Background: Mutations in SERCA2, an ER calcium pump, were discovered to cause Darier disease (DD) more than twenty years ago, yet there still remain no FDA-approved therapies for this rare disorder. Disease-linked mutations are predicted to cause SERCA2 haplo-insufficiency, but the downstream effects that directly cause epidermal breakdown in DD have not been sufficiently delineated to allow design of targeted therapies. *Our work funded by FIRST addresses this knowledge gap by harnessing novel in vitro DD models compatible with live microscopy and real-time biosensors, which we use to define DD pathogenic mechanisms and evaluate new treatment approaches.* Our RNAseq and proteomics datasets identified EGFR signaling and ER proteotoxic stress as candidate drivers of DD pathology; in ongoing work, we aim to define the role of these pathways in DD.

Simpson Lab Updates: Our recent open-access publication in *JCI Insight* identified a novel role for ERK hyperactivation in DD pathogenesis (Zaver *et al.* 2023; https://insight.jci.org/articles/view/170739) and suggests MEK inhibitors (already FDA-approved for other indications) could be effective for treating DD, including via topical delivery. Of note, this publication was featured in the FIRST newsletter and has led to multiple patients with DD reaching out to ask how they can get involved in our research. Building on momentum from our published DD model, I worked with Dr. Kathleen Green to organize the inaugural Darier and Hailey-Hailey Disease (DHHD) Symposium (https://www.pachyonychia.org/2024symposiums/) to be held just prior to the SID meeting in Dallas with the goal of summarizing ongoing translational research to inform future clinical trials for these skin blistering disorders. At the DHHD Symposium, I will have the opportunity to present my lab's FIRST-funded work on DD to an international gathering of experts interested in these rare diseases, including physician-scientists, basic researchers, clinical trialists, and industry representatives as well as a few patients and families. We hope this unique opportunity to share the latest research related to Darier and Hailey-Hailey disease will catalyze synergistic collaborations to help deliver the first FDA-approved drug for these orphan dermatologic disorders.

Research Progress: At 6 months into our FIRST grant funding period, most of our progress has been focused on **Aim 1** and is summarized below. Excitingly, we recruited a medical student to work alongside my technician on this FIRST-funded project full-time for 3 months this summer to accelerate our progress.

Aim 1: To determine if targeted inhibitors of the EGFR pathway or calcium signaling normalize desmosome assembly to restore intercellular adhesion in SERCA2-deficient keratinocytes. Epidermal growth factor receptor (EGFR) is a major regulator of keratinocyte differentiation and intercellular adhesion. Our bulk RNAseq dataset from *ATP2A2* heterozygous (HET) keratinocytes identified hyper-activation of the EGFR pathway, which we validated in DD biopsies. These data support *our hypothesis that existing drugs able to blunt EGFR activity or its downstream signaling mediators (like MEK or MAP kinases) may rescue cell-cell adhesion in SERCA2-deficient keratinocytes and could serve as novel DD therapies.*

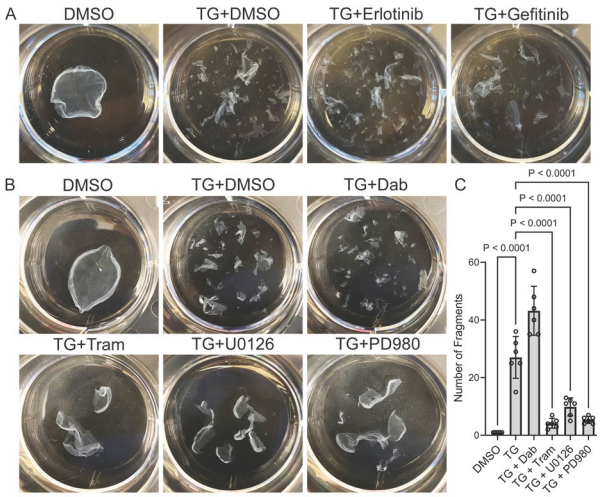

Fig. 1: MEK inhibitors overcome SERCA2 inhibition to rescue integrity of thapsigargin-treated cell sheets. (**A**) Treatment of normal human epidermal keratinocyte (NHEK) monolayers with thapsigargin (TG) disrupted intercellular adhesion to cause increased fragmentation upon mechanical stress; co-treatment with erlotinib or gefitinib failed to restore monolayer integrity. (**B**) While the BRAF inhibitor dabrafenib (Dab) did not overcome the effect of TG on NHEK monolayers, MEK inhibition using trametinib (Tram), U0126, or PD98059 reduced the fragmentation of SERCA2-inhibited monolayers. (**C**) Quantification of drug-treated monolayer fragments after mechanical stress (N=6).

Interestingly, our results indicate that direct inhibition of EGFR with the FDA-approved compounds erlotinib or gefitinib was *not* able to rescue intercellular adhesion in human keratinocyte monolayers treated with thapsigargin (TG), a selective SERCA2 inhibitor (**Fig. 1A**). However, we found that inhibition of MEK, a kinase downstream of EGFR that activates ERK, restored the integrity of SERCA2-inhibited keratinocyte monolayers (**Fig. 1B-C**). In fact, we confirmed that 3 different MEK inhibitors (trametinib, U0126, PD98059) were all able to strengthen intercellular adhesion despite TG treatment. Importantly, inhibiting the kinase upstream of MEK (BRAF) with dabrafenib failed to rescue cell-cell adhesion. These results support our proposal that selective inhibition of MEK, but not its upstream activators, is sufficient to overcome deficiency of SERCA2 and could serve as a novel therapeutic strategy for DD. We propose this may be related to off-target effects of EGFR or BRAF inhibition on other downstream pathways not affected by SERCA2 deficiency or due to compensatory cellular feedback responses to inhibition of upstream kinases.

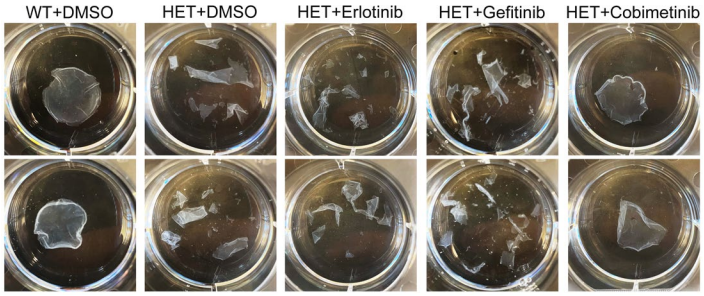

Fig. 2: Inhibition of MEK but not EGFR rescues integrity of SERCA2-deficient cell sheets. Compared to control cells (WT) two lines of *ATP2A2* heterozygous (HET) keratinocytes grown as monolayers exhibited reduced integrity upon mechanical stress. While treatment with the EGFR inhibitors erlotinib or gefitinib failed to rescue cohesion of HET cells, the MEK inhibitor cobimetinib bolstered cell-cell adhesion to levels comparable to WT cells.

To validate these results, we treated *ATP2A2* heterozygous (HET) cells haplo-insufficient for SERCA2 with similar inhibitors (**Fig. 2**). Our data again demonstrate that inhibiting EGFR did ***not*** rescue intercellular adhesion in HET cells. However, in our recent publication, we demonstrated rescue of the integrity of SERCA2-deficient epithelial sheets upon treatment with trametinib (Zaver *et al.* 2023, *JCI Insight*), which is already FDA-approved for treatment of BRAF mutant cancers and has been successfully used topically for treatment of a MAP kinase-driven dermatosis (Fay *et al*. 2023, *JAAD Case Reports*: https://doi.org/10.1016/j.jdcr.2023.06.038). Our more recent data from this funding period revealed that two additional MEK inhibitors that are also FDA-approved for other disease indications, cobimetinib (**Fig. 2**) and selumetinib (not shown), were similarly able to suppress ERK overactivation in keratinocytes and enhanced intercellular adhesive strength. These results have allowed us to hone our model of DD pathogenesis to propose that suppressing overactivation of MEK signaling downstream of EGFR, but not inhibiting EGFR itself, remains a promising strategy for promoting keratinocyte cohesion to potentially rescue epidermal integrity in DD.

Further supporting the critical role of ERK signaling in regulating intercellular adhesion in keratinocytes, we found that overactivation of ERK is sufficient to weaken cell-cell junctions. Based on prior data showing BRAF inhibitors can actually lead to paradoxical *activation* of downstream signaling via MEK, we treated keratinocytes with dabrafenib or vemurafenib to inhibit BRAF and showed they disrupted desmosomes to weaken monolayer integrity (**Fig. 3**) and confirmed both drugs led to *increased* ERK phosphorylation (**Fig. 4A-B**). Intriguingly, this is consistent with the observation that patients treated with BRAF inhibitor monotherapy for melanoma developed DD-like skin eruptions (diagnosed as Grover disease, which can be identical to DD on pathology) as a side effect of treatment. This is likely due to weakening of cell-cell adhesion from over-activation of MEK and ERK, which we have shown to drive DD pathology. To validate our ERK immunoblot results in live cells, we obtained a GFP-based kinase translocation reporter (ERK-KTR-Clover), which serves as a real-time ERK biosensor. Upon activation of endogenous ERK, the ERK biosensor is localized to the cytoplasm, but during ERK inhibition, the biosensor becomes concentrated in the nucleus through a phosphorylation-sensitive nuclear localization sequence (**Fig. 4C-D**). Indeed, we saw that treatment with BRAF inhibitors caused activation of ERK, which could be mitigated by co-treatment with trametinib (**Fig. 4E**). Use of this fluorescent biosensor in live microscopy experiments will allow us to delineate how aberrant ERK activation in DD disrupts cell-cell junctions within live SERCA2-deficient keratinocytes and organotypic epidermis.

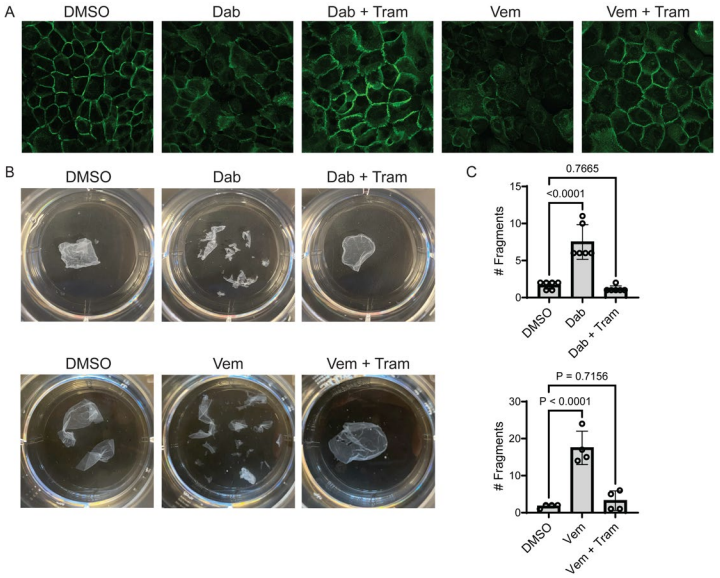

Fig. 3: Excess ERK activation weakens cell-cell adhesion in human keratinocytes. (**A**) Treatment of NHEK monolayers with the BRAF inhibitors dabrafenib (Dab) or vemurafenib (Vem) impaired organization of cell-cell junctions as shown by immunostaining for the desmosome marker plakoglobin (green). (**B**) The disruption of cell-cell junctions due to BRAF inhibition was paralleled by a reduction in integrity in Dab- or Vem-treated keratinocyte monolayers. (**C**) Quantification of drug-treated monolayer fragments after mechanical stress (N=6 for Dab; N=4 for Vem).

Planned Research: During the remaining funding period, we will continue our work on **Aim 1** to confirm that the therapeutic effect of MEK inhibition occurs via downstream ERK suppression. Our initial results indicate that treatment with selective inhibitors of *other* MAP kinases, p38 (SB203580) or JNK (SP600125), actually *weakened* keratinocyte monolayer integrity in the mechanical dissociation assay, underscoring the specificity of the therapeutic effect of MEK and ERK inhibition. As originally proposed in **Aim 1**, we will expand our assessment to evaluate whether mitigating the effects of excess cytosolic calcium due to SERCA2 deficiency will rescue cell-cell adhesion in our model of DD.

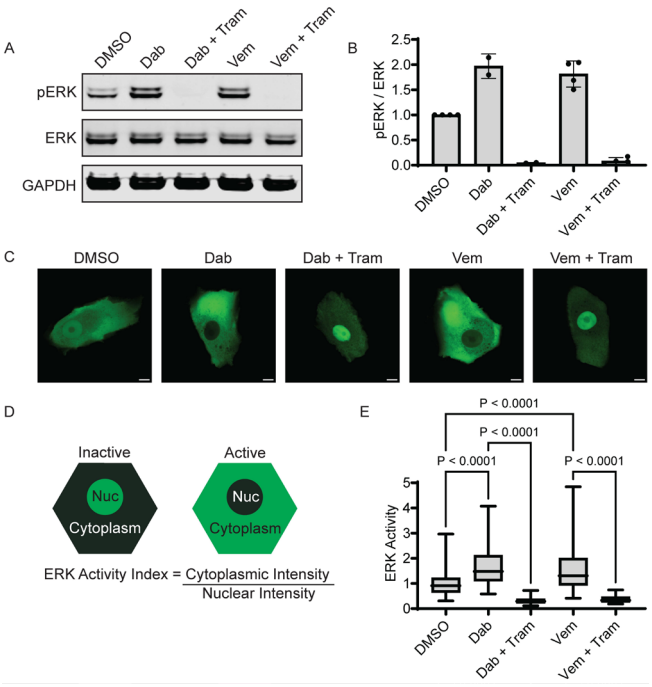

Fig. 4: Validation of an ERK biosensor in human keratinocytes. (**A**) Immunoblotting of protein lysates from keratinocytes treated with dabrafenib (Dab) or vemurafenib (Vem) revealed that BRAF inhibition led to a paradoxical *increase* in ERK phosphorylation (pERK), which was suppressed by co-treatment with the MEK inhibitor, trametinib (Tram). (**B**) Quantification of pERK/ERK intensity in replicate immunoblots. (**C**) We obtained a GFP-based ERK biosensor (ERK-KTR-Clover) to confirm the role of this signaling pathway in DD pathogenesis; confocal microscopy images of human keratinocytes transduced to express ERK-KTR-Clover confirm that the biosensor is concentrated in the cytoplasm upon paradoxical *activation* of MEK by the BRAF inhibitors Dab or Vem, which are known to induce a DD-like eruption in patients while co-treatment with Tram led to its nuclear localization. (**D**) Diagram of ERK biosensor localization and the ERK activity index. (**E**) Quantification of ERK activity in drug-treated keratinocytes (N=3 experiments).

Toward understanding the role of reactive oxygen species (ROS) in DD, as proposed in **Aim 2**, we recently established a collaboration with Dr. Robert Harmon, PhD, Research Assistant Professor in the lab of Dr. Kathleen Green, PhD, at Northwestern University. The Green Lab has performed metabolomic analysis on our *ATP2A2* HET keratinocytes to determine the global effects of SERCA2 deficiency on keratinocyte metabolism, including pathways driving ATP consumption and oxidative stress. In a preliminary experiment to test the effect of ROS on cell-cell adhesion, we treated keratinocytes with menadione to induce ROS and this resulted in marked disorganization of desmosomes as well as a reduction in intercellular adhesive strength of keratinocyte monolayers. These data support our hypothesis that excess oxidative stress impairs cell-cell junctions in keratinocytes and could drive DD pathogenesis.

Summary and Outlook: The above summarized work from my lab with support from FIRST has validated MEK as a particularly promising target for drug development for DD, potentially through prescription of oral MEK inhibitors that are already in clinical use mostly in oncology for BRAF-driven malignancies. However, we are also encouraged that DD may be an optimal disease for *topical* delivery of compounded MEK inhibitors given the epidermal location of DD pathology and the already-breached cutaneous barrier inherent to the acantholytic phenotype of DD. This could significantly reduce the risk of systemic side effects. Our FIRST-funded work has confirmed that specific suppression of MEK, but not upstream EGFR or RAF, robustly increases cell-cell adhesion in our *in vitro* model of DD, suggesting it could have potential to rescue epidermal integrity in DD patients. Importantly, our experiments in SERCA2-deficient or SERCA2-inhibited cells demonstrated therapeutic effects of *multiple* MEK inhibitors already FDA-approved for use in patients, including trametinib, cobimetinib, and selumetinib, any of which might be suitable for drug repurposing since the rarity of DD makes initiation of Phase 1 clinical trials for totally new drugs more challenging. Finally, results from our FIRST-funded work are being included as preliminary data to support my first NIAMS R01 early-stage investigator (ESI) application, which proposes to elucidate the role of ER stress and downstream signaling via calcium and MEK in modulating cell-cell adhesion in keratinocytes and how dysregulation of these pathways may drive DD. However, I plan to apply for a second year of FIRST grant support to help continue to fund our DD project, which is not directly funded by my other current grants, while I work to obtain NIH R01 funding.