ATF4-high stressed cells, and results in supporting mitochondrial metabolism of surrounding cells (**Fig. 1a**).

The master stress response transcription factor ATF4 is activated in response to numerous stressors. These include the unfolded protein response, endoplasmic reticulum stress, viral infection, and amino acid restriction. Assessing the activation of the critical nodes mediating these pathways, we observed that only the amino acid response kinase General control nonderepressible 2 (GCN2) activation separated the clonal groups.

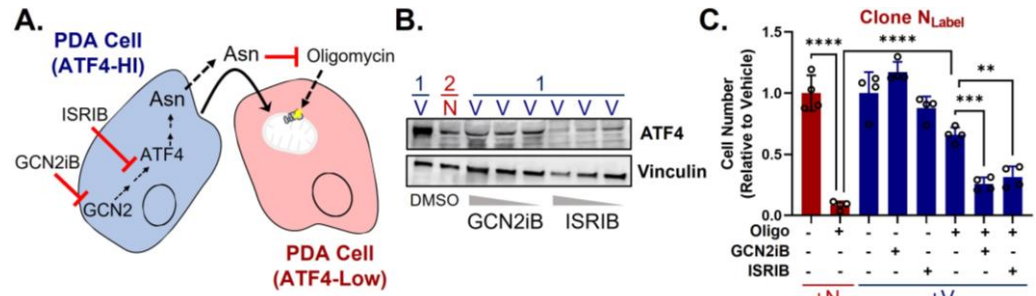


Figure 1: Constitutively activated integrated stress response (ISR) fuels rescue in co-culture models. **A.** Activated GCN2 in oligomycin resistant (ATF4-HI) PDA cells drives expression of ATF4 to produce asparagine and support mitochondrial metabolism of oligomycin sensitive (ATF4-low) cells, which can be targeted with pharmacological inhibitors to GCN2 (GCN2iB) or ATF4 activation (ISRIB). **B.** ATF4 levels of ISR-HI (Group 1) clone V and reduced to levels of labeled ISR-low (Group 2) clone N with ISRIB and GCN2iB treatment in a dose dependent manner. **C.** Co-culture rescue of sensitive clone N by insensitive clone V is reduced with ISRIB and GCN2iB treatment.

Given the differential expression of the ISR between ETC inhibitor sensitive and insensitive PDA clones, we confirmed that targeting the GCN2-ATF4-ASNS axis would abrogate the metabolic crosstalk mediating the rescue of sensitive clones in co-cultures (**Fig. 1a**). Indeed, we observed that treating an insensitive, ISR-HI group 1 clone V with pharmacological a GCN2 inhibitor (GCN2iB) or ATF4 (ISRIB) inactivator reduced ATF4 levels to that of sensitive ISR-low group 2 clone N (**Fig. 1b**). Importantly, treatment of either GCN2iB or ISRIB in a fluorescent co-culture model blocked rescue of proliferation of labeled sensitive clone N treated with oligomycin in the presence of insensitive clone V (**Fig. 1c**). Taken together, these data demonstrate that targeting the GCN2-ATF4 axis to block asparagine production abrogates PDA crosstalk supporting proliferation during mitochondrial inhibition.

GCN2 activation of ATF4^{HI} indicates cells engaged in the amino acid stress response, indicating they are starved and poised on the edge of a metabolic crisis. Reduction-oxidation factor 1 (Ref-1) inhibitors recently developed by Melissa Fishel's group at the University of Indiana been shown to kill PDA cells undergoing a GCN2-driven stress response. Through a collaboration with Dr. Fishel, we obtained these inhibitors and observe a set of ATF4^{HI}

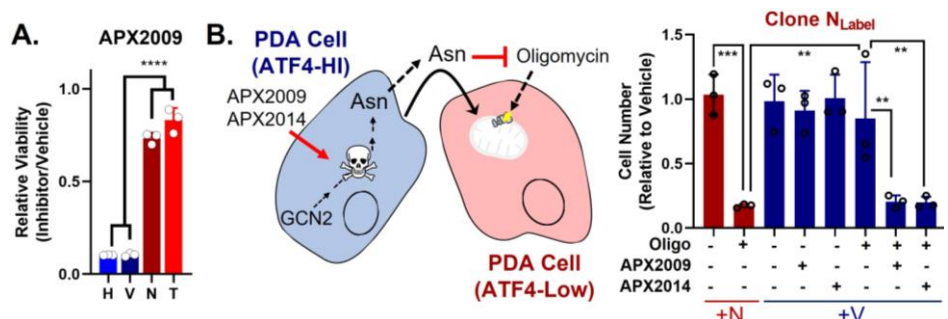


Figure 2: Ref-1 inhibitors potentially impair growth of ATF4^{HI} clones. **A.** Reduction-oxidation factor 1 (Ref-1) inhibitors APX2009 at 16μM shows selectivity for ATF4^{HI} (Group 1) clones. **B.** Co-culture rescue of Group 2 sensitive clone N by Group 1 insensitive clone V during oligomycin treatment is abrogated by addition of either APX2009 or APX2014.

clones are more sensitive to the Ref-1 inhibitors APX2009 and APX2014 (**Fig. 2a,b**). Given this, we tested if combining the APX compounds could act to impair metabolic crosstalk. Indeed, we observe that drug concentrations insufficient to impair the growth of group 2 clones can act to completely disrupt oligomycin co-culture rescue by group 1 (ATF4^{HI}) clones (**Fig. 2c**).

It has been postulated that these Ref-1 inhibitors act in synergy with induction of the ATF4 stress response, and we have seen that the group 2 (ATF4^{Low}) clones activate GCN2 when treated with oligomycin. Accordingly, in addition to targeting group 1 clones that have constitutive GNC2 activation, we hypothesize that combining mitochondrial inhibitors with APX will also eliminate the growth of group 2 (ATF4^{Low}) clones, and by extension, polyclonal tumors where these populations co-exist.

Moving forward on this aim, we will treat our other sets of human and murine clonal PDA lines with the APX inhibitors alone and in combination with ETC inhibitors to confirm the clonal selectivity of monotherapy for group 1 clones and synergism with mitochondrial inhibitors in group 2 clones, KPC cell lines, and our established patient-derived organoids (PDOs). Co-culture rescue experiments as in **Fig. 2b** will be repeated in our other independent murine and clonal combinations. We will perform synergy assays in polyclonal human and murine cells to determine if a combination treatment with these approaches is warranted. If we observe either an additive or synergistic relationship between APX compounds and electron transport chain inhibitors, we will move to treating murine tumor models and patient-derived organoids with this combination.

It is unclear how if the APX inhibitors (with or without mitochondrial inhibitors) are mediating growth inhibition, so we will determine the mechanism of cell death or cell cycle arrest. To test different forms of cell death we aim to co-treat group 1 clones with APX2009 and Necrostatin-1, ZVAD-FMK, bafilomycin A1, or ferrostatin treatment. As the activity of Ref-1 inhibition on cell growth is reported to require ATF4 activation, we will examine if treatment with GCN2iB or ISRIB rescues cells APX treated cells in each of these experiments.

Progress toward long-term funding for this work:

The data obtained shown above with the support of the APA Young Investigator in Pancreatology Award were included in a revised NIH R01 application (R01CA283575-01A1). The application was scored at an 8th percentile at the National Cancer Institute Cancer Cell Biology study section, leading to a high likelihood of sustained funding for this work.