Title

Ubiquitin-Dependent Control of Myogenic Development: Mechanistic Insights into Getting Huge, and Staying Huge

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Ubiquitin-Dependent Control of Myogenic Development: Mechanistic Insights into Getting Huge, and Staying Huge

By
Fernando Rodríguez Pérez

A dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy
in
Molecular and Cell Biology
in the
GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, BERKELEY

Committee in charge:

Professor Michael Rape, Chair
Professor Roberto Zoncu
Professor Jeffery Cox
Professor Daniel Nomura

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ABSTRACT

Ubiquitin-Dependent Control of Myogenic Development: Mechanistic Insights into Getting Huge, and Staying Huge
by
Fernando Rodriguez Perez
Doctor in Philosophy in Molecular and Cell Biology
University of California, Berkeley
Professor Michael Rape, Chair

Metazoan development is dependent on the robust spatiotemporal execution of stem cell cell-fate determination programs. Although changes in transcriptional and translational landscapes have been well characterized throughout many differentiation paradigms, their regulatory mechanisms remain poorly understood. Ubiquitin has recently been found to be a key modulator of developmental programs. Ubiquitylation of target proteins occurs through a cascade of enzymatic reactions beginning with a ubiquitin activating enzyme (E1) which transfer the ubiquitin moiety to a ubiquitin conjugating enzyme (E2). The reaction is finalized by the transfer of ubiquitin to its target protein by a ubiquitin ligase (E3). Post-translational modification of proteins can lead to several different outcomes, depending on the context of the modification, known as the ubiquitin code. The precise spatiotemporal execution of ubiquitylation is critical for organismal development and homeostasis. Due to the modular and reversible nature of ubiquitylation, it is an ideal moiety in the control of a plethora of cellular processes.

Cell-cell fusion is a frequent and essential event during development, whose dysregulation causes diseases ranging from infertility to muscle weakness. Critical to this process, cells repeatedly need to remodel their plasma membrane through orchestrated formation and disassembly of cortical actin filaments. In Chapter 2, I describe the identification of a ubiquitin-dependent toggle switch that establishes reversible actin bundling during mammalian cell fusion. My work identified KCTD10 as a modulator of the EPS8-IRSp53 complex, which stabilizes cortical actin bundles at sites of cell contact to push fusing cells towards each other. This work highlights how cytoskeletal rearrangements during development are precisely controlled, raising the possibility of modulating the efficiency of cell fusion for therapeutic benefit.

Organismal development must rely on the timely and robust execution of quality control responses. However, how these responses modulate metazoan development is poorly understood. Showcasing the versatility of ubiquitin signaling, Chapters 3 and 4 provide insight into the role of ubiquitin in controlling stress and quality control responses. Chapter 3 describes the reductive stress response, in which FEM1B senses and reacts to persistent depletion of reactive oxygen species. Loss of ROS is detrimental for development, as it inhibits myogenesis. Concomitant to this stress response is the identification of multimerization quality control, regulated by BTBD9. MQC surveys multimeric BTB complex composition, ensuring that multimeric complexes contain the correct stoichiometries and compositions. MQC is critical for development, as loss of MQC als prevents myogenesis. These two chapters showcase the integration of ubiquitin signaling, stress/quality control pathways, and development. These writings provide a more holistic understanding into the robust regulatory underpinnings of organismal formation

DEDICATION

Esta tesis y este trabajo, esfuerzo, sangre y sudor, se lo dedico a mi mamá Guadalupe (Jefa) y mi papá Fernando (Jefe). Gracias por todo lo que sacrificaron por mí, y por todo el apoyo que me han dado durante toda mi vida. Y claro, ¡gracias por todas las conchas y paquetitos que me mandaron y me mantuvieron vivo! Los amo.

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I want to dedicate this work to all immigrants (documented and undocumented) that have come before me, and those that will come after me.

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This work would not have been possible without the help of countless of people. Mentorship has been paramount for my growth as a scientist, and my work during grad school. I could not have asked for a better mentor than Michael Rape, who provided unmeasurable guidance and advice during my years here in Berkeley. You made me the scientist I am today. I'm sorry your soccer team couldn't beat my soccer team.

I am also forever thankful to the members of my thesis committee: Roberto Zoncu, Jeffery Cox, and Daniel Nomura. Your extensive guidance throughout my journey allowed me to get to where I am today. Speakin of mentors, I have to give a massive thanks to Jennifer Shepherd and Marianne Poxleitner from Gonzaga University (GO ZAGS!). You two saw my true potential and pushed me to places I did not think I could reach. Thank you.

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UC Berkeley MCB is a premier research program worldwide, but the reason I decided to come here was because of the people and the sense of community. I am fortunate of being part of the MCB Class of 2014 cohort (AKA as Dankest and Tighters cohort in the history of MCB). I especiall want to thank George Otto and Rose Hill, my first roommates in Berkeley. Like the One Ring, our friendship was forged in the Fires of Casa Bonita, making it as everlasting ast he One Ring itself. George Otto, you're a true homie, you saw me at my lowest and my highests. I could not have survived grad school without you. Could not have asked for a better friend. Robert Nichols, you brought so much laughter and joy to our whole cohort. Your energy is firkin contagious bro, thanks for Squanching with me and being a true friend. No doubt I will be seeing much more of you in the future.

Don't think I fogto about you Jesse Garcia Castillo and Santio Yori Restrepo. Ya'll are the true homebois. All of things we went through together, and are still going through, I will never forget. I am so greatful you guys decided to come to Berkeley. Ya'll are mi familia here.

A huge thanks to the HHMI Gilliam Fam. You guys made me realize my true potential and made me feel welcome in an environement where is hard to do just that.

These are trying times, as this thesis was written during a global pandemic (SARS-CoV-2), and there is no way I could have survived the last months of my PhD without some amazing groups of people. A special shoutout to the COVFee Slack Channel. Everything about you guys is hella special, and just showcases the thight-knit community of MCB here at Berkeley. The CoD Boiz and Bike Crew, so much yeetness happened with these crews. Thanks for making this quarantine livebale, and actually fun. Special shoutouts to Justin Zhang, Oscar Whitney, and Hannah Nilsson.

There are many more people that have made my PhD and tenure as a graduate student possible. To all of you, thank you from the bottom of my heart.

Finally, one last shout out to "Australia" by The Shins. This song got me through my darkest times, and is the most played song on my playlist, so there is that. And without further ado, here's "Wonderwall".

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PUBLICATIONS

Rodríguez-Pérez F, Manford A, Pogson A, Ingersoll A, Martínez-González M, Rape M (2020). Ubiquitin-dependent remodeling of the actin cytoskeleton drives cell fusion. Developmental Cell (in revision)

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RESEARCH PRESENTATIONS

Talk: *Ubiquitin-dependent remodeling of the actin cytoskeleton drives cell fusion*

Keystone Symposia on Ubiquitin Biology, Snowbird, UT, Mar 2020 (CANCELLED DUE TO SARS-CoV2)

Talk: *Ubiquitin-Dependent Regulation of Actin Dynamics*
UC Berkeley MCB Quality Control Supergroup. Berkeley, CA, Jan 2020

Talk: *Control of cell fusion during development*
Berkeley's MCB – Cell and Developmental Biology Annual Retreat. Monterrey, CA. September 14, 2018.

Poster: *Ubiquitin-Dependent Regulation of Myogenesis*
HHMI Annual Gilliam Meeting. Chevy Chase, MD. September, 2015

Poster: *Identifying Roles of Cullin-RING Ligases During Cellular Differentiation*.
Berkeley's MCB – Cell and Developmental Biology Annual Retreat. Tahoe City, CA. September 19, 2015.

Poster: *Using Drosophila melanogaster as a model for tubular organ development and angiogenesis during tumor growth*. Presented at the 2011 HHMI EXROP Meeting, HHMI Headquarters, Chevy Chase, MD. June 2011; and presented at Annual Murdock Conference, Linfield, WA. November 2011

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Poster: *Screening of rhodoquinone biosynthetic gene targets using Rhodospirillum rubrum deletion mutants and characterization of gene products*. Presented at the 2012 NCUR conference, Weber State University, Ogden, UT. March 28-30, 2012

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CHAPTER 1:

Now I’ve Become Ubiquitin, Destroyer of Proteins

1.1 The Ubiquitin Code: Ubiquitin and Development, an Overview

1.1.1 Ubiquitin: What's in a name?

What is the "essence" of a protein? Besides the philosophical and metaphysical implications of this question, proteins (one of the basic building blocks of life) possess a plethora of biochemical and biophysical properties that confer them their abilities to perform their biological roles. Up until recently, proteins were thought to be static entities with long half-lives (Varshavsky, 2006; White, 1957). This could not be further from reality, as it has become evident that proteins can be post-translationally modified to modulate their biochemical and cellular functions. These modifications happen with surgical precision, and allows living systems to tailor protein functions according to cellular and organismal needs (Dalle-Donne, et al, 2006; Khoury, et al, 2011; Ubersax and Jr, 2007; Wang, et al, 2014; Williamson, et al, 2013).

In the middle of the 20th century, it was thought that proteins were long lived entities. It was not until the late 1970s and early 1980s when it was shown by Hershko, Ciechanover (his graduate student!) and colleagues that the small protein APF-1 was able to promote the ATP-dependent turnover of proteins in rabbit reticulocyte lysates (Ciehanover, et al, 1978). Around the same time, Alexander Varshavsky (one of the founding fathers of the ubiquitin field that did not get to share the Noble Prize) identified and isolated histones modified with a protein that had previously been described as ubiquitin (Behuliak, et al, 2005). Ubiquitin itself was initially identified as a free protein from bovine thymus cells that had lymphocyte-differentiating properties (Goldstein, et al, 1975). Its ubiquitous presence in eukaryotic organisms that ranged from yeast to plants to mammals gave ubiquitin its very appropriate name by which we now know it. This APF-1 protein was later shown to be the ubiquitin, bridging together the fields of protein degradation and ubiquitin-modified histones (Levinger and Varshavsky, 1980; Levinger and Varshavsky, 1982; Wilkinson, et al, 1980). This set the stage for what would later become an explosive and exciting field exploring the regulatory underpinning of ubiquitin signaling, and the birth of the study of ubiquitin biology.

1.1.2 Compiling the Code: The "Programmers" of Ubiquitylation

When ubiquitin was first discovered in 1975 as a free protein with T-cell and B-cell differentiation potential, its biological role and function were still a mystery (Varshavsky, 2006). Varshavsky's observations that ubiquitin-modified histones were found on actively transcribed genes, and that ubiquitin-modified proteins were targeted for degradation in a lysate system, provided little mechanistic insight as to the true role of ubiquitin (Ciehanover, et al, 1978). Along comes the identification of cyclins, master regulators of the cell cycle (Johnson and Walker, 1999; Malumbres and Barbacid, 2001). Work by Glotzer and Kirschner (and independently validated by Hershko) showed that these short-lived proteins were degraded by the ubiquitin system (Glotzer, et al, 1991; Hershko, et al, 1991). This was a critical discovery, as it provided functional significance to the regulatory functions of ubiquitin. Indeed, showing that the spatiotemporal regulation and degradation of proteins was necessary for the fateful progression through the cell cycle, allowing life as we know it to exist.

Altough a regulatory molecule, ubiquitin was only thought to play a role in the targeted degradation of proteins (i.e. cyclins). Unsurprisingly, it is more complex than that. Post-translational modification of proteins with ubiquitin can take on many different flavors (Komander and Rape, 2012; Williamson, et al, 2013; Yau and Rape, 2016). Proteins can be modified with a single ubiquitin moiety, or decorated with polymeric ubiquitin chains. Ubiquitin chains can be linked through any of its seven lysine (Lys) residues or its amino-terminal NH2-group, providing different topologies. These different topologies "code" for different sets of instructions that can change the biophysical and biochemical properties of target substrates (Hicke, 2001; Williamson, et al, 2013). Much like a computer program, this "ubiquitin code" can be executed by biological systems in a spatiotemporal way in order to fit their specific needs. The "programmers" of this "code" are a collaborative set of enzymes that form the ubiquitylation enzymatic cascade. The ubiquitin-activating enzyme (E1) catalyzes the initial attachment on one ubiquitin moiety, followed by the transfer to a ubiquitin-conjugating enzyme (E2), and finalized by transferring the ubiquitin moiety to its target substrate by a ubiquitin ligase (E3). Substrate specificity is conferred by the E3 ubiquitin ligase, making E3 ubiquitin ligases an exciting handle to study and discover biological processes (Komander and Rape, 2012). There are three major classes of E3 ubiquitin ligases: HECT, RING, RING-Between-RINGs, and RING-Cys-Relays (Mabbitt, et al, 2020; Pao, et al, 2018). About 30% of a cell's ubiquitylation load is performed by cullin- RING ligases (CRLs). Indeed, several developmental and pediatric disorders have been linked to cullin-RING ligases, and mutations in several of these ligases have been shown to be cancer drivers.

Cullin-RING ligases are modular entities that are themselves tightly controlled. At the core of these complexes is the cullin-fold containing scaffolding protein, which serves as a platform that brings together other a substrate-substrate receptor (SR) complex and the catalytic component containing the E2-Ubiquitin conjugate. There are six of these scaffolds, each with their own set of SRs that target their own specific substrates (Lydeard, et al, 2013; Petroski and Deshaies, 2005). Several disease-causing mutations have been linked to CRLs, underscoring the importance of CRLs in metazoan development and homeostasis (Andérica-Romero, et al, 2013; Gupta and Beggs, 2014; Papizan, et al, 2018). Mutations in KEAP1, a CUL3 SR, are found in 3% of all cancers, and are widely prevalent in lung malignancies (Singh, et al, 2006). VHL, a SR of CUL2, has been found to be critical for oxygen sensing (Stebbins, et al, 1999). Mutations in the CUL3 SRs KLHL40/41 have been linked to nemaline myopathies (Ravenscroft, et al, 2013). The CUL4 SR Cereblon is the target of the immunomodulator drug thalidomide, which was responsible for one of the largest pharmaceutical disasters in history (but it has been instrumental to the field of targeted protein degradation as a therapeutic) (Rodríguez-Pérez and Rape, 2018). These are just few of the many examples of ubiquitin ligases that are implicated in a myriad of biological processes that are critical for metazoan development and homeostasis. Being able to identify the substrates regulated by SRs of CRLs can help to better understand biological pathways, and even elucidate novel regulatory mechanismsd (McGourty, et al, 2016; Mena, et al, 2018; Oh, et al, 2020; Werner, et al, 2015). This can provide therapeutic avenues for disease, making the study of E3 ubiquitin ligases an exciting area of research.

In the writings of chapters 2 – 4, we delve into the regulatory underpinnings of CRL3^{KCTD10}, CRL2^{FEM1B}, and CRL3^{BTBD9}. Using these E3 ligases as handles, we have identified exciting new biology. In chapter 2, we hone in onto CRL3^{KCTD10} as a key regulator of cell fusion during myogenesis, an elusive process that has remained poorly understood. In chapter 3, we describe work that led us to identify CRL2^{FEM1B} as a master regulator the reductive stress response, a novel pathway that monitors the reductive environment of a cell analogous to the oxidative stress response (also regulated by a CRL, CRL3^{KEAP1}). Lastly, in chapter 4 we delve into the regulatory workings of CRL3^{BTBD9}, an essential quality control enzyme of multimeric protein complex formation that is responsible for correct multimeric BTB (Broad-complex, Bric-a-brac, Tramtrack). Overall, the work in this tome of wisdom and knowledge adds new understandings to the harmonious regulatory mechanisms of ubiquitin signaling. Furthermore, it underscores the importance of understanding the regulation of fundamental cellular processes, which can aid in the discovery of novel biology and understanding disease.

1.1.3 Executing the Code: Ubiquitin as a Regulator of Metazoan Development

Life is a complex and mind-boggling process. Adult metazoans contain about 37 trillion (!) cells that arise from countless rounds of cell division cycles (Sender, et al, 2016). Human bodies are not made up of just one cell type, but are a beautiful and harmonious orchestration of several organs made up of specialized cell types (Bartsch, et al, 2015). How the cell-fate determination decisions creating organs are made, and how they are executed at exactly the right time and place to allow for successful organismal formation is not very well understood (Carvalhal Marques, et al, 2015; Erwin, 1993; Gilbert, 2000). Recent advancements have been made that highlight the importance of ubiquitin signaling in development (Ciechanover and Schwartz, 2004; Rape, 2018). Ubiquitin is now known to control progression of several developmental programs, such as neural crest specification, muscle formation, and neuronal system development (Gupta and Beggs, 2014; Mena, et al, 2018; Papizan, et al, 2018; Werner, et al, 2015). One of the most well-known roles of ubiquitin is limb development, which came to light by a medical tragedy created by thalidomide, a drug once used to treat morning sickness (Rodríguez-Pérez and Rape, 2018). This tragedy, along with the fact that mutations in integral components of the ubiquitin machinery lead to developmental and pediatric disorders, have created a great deal of interest in trying to decipher the ubiquitin code and the cellular pathways that it regulates (Andérica-Romero, et al, 2013; Ciechanover and Schwartz, 2004; Rape, 2018).

The intricacies of developmental processes and cell-fate determination paradigms means there must be tight spatiotemporal control to successfully execute these decisions (Gilbert, 2000; Rape, 2018). One of the most dramatic differentiation programs is myogenesis, or muscle formation (Bentzinger, et al, 2012; Deng, et al, 2017; Doherty, et al, 2011). Myogenesis is an essential differentiation process required for organismal survival and homeostasis. Humans face a harsh world full of perils, and injuries to tissues will occur. Muscle tissues undergo a tremendous amount of stress every day, and so they must be able to be replaced rapidly and efficiently (Bentzinger, et al, 2012; Hernández-Hernández, et al, 2017; Petrany and Millay, 2019). Although muscle tissues have been studied for many decades, and the proteomics and transcriptional landscapes of differentiating muscles have been widely studied, how this process is regulated at the molecular level remains poorly understood (Heffner, 1975; Hudgson, 1970; Ong, et al, 2002; Ranatunga, et al, 1987). Myogenesis, both *in vivo* and *in vitro*, is a fascinating process that happens with great speed and is accompanied by dramatic changes. *In vitro*, a precursor cell, known as a myoblast, can differentiate in to a full muscle fiber, or myotube, in just 72 hrs. In the course of this process, the myoblast must completely reorganize and reform its cellular architecture to generate a new and specialized cell type capable of moving an adult body (Bentzinger, et al, 2012; Hernández-Hernández, et al, 2017; Ong, et al, 2002). One of the hallmarks of muscle cells is the fact that they are multinucleated, that is to say that one muscle fiber can contain thousands of nuclei all in one cellular unit (Deng, et al, 2017; Petrany and Millay, 2019). The longest muscle in the human body, the Sartorius muscle, can be as long as 60 cm (longer in taller individuals) and have muscle fibers that run the entirety of the muscle (Ashaolu, et al, 2015; Dziedzic, et al, 2014; Walters and Varacallo, 2020). This means that one cell can be up to 60 cm long! These multinucleated cells arise through a fascinating, yet elusive, process known as cell fusion. Especially the regulation of cell fusion has remained a mystery (Deng, et al, 2017; Lehka and Redowicz, 2020; Millay, et al, 2013; Petrany and Millay, 2019; Quinn, et al, 2017; Sens, et al, 2010). In chapter 2, we describe an approach that we took to uncover CRL3^{KCTD10} as an essential regulator of cell fusion during myogenesis. This led us to identify the actin bundling complex EPS8-IRSp53 as a key substrate of CRL3^{KCT10}. CRL3^{KCTD10} regulates the cellular localization of EPS8-IRSp53, and it negatively regulates its actin bundling activity. This precise spatiotemporal regulation by CRL3^{KCT10} allows for robust actin dynamics and faithful cell fusion.

1.1.4 Debugging the Code: Ubiquitin, Stress and Quality Control

Life is not perfect, and its complexity makes it error-prone. However, part of life is identifying mistakes and taking care of them. Cells will make mistakes from time to time, and they must be able to ameliorate these errors in order to survive (Hwang and Qi, 2018; Lans, et al, 2019; Mena, et al, 2018; Tyedmers, et al, 2010). Protein folding is a fascinating process that is critical for life to exist. As robust as this process is it is not perfect, and mistakes in protein folding can be detrimental for life and lead to disease. To combat this, cells have evolved quality control mechanisms (Brandman and Hegde, 2016; Hwang and Qi, 2018). Furthermore, many proteins need to form multimeric complexes in order to function properly (Huttlin, et al, 2015; Huttlin, et al, 2017). How proteins form the correct complex is a process that must be actively monitored (Mena, et al, 2018). The formation of wrong proteins complexes can be detrimental for organismal development and homeostasis (Mena, et al, 2018; Smith, et al, 2003; Tyedmers, et al, 2010). This can be best exemplified by the cancer driver fusion protein BCR-ABL1, which aberrantly dimerizes with itself to yield a constitutively active kinase driving cell transformation (Smith, et al, 2003). In chapter 4 we describe the discovery of the ubiquitin ligase BTBD9, an enzyme involved in quality control of multimeric complex formation, which we have named multimerization quality control (MQC). This discovery further highlights the integral role ubiquitin signaling plays in "debugging" protein complex formation, and protein homeostasis as a whole.

Living systems have to do more than just survey their inner workings. They are constantly bombarded with forces from the outside world and a plethora of environmental stressors and insults (Denhardt, et al, 2001; Edwards, et al, 2001). To maintain cellular and organismal homeostasis, cells have evolved stress responses that help the ameliorate these stressors. These include, but are not limited to: the unfolded protein response, the hypoxic stress response, and the oxidative stress response (Brandman and Hegde, 2016; Denko, 2008; Duleh, et al, 2016; Harding, et al, 2003; Kroemer, et al, 2010; Maity, et al, 2016; Tyedmers, et al, 2010). Many of these stress responses take advantage of the versatility of ubiquitin signaling to execute their debugging properties (CUL2^{VHL} in the hypoxic stress response, and CUL3^{KEAP1} in the oxidative stress response). In chapter 3 of this work, we identify CUL2^{FEM1B} as a sensor for reductive stress caused by persistent loss of reactive oxygen species. Our findings put forward a mechanism by which cells are able to quickly respond to changes in redox homeostasis. Furthermore, this work provides evidence of the intersection between ubiquitin and metabolic signaling, paving the way for exciting new discoveries.

By no means is the work laid out in this thesis able to answer all pressing questions. However, it has shed light onto a dark abyss of the unknown, providing insights into novel regulatory underpinning of ubiquitin signaling. With the screening platforms and unbiased approaches I present here, this work has laid the foundation on which more exciting discoveries are waiting to happen.

CHAPTER 2:

Ubiquitin-Dependent Remodeling of the Actin Cytoskeleton Drives Cell Fusion

Fernando Rodríguez-Pérez, Andrew G. Manford, Angela Pogson, Andrew J. Ingersoll, Brenda Martínez-González, Michael Rape

Developmental Cell (In revision)

2.1 Summary

Cell-cell fusion is a frequent and essential event during development, and its dysregulation causes diseases ranging from infertility to muscle weakness. Critical to this process, cells repeatedly need to remodel their plasma membrane through orchestrated formation and disassembly of cortical actin filaments. While filament formation has long been studied, how cells dismantle existing actin structures to complete tissue formation is still poorly understood. Here, we identified a ubiquitin- dependent toggle switch that establishes reversible actin bundling during mammalian cell fusion. We found that EPS8-IRSp53 complexes stabilize cortical actin bundles at sites of cell contact to push fusing cells towards each other. Monoubiquitylation by CUL3^{KCTD10} then displaces EPS8- IRSp53 from cellular interfaces and triggers disassembly of actin bundles, a dual activity that allows apposed cells initiate membrane mixing. We conclude that cytoskeletal rearrangements during development are precisely controlled by ubiquitylation, raising the possibility to modulate the efficiency of cell-cell fusion for therapeutic benefit.

2.2 Introduction

Approximately one third of the cells in our bodies have fused with other cells and contain multiple nuclei (Brukman, et al, 2019). Cell-cell fusion (henceforth referred to as cell fusion) occurs at the beginning of life during fertilization and continues to be required for placenta formation, generation of bone-resorbing osteoclasts, and the development of myofibers or giant cells of the macrophage lineage (Petrany and Millay, 2019). As a consequence of its role during tissue formation, loss of cell fusion is incompatible with life. However, mutations that render cell fusion less efficient can cause a range of diseases, including infertility (Yu, et al, 2018), muscle weakness (Di Gioia, et al, 2017), or osteopetrosis (Pereira, et al, 2018).

Many insights into molecular mechanisms of cell fusion have been gained from studies of multinucleated myotubes, the most prevalent tissue in mammals (Deng, et al, 2017; Lehka and Redowicz, 2020). Both growth and regeneration of myotubes rely on their fusion with myoblasts derived from satellite precursors under the basal lamina of muscle fibers. Fusion-competent myoblasts first migrate towards a growing myotube to establish adhesion, which keeps the plasma membranes of fusing cells ~45nm apart from each other (Galletta, et al, 2004; Rosen, et al, 1992; Ruiz-Gomez, et al, 2000). The myoblast membrane then flattens onto the myotube to achieve a closer contact that is referred to as membrane apposition (Dhanyasi, et al, 2015). While this step enlarges the contact area between cells, myoblasts have to overcome repulsion exerted by the hydrated lipid head groups of apposing plasma membranes. The close juxtaposition of cells allows fusogens, such as the mammalian transmembrane proteins Myomaker and Myomixer, to form a fusion pore that initiates membrane and cytoplasmic mixing (Bi, et al, 2017; Millay, et al, 2013; Quinn, et al, 2017; Zhang, Q., et al, 2017).

To progress through the successive steps of the fusion program, cells frequently need to reshape their plasma membrane, which they accomplish through orchestrated remodeling of their actin cytoskeleton (Deng, et al, 2017). The critical contribution of actin polymers to cell fusion is best understood in Drosophila. In these organisms, myotubes employ filopodia filled with actin bundles to capture approaching myoblasts (Segal, et al, 2016). Fusion-competent myoblasts then use branched actin networks to establish membrane apposition (Dhanyasi, et al, 2015), and they generate a large actin focus composed of branched filaments to sprout podosomes towards the myotube (Sens, et al, 2010). These actin-filled protrusions are met by a thin actomyosin network beneath the plasma membrane of the myotube that provides mechanical resistance needed for fusion to occur (Kim, et al, 2015). Genetic experiments in flies have accordingly identified several regulators of actin filament nucleation, branching, or bundling, including formins, the Scar/WAVE complex, or Dynamin, as components of the fusion machinery (Dhanyasi, et al, 2015; Segal, et al, 2016; Zhang, R., et al, 2020). Whether and how similar players shape the actin cytoskeleton during mammalian cell fusion is less well understood.

While filament assembly has long been studied, the cytoskeletal rearrangements during cell fusion also require the timely dismantling of actin structures that have fulfilled their functions. Indeed, after Drosophila myotubes used filopodia filled with actin bundles to capture myoblasts, they remodel their cytoskeleton into a cortical actomyosin network that provides resistance against invading podosomes. The actin focus in myoblasts also persists for only ~11 minutes (Deng, et al, 2017). If disassembly of filaments or bundles is prevented, the fusion program cannot proceed through its multiple steps and the merger of cells is aborted (Deng, et al, 2015; Deng, et al, 2016; Geisbrecht, et al, 2008; Haralalka, et al, 2014). While actin polymerization or bundling can be tuned down in fusing cells by GTP hydrolysis (Doherty, et al, 2011; Zhang, R., et al, 2020), whether existing actin structures are actively dismantled in a regulated manner is still unclear. How the actin cytoskeleton can be rapidly adapted to the dynamic needs of cell fusion therefore remains to be elucidated.

Here, we identified the conserved E3 ligase CUL3^{KCTD10} and its monoubiquitylation target EPS8 as key regulators of reversible actin bundling and cell fusion. When bound to IRSp53 (also known as BAIAP2), EPS8 promotes the formation of cortical actin bundles that push neighboring cells towards each other for successful membrane apposition. CUL3^{KCTD10} then displaces EPS8-IRSp53 from sites of cell contact, shuts down its bundling activity, and helps dismantle existing actin bundles even if stabilized by distinct factors. Its dual activity in controlling localization and function of EPS8-IRSp53 allows CUL3^{KCTD10} to establish a ubiquitin-dependent toggle switch that controls actin bundling and guides cell fusion beyond membrane apposition. Our findings reveal ubiquitin-dependent regulation of cytoskeletal rearrangements during development, which may provide opportunities for counteracting aberrant actin bundling or cell fusion during disease.

2.3 Results

2.3.1 KCTD10 is required for myoblast fusion

Although core components of the cell fusion machinery have been identified, less is known about proteins that ensure accurate temporal and spatial execution of this process during development. C2C12 cells provide a powerful system to discover such regulators of cell fusion, as they can be induced to undergo rapid differentiation from a myoblast stage with single nuclei into multinucleate myotubes. We noted that specific inhibition of myoblast fusion upon depletion of Myomixer caused the formation of cells that were mononucleate yet expressed the late differentiation marker Myosin Heavy Chain (MyHC) (Figure S1A). This phenotype could be quantified as an increased average ratio between the short and long axes of MyHC-positive cells (R^{MyHC}), which provided a robust read-out for genetic screens dissecting cell fusion (Figure S1B).

Having established a genetic platform, we depleted myoblasts of ~150 substrate adaptors of CUL2 and CUL3 E3 ligases and searched for the increase in R^{MyHC} that is indicative of defective cell fusion. We focused on these E3 ligases, as they are known to control metazoan development and are mutated in distal and nemaline myopathies (Rape, 2018; Ravenscroft, et al, 2013). Moreover, tissue-specific deletion of CUL3 prevented myoblast fusion in mice (Blondelle, et al, 2019; Papizan, et al, 2018). Our screen identified a single CUL3 adaptor, KCTD10, whose loss dramatically inhibited myoblast fusion (Figure 1A). We confirmed with individual siRNAs that KCTD10 depletion sharply increased the number of mononucleate MyHC positive cells (Figure 1B), similar to what we observed for loss of Myomixer (Figure S1A, B). Highlighting the specificity of these results, the close KCTD10 homologs KCTD13 and TNFAIP1 were not required for cell fusion (Figure S1C). Moreover, fusion of KCTD10-depleted cells was fully restored by the expression of siRNA-resistant KCTD10, even if it was induced days after differentiation had begun and MyHC had already been expressed (Figure 1B, C). A KCTD10 variant that was unable to bind CUL3, KCTD10$^{\Delta CUL3}$, did not support myotube formation, indicating that ubiquitylation is required for the cell fusion process (Figure 1B, C; Figure S1D).

Despite its striking effects on cell fusion, depletion of KCTD10 did not strongly impact the gene expression program of myotube specification (Figure 1D). Protein levels of differentiation markers, such as Myogenin, MyHC, or Myomixer, were accordingly unaffected by the loss of this CUL3 adaptor (Figure S1E, F), and KCTD10 was not required for myoblast division or survival (Figure S1G). We therefore conclude that the E3 ligase CUL3^{KCTD10}, whose specificity component KCTD10 is highly conserved from flies to humans (Figure S1H), is an important regulator of myoblast fusion.

2.3.2 CUL3^{KCTD10} targets the EPS8-IRSp53 complex

We developed an integrated approach to identify proteins that CUL3^{KCTD10} must ubiquitylate for robust myoblast fusion. First, we appended a 3xFLAG epitope to the myoblast KCTD10 locus and confirmed expression of the tagged protein (Figure S2A). We then affinity-purified KCTD10^{3xFLAG} from cells treated with MLN4924, an inhibitor of neddylation that prevents substrate turnover by Cullin-RING E3 ligases (CRLs) and should trap targets on KCTD10 (Bennett, et al, 2010). We also isolated interactors of KCTD10$^{\Delta CUL3}$, which can bind, but not ubiquitylate, its substrates, a feature that we had previously found to improve association of other CRLs with their targets (Mena, et al, 2018; Oh, et al, 2020; Werner, et al, 2018). Finally, we searched for interactors of KCTD10^{UBA}, whose ubiquitin-binding domains provided KCTD10 with an additional recognition site for ubiquitylated substrates (Mark, et al, 2016; Oh, et al, 2020). All affinity-purifications from myoblasts were analyzed by semi-quantitative CompPASS mass spectrometry(Bennett, et al, 2010). We expected that substrates of CUL3^{KCTD10} were enriched in purifications of endogenous KCTD10 in the presence of MLN4924, by KCTD10$^{\Delta CUL3}$ compared to wildtype KCTD10, as well as by KCTD10^{UBA}.

Our experiments converged on a small set of candidate CUL3^{KCTD10} substrates that was dominated by regulators of the actin cytoskeleton (Figure 2A). Among these, we noted most members of the α-actinin family of actin bundlers (ACTN1, ACTN3, ACTN4), whose aberrant regulation was known to interfere with myoblast fusion (Blondelle, et al, 2019). We also detected two proteins, EPS8 and IRSp53, which preferentially bound KCTD10 in the presence of MLN4924 and were enriched in purifications of KCTD10$^{\Delta CUL3}$ and KCTD10^{UBA}. The highly conserved EPS8 and IRSp53 interact with each other to form a complex that, similar to α-actinin, promotes actin-bundling (Disanza, et al, 2006; Funato, et al, 2004).

To determine which of its candidate targets mediated the effects of CUL3^{KCTD10} onto cell fusion, we considered two potential outcomes of ubiquitylation: if CUL3^{KCTD10} inhibited a key target, loss of this protein should rescue myoblast fusion in the absence of KCTD10. By contrast, if ubiquitylation activated a substrate, depletion of this protein by itself should phenocopy loss of KCTD10. Consistent with an inhibitory role of ubiquitylation, we found that depletion of EPS8, IRSp53, or the IRSp53 homolog IRTKS (also known as BAIAP2L1), rescued fusion of KCTD10- deficient myoblasts (Figure 2B). These results were specific, as siRNA-resistant EPS8 corrected all effects of depleting the endogenous protein (Figure 2C). Loss of the GTPase CDC42, which activates EPS8 and IRSp53 to bundle actin filaments (Kast, et al, 2014; Krugmann, et al, 2001), also improved fusion of myoblasts lacking KCTD10, while depletion of Mena or SOS, two EPS8- and IRSp53-binding partners that do not bundle filaments along their length, did not have this outcome (Figure S2B). In contrast to its effects onto KCTD10-deficient myoblasts, depletion of EPS8 did not rescue fusion of cells lacking Myomixer (Figure S2C).

Depletion of EPS8 or IRSp53 by themselves did not interfere with myoblast differentiation nor did such treatment inhibit fusion of myoblasts into multinucleated myotubes (Figure 2B, D). This was mirrored by the α-actinin proteins, which also bind KCTD10 and were not required for myoblast fusion. However, consistent with actin bundlers compensating for each other (Mogilner and Rubinstein, 2005), co-depletion of EPS8 and α-actinin strongly inhibited myoblast fusion (Figure 2D). We conclude that actin bundling by either EPS8-IRSp53 or α-actinin as well as negative regulation of EPS8-IRSp53 by CUL3^{KCTD10} play important roles during myoblast fusion.

2.3.3 CUL3^{KCTD10} monoubiquitylates EPS8

To understand how CUL3^{KCTD10} regulates EPS8-IRSp53, we reconstituted substrate binding by KCTD10 in vitro. We purified KCTD10 from bacteria or C2C12 cells and incubated it with its candidate targets produced by in vitro transcription and translation. These experiments showed that EPS8, IRSp53, and IRTKS, but not control proteins, were efficiently retained by KCTD10 (Figure 3A; Figure S3A). To validate this interaction *in vivo*, we performed endogenous immunoprecipitations of EPS8. This validated the interaction bweteen KCTD10 and EPS8, providing evidence of EPS8 being a candidate subsrate (Figure 3A) In addition, we mixed KCTD10, EPS8, and IRSp53 that had been purified from bacteria and found by size exclusion chromatography that they formed a stable ternary complex containing all recombinant proteins (Figure 3B). Truncation analyses revealed that EPS8 bound KCTD10 through either half of its lipid-binding split-pleckstrin homology (PH), while IRSp53 employed its amino-terminal BAR-like IMD domain for the same purpose (Figure 3C-E). The tetrameric KCTD10 therefore directly engages the EPS8-IRSp53 complex through domains that are typically involved in membrane recruitment.

Having reconstituted the interaction between KCTD10 and EPS8-IRSp53 allowed us to investigate whether CUL3^{KCTD10} ubiquitylated these proteins. Importantly, recombinant NEDD8-modified CUL3^{KCTD10} efficiently decorated EPS8 with one or two ubiquitin molecules (Figure 3F). IRSp53 was not ubiquitylated, even though it was present in the same reaction. Experiments with methylated ubiquitin, which cannot form ubiquitin polymers, confirmed that CUL3^{KCTD10} primarily monoubiquitylated EPS8 (Figure 3G), with one site being found within the substrate's split-PH domain (Figure S3B). Using the same approach, we found that CUL3^{KCTD10} also catalyzed monoubiquitylation of α-actinin (Figure S3C), the actin bundler that collaborates with EPS8- IRSp53 during cell fusion. Together with our proteomic analyses, these results showed that CUL3^{KCTD10} binds and monoubiquitylates two actin bundlers, EPS8 and α-actinin. As our genetic experiments had identified EPS8 as the critical CUL3^{KCTD10} target during cell fusion, we focused subsequent analyses on the role of the EPS8-IRSp53 complex, as well as of EPS8 ubiquitylation, in this process.

2.3.4 CUL3^{KCTD10} restricts membrane localization of EPS8

Using live cell imaging of stably expressed EPS8eGFP, we noted a dynamic localization of this actin bundler during myoblast differentiation and fusion. While EPS8 was initially concentrated in the cytoplasm of myoblasts, it rapidly accumulated at interfaces of differentiating cells, once contact between fusion partners had been established (Figure 4A). EPS8 was retained at sites of cell contact, as the area shared between cells slowly grew. At the time of cell fusion, EPS8 became diffuse in the now shared cytoplasm. Thus, EPS8 localizes to sites of cell contact in a dynamic, and thus likely regulated, manner.

Depletion of KCTD10 did not affect EPS8 or IRSp53 stability (Figure S4), as expected for monoubiquitylation (Hicke, 2001). This prompted us to ask by immunofluorescence microscopy whether CUL3^{KCTD10} regulates membrane targeting of the endogenous EPS8-IRSp53 complex. Consistent with our live cell imaging, we detected most EPS8 and IRSp53 within the cytoplasm of undifferentiated myoblasts (Figure 4B). This dramatically changed upon KCTD10 depletion, when EPS8 and IRSp53 strongly and specifically accumulated at sites of cell contact (Figure 4B). Acute CRL inactivation by MLN4924 also led to an enrichment of EPS8 and IRSp53 at sites of cell contact, while proteasome inhibition had no effect (Figure 4C). Underscoring the specificity of these results, expression of siRNA-resistant KCTD10, but not inactive KCTD10$^{\Delta CUL3}$, prevented the aberrant localization of EPS8 in the absence of endogenous KCTD10 (Figure 4D). We conclude that ubiquitylation by CUL3^{KCTD10} restricts the localization, but not the stability, of EPS8-IRSp53 at sites of cell contact. In line with this notion, we found that KCTD10 accumulated in myoblasts specifically at interfaces between cells (Figure 4E).

Combined with our screen results, these findings suggested that EPS8 recruitment to sites of cell contact promotes cell fusion, while its persistent membrane accumulation might interfere with the same process. Indeed, constitutive localization of EPS8 to plasma membranes strongly inhibited myoblast fusion (Figure 4F). By contrast, if we fused ubiquitin to EPS8 to mimic monoubiquitylation, EPS8 was displaced from sites of cell contact even in KCTD10-depleted cells (Figure 4G). In contrast to wildtype EPS8, introduction of Ub-EPS8 did not prevent the fusion of myoblasts devoid of endogenous KCTD10 and EPS8, showing that the ubiquitylated protein is inactive (Figure 4H, I). We conclude that cell fusion requires the orchestrated recruitment and displacement of EPS8-IRSp53 from sites of cell contact, as regulated $CUL3^{KCTD10}$.

2.3.5 KCTD10 inhibits actin bundling by EPS8-IRSp53

While re-localization of EPS8-IRSp53 should turn off actin bundling at sites of cell contact, it might lead to deleterious stabilization of actin filaments at other locations. This led us to speculate that release of EPS8-IRSp53 from cellular interfaces should be coupled to inhibition of its activity in actin bundling. To test this hypothesis, we added recombinant EPS8-IRSp53 complexes to pyrene labeled actin for analysis by microscopy. We found that, while EPS8-IRSp53 had little effect on actin polymerization (Figure S5A), it drove formation of parallel actin bundles in vitro (Figure 5A; Figure S5B). These bundles were different in appearance from those produced by α-actinin, which stimulated assembly of thicker, yet less parallel, cables (Figure 5A). Strikingly, if we treated EPS8-IRSp53 with $CUL3^{KCTD10}$ to induce EPS8 monoubiquitylation, actin bundling was prevented (Figure 5B).

Our proteomic and biochemical analyses showed that KCTD10, EPS8 and IRSp53 form a stable ternary complex, reminiscent of persistent interactions between other CUL3 ligases and their substrates (McGourty, et al, 2016; Tong, et al, 2006; Werner, et al, 2015). We therefore also dissected the behavior of recombinant KCTD10-EPS8-IRSp53 towards actin. We noted that the ternary complex containing KCTD10 reduced the amount of actin polymer in a concentration- dependent manner, even if no ubiquitylation could occur (Figure 5C; Figure S5A). As the ternary complex predominantly impacted the maximum extent of actin polymerization, but less the initial reaction rates, KCTD10 might unlock the actin-capping function of EPS8 that is masked by IRSp53 (Disanza, et al, 2006; Kast, et al, 2014; Vaggi, et al, 2011). The ternary complex also failed to bundle actin filaments and even prevented actin bundling by α-actinin in trans (Figure 5D, E). Most strikingly, when we added EPS8-IRSp53-KCTD10 to bundles after these had been stabilized by α-actinin, these structures were completely dismantled (Figure 5E).

$CUL3^{KCTD10}$ therefore not only displaces EPS8-IRSp53 from sites of cell contact, but also switches its activity from actin bundling into unbundling. Genetic experiments suggested that the latter role of $CUL3^{KCTD10}$ is critical for cell fusion: if $CUL3^{KCTD10}$ were to terminate actin-bundling by EPS8-IRSp53, we expected that KCTD10-depleted cells that selectively expressed a bundling deficient variant, $EPS8^{RAFA}$, should be able to fuse; this was the case (Figure S5C). Conversely, a variant that is more efficient in actin bundling, $EPS8^{SATA}$ (Menna, et al, 2009), should prevent cell fusion even in the presence of KCTD10, which was observed as well (Figure 5F). We conclude that

$CUL3^{KCTD10}$ possesses dual ability in reversing localization and function of EPS8- IRSp53, thereby establishing a toggle switch for actin bundling that drives regulated cytoskeletal rearrangements needed for cell fusion.

2.3.6 Cortical actin bundles push fusing cells towards each other

Having identified $CUL3^{KCTD10}$ as a regulator of cell fusion, we wished to determine the function of the actin structures under its control. We therefore depleted KCTD10 to prolong EPS8-IRSp53 activity in fusing myoblasts and used transmission electron microscopy to search for changes in the actin cytoskeleton during. Strikingly, the KCTD10-depleted cells formed prominent linear actin bundles just below the plasma membrane at sites of cell contact (Figure 6A), a distinctive structure reminiscent of transverse arcs assembled during cell migration (Burnette, et al, 2011). While KCTD10-deficient cells became closely aligned with neighboring cells over large membrane areas, they did not initiate fusion as observed under control conditions. These findings implied that $CUL3^{KCTD10}$ restricts formation of cortical actin bundles that are specifically formed at cellular interfaces and may provide a force to align partner membranes. Yet, persistent formation of these bundles appeared to inhibit steps of the fusion program subsequent to membrane apposition.

To probe the above hypothesis, we plated myoblasts at low densities to prevent formation of cell-cell contacts, thereby relying on filament formation at random sites. We noted by live-cell imaging that KCTD10-depleted myoblasts ceased to move, an outcome that was dependent on EPS8 accumulation and revealed that undirected actin bundling is deleterious for cell migration (Figure 6B). Importantly, the KCTD10-depleted cells also formed dramatic actin structures that spontaneously assembled in a circular fashion, grew upwards for hours until they extended several cell diameters above the myoblast, and then collapsed (Figure 6C, D). These protrusions contained EPS8 and required both EPS8-IRSp53 as well as actin polymerization for their formation (Figure 6E). Extensive outward membrane growth caused by EPS8-IRSp53 activity was also prominently seen by scanning electron microscopy of KCTD10-depleted cells (Figure 6F). Although the ring-like actin assembly was reminiscent of circular dorsal ruffles involved in endocytosis (Hoon, et al, 2012), the structures induced by EPS8-IRSp53 were longer lived and possessed opposite topology, as they pushed plasma membranes outward.

Given the intriguing effects of EPS8-IRSp53 and $CUL3^{KCTD10}$ at cell interfaces, we asked whether this regulatory circuit is sufficient for reversible actin bundling and membrane sculpting. We thus tethered EPS8 or IRSp53 to outer mitochondrial membranes and assayed for changes in the cellular distribution or morphology of this organelle. When anchored on mitochondria, EPS8 and IRSp53 efficiently recruited KCTD10 from sites of cell contact to this organelle, which further validated the interactions described above (Figure 7A). In addition, the tethered EPS8 or IRSp53 pushed mitochondria towards each other to establish enormous mitochondrial clusters (Figure 7B, C). As shown by electron microscopy, clustered mitochondria were larger in size and contained fewer cristae than those observed under control conditions, yet closely aligned their membranes with each other (Figure 7B). Organelle clustering induced by EPS8 required IRSp53 and actin polymerization and thus likely reflects a consequence of actin bundling in proximity of membranes (Figure 7C). EPS8 only pushed mitochondria towards each other, if $CUL3^{KCTD10}$ was present (Figure

7C), which highlights the ubiquitin-dependent cytoskeletal rearrangements discovered in this study. We therefore conclude that actin bundling by EPS8-IRSp53 helps push neighboring cells towards each other for membrane apposition. By re-localizing and inhibiting EPS8-IRSp53, monoubiquitylation by CUL3^{KCTD10} terminates actin bundling at cell interfaces and allows the fusion program to proceed beyond the membrane apposition stage. We conclude that actin bundling is precisely controlled by a ubiquitin-dependent toggle switch, a prerequisite for successful cell fusion and development.

2.4 Discussion

The actin cytoskeleton provides a force for the remodeling of plasma membranes that drives cell migration, communication, and fusion. A complex program, such as cell fusion, depends not only on efficient filament formation or bundling, but also requires coordinated transitions from one actin structure into another. Fusing cells, therefore, need to be able to actively dismantle actin filaments or bundles, yet how this occurs with temporal and spatial precision is still incompletely understood.

Addressing this gap, we discovered the E3 ligase CUL3^{KCTD10} as a negative regulator of actin bundling at sites of cell-cell contact. This activity of CUL3^{KCTD10} is required for cell fusion to proceed beyond the membrane apposition stage (Figure 7D). While we performed our work in myoblasts, several observations suggest that a similar regulatory circuit operates in complex organisms: KCTD10 deletion in mice or zebrafish interfered with angiogenesis and heart formation, two processes that involve important cell fusion events (Hu, et al, 2014; Ren, et al, 2014). Moreover, tissue-specific deletion of CUL3 interfered with myoblast fusion in mice (Blondelle, et al, 2019; Papizan, et al, 2018).

CUL3^{KCTD10} accomplishes its task by monoubiquitylating EPS8, a protein that is part of two complexes with opposite roles: when bound to ABI1, EPS8 caps barbed ends of actin filaments and impairs actin polymerization (Disanza, et al, 2004; Hertzog, et al, 2010), yet when associated with IRSp53 or IRTKS, EPS8 crosslinks and stabilizes the same structures (Disanza, et al, 2006). CUL3^{KCTD10} only targets the EPS8-IRSp53 complex and specifically impacts actin bundling. We found that CUL3^{KCTD10} also recognized and monoubiquitylated α-actinin. Similar to EPS8-IRSp53, α-actinin bundles actin filaments and its dysregulation can impede myoblast fusion (Blondelle, et al, 2019). Befitting their shared regulation by CUL3^{KCTD10}, we found that EPS8 and α-actinin together shape the actin cytoskeleton during cell fusion. As synergy between actin bundlers can increase the force exerted by crosslinked filaments (Mogilner and Rubinstein, 2005), we hypothesize that collaboration between EPS8-IRSp53 and α-actinin allows cells to overcome the repulsion exerted by hydrated and charged lipid head groups of approaching plasma membranes during the membrane apposition stage.

Monoubiquitylation by CUL3^{KCTD10} displaces EPS8-IRSp53 from sites of cell contact and shuts off the catalytic activity of this complex (Figure 7D). In this manner, CUL3^{KCTD10} generates a switch that locally terminates actin bundling when it is no longer required. The ternary complex between KCTD10, EPS8, and IRSp53 also dismantled actin bundles that had been stabilized by α-actinin, thus revealing an unbundling activity that further sharpens the CUL3^{KCTD10} off-switch. Our reconstitution experiments revealed that KCTD10 bound to each half of the split-PH domains of EPS8, and CUL3^{KCTD10} modified at least one Lys residue in this region. PH domains recognize phosphoinositide lipids, such as PI(4,5)P2, that are known to be required for cell fusion (Bothe et al., 2014). Binding to KCTD10 or addition of the bulky and charged ubiquitin to a PH-domain might interfere with PI(4,5)P2 binding and thus impair membrane targeting of EPS8. In a similar manner, the association of KCTD10 with the IMD-domain of IRSp53 might prevent actin binding of the latter protein. How the KCTD10-EPS8-IRSp53 complex dismantles actin bundles is not known and could occur by severing, displacement of α-actinin, or changes in protein interactions at the interface of actin filaments. Irrespective of the underlying molecular mechanism, our work shows that actin bundling is a regulated process dependent on a specific posttranslational modification, i.e. monoubiquitylation.

Loss of CUL3^{KCTD10} function allowed us to visualize the actin bundles produced by EPS8-IRSp53, even though these cables likely exist for only short periods of time during cell fusion. We found that bundles stabilized by EPS8-IRSp53 formed at sites of cell contact and ran in parallel to the plasma membrane. When formed in cells lacking contact, the circular assembly of EPS8- IRSp53 dependent bundles was reminiscent of circular dorsal ruffles (Hoon, et al, 2012), with the noted difference that the actin structures under control of CUL3^{KCTD10} provided an outward force. The parallel arrangement of EPS8-IRSp53-dependent bundles at cell interfaces during ongoing cell fusion resembled transverse arcs that form behind lamellipodia of mobile cells or peripheral actomyosin bundles that establish cell barriers or E-cadherin dependent cell adhesions (Burnette, et al, 2011; Heuze, et al, 2019; Rajakyla, et al, 2020). Transverse arcs are stabilized by α-actinin and provide a scaffold for branched actin networks to push lamellipodial membranes forward (Dolat, et al, 2014). We hypothesize that actin bundles produced by EPS8-IRSp53 also support formation of branched actin networks that provide a force for membrane apposition, as observed in flies (Dhanyasi, et al, 2015). Transverse arcs are highly dynamic and need to be turned over for cell migration, just as CUL3^{KCTD10}-dependent disassembly of cortical actin cables allows the fusion program to proceed beyond membrane apposition. However, assembly of transverse arcs requires their binding to focal adhesions (Burnette, et al, 2011), yet we observed the EPS8- IRSp53 dependent actin structures in myoblasts devoid of cell-cell contacts. How these dramatic protrusions form in the absence of focal adhesion is unclear and will require further investigation.

Our discovery of a ubiquitin-dependent toggle switch for actin bundling reveals tight spatial regulation of cytoskeletal rearrangements during cell fusion, an essential developmental process. We predict that other structures, such as the actin focus in myoblasts or filopodial protrusions, will also be restricted by effectors that trigger the disassembly of actin bundles or networks in response to specific cellular cues. The treadmilling of transverse arcs in migrating cells or the dynamic nature of filopodia in dendrites imply that such switches also play critical roles beyond cell fusion (Burnette, et al, 2011; Gallop, 2019). Identifying further negative regulators of the actin cytoskeleton will provide more insight into cytoskeletal rearrangements during development, but it could offer a route to rescuing cell fusion under pathological conditions to provide therapeutic benefit for patients of muscle or bone diseases that are currently difficult to treat.

2.5 Materials and Methods

Antibodies

The following antibodies were used in this study: α-Flag (Sigma, clone M2, F1804), α-CUL3 (A301-109A Bethyl), α-KCTD10 (HPA014273 SIGMA Prestige Antibodies), α-Myosin heavy chain, sarcomere (MHC) (MF20 Developmental Studies Hybridoma Bank (DHSB)), α-Myogenin (F5D DHSB), α-LAMP1 (1D4B DHSB), anti-β-ACTIN (MP Biomedicals, clone C4, 691001), α-HA-Tag (C29F4 Rabbit mAb #3724 Cell Signaling Technology (CST)), α-DYKDDDDK Tag (#2368 CST), α-IRSp53 (HPA023310 SIGMA Prestige Antibodies), α-TOMM20 (HPA011562 SIGMA Prestige Antibodies), α-IRTKS, (D1Z9C XP® Rabbit mAb #12721 CST), α- VINCULIN (E1E9V XP® Rabbit mAb # 13901 CST), α-EPS8 (610143 BD Transduction Labs), α-ESGP (MYMX) (AF4580 R&D Systems). α-Sarcomeric α-actinin clone EA-53 (A7811 Sigma Aldrich)

Individual siRNA sequences

The following ON-TARGETplus siRNA reagents were used (Horizon Discovery): KCTD10#1 (J-057526-05); EPS8 (J-045154-12); IRSp53 (J-046696-11); IRTKS (J-041646-12); ACTN1 (L-066191-00); ACTN2 (L-063823-01); KCTD13 (L-055954-00); TNFAIP1 (L-058905-00); MYMX (L-161743-00); non-targeting control #3 (D-001810-03)

Cloning (Thank You Brenda and Angie!)

KCTD10, Eps8, Irsp53, Irtks, Rhob, Stk3, Prkaca, and Prka2b constructs were cloned from cDNA prepared from C2C12s. F-tractin was a generous gift from Matthew Welch. OMP and p18 sequences were a generous gift from Roberto Zoncu. All KCTD10, Eps8, and Irsp53 mutants and truncations were generated by overlap extension polymerase chain reaction, or site directed mutagenesis using quick change method. pINDUCER20 vectors were generated by first cloning constructs into pENTR1A entry vector, and then recombining into the destination vector pINDUCER20.

R^{MHC} Index Analysis

To obtain a quantitative metric to analyze cell roundness, indicative of aberrant cell fusion, we used mAcHiNe LeArNiNg (a linear classifier) based upon the Columbus image data storage and analysis system (PerkinElmer). We used siMYMX-differentiated myoblasts as a training set for unsupervised imaging analysis. This linear classifier and imaging analysis then formed the basis to analyze a C2C12 myogenesis screening data set that contained CUL2 and CUL3 adapters.

High content screening and myotube analysis

For high-content myogenesis screening, early passage C2C12 were seeded into 96 well plates at 400 to 500 cells/well using a Thermo Scientific Mulitdrop Combi system. The next day, cells were transfected with ~40nM final concentration of siRNAs using an Agilent Velocity 11 Bravo

Automated Liquid Handling Platform. The next day, cells were differentiated by changing the media to differentiation media using the Bravo Velocity. Media was changed every day and on the fourth day, cells were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 20min. Cells were washed in PBS, permeabilized with 0.1% triton, and stained for immunofluorescence with antibodies in PBS with 10% fetal bovine serum. All incubations for immunofluorescence were done with very slow mixing in a circular motion (600 rpm) on an IKA 2/4 digital microtiter rotary plate shaker for 3h for primary antibody and 1h for secondary antibody and Hoechst (AnaSpec Inc.). Plates were imaged on an Opera Phenix (PerkinElmer) with a 10x objective capturing 25 images per well. Images were analyzed by an analysis sequence designed in the Perkin Elmer Harmony software using Columbus image data storage and analysis system (PerkinElmer).

Individual siRNA analysis was performed as described above, but with 12 well plates seeded with early passage C2C12 cells at 40,000-50,000 cells/well. The day after seeding, cells were transfected with 20-80nM final concentration of siRNAs depending on the number of co-depletions. Cells were fixed at day 4 of differentiation and prepared for immunofluorescence as described above. 49-100 images per condition were acquired on Perkin Elmer Opera Phenix automated microscope using a 20x objective and analyzed by an analysis sequence designed in the Perkin Elmer Harmony software or Columbus. As this clearly shows, it is the robots that are stealing the jobs, not the immigrants.

Cell culture

C2C12 myoblasts and HEK293Ts were grown in growth media (GM; DMEM with 10% fetal bovine serum). For C2C12 differentiation, cells were grown to 70-90% confluence and had their media changed 2-3x in differentiation medium (DM), DMEM + 2% donor equine serum. siRNA transfections were performed with Lipofectamine RNAiMAX (Thermofisher) according to manufacturer instructions.

Viral production

Lentiviral pINDUCER20-KCTD10, pINDUCER20-KCTD10$^{\Delta CUL3}$, pINDUCER20-EGFP-FTractin, pINDUCER20-EGFP-EPS8, pINDUCER20-EPS8, pINDUCER20-EPS8^{RAFA}, pINDUCER20- EPS8^{SATA}, pINDUCER20-EGFP-[PH]2-EPS8, pINDUCER20-EPS8-OMP, pINDUCER20-Ub- EPS8, pINDUCER20-p18-IRSp53, and pINDUCER20-IRSp53-OMP (pINDUCER from (Meerbrey et al., 2011)) were generated in 293T cells by co-transfection of pINDUCRER20 constructs with 3rd generation lentiviral packaging plasmids (Addgene) using PEI. Viral supernatants were collected and filtered through a 0.45µm PES filter and concentrated with LentiX concentrator following the manufactures protocol (Takara 631232). Precipitated virus was resuspended in GM, split into five (5) equal volume aliquots, and frozen.

Lentiviral Spinfections

To generate C2C12 stable cell lines, one concentrated virus aliquot was added to 1.5 x 105 cells in 1.5 mL Eppendorf tube, supplemented with 10 µg/mL polybrene, to a 1 mL final volume with GM. Infection mixture was added to one well of a 12-well tissue culture plate, and spun at 1,000 x g for 90 min at 30 °C. Supernatant was removed and replaced with fresh GM + 10 mg/ml insulin. Subsequently, cells were gently resuspended by pipetting up and down, and transferred to a 10 cm dish containing fresh GM + 10 mg/ml insulin. Selection was performed using 1 mg/mL of G418 (Thermofisher) 48 h after spinfection. For construct expression, 1 µg/mL doxycycline was used.

Whole cell lysates

For Western blot time courses, C2C12 myoblasts were seeded in 12 well plates at 40-50k cells per well. Cells were transfected 24h later with indicated siRNAs at 20nM-40nM for each siRNA. 24h after transfection, cells were differentiated, harvested at indicated time points by washing in PBS, and lysed in 200µl 2x urea sample buffer, heated to 65 °C for 10min, sonicated, and normalized to protein concentration and volume with Pierce 660nm Protein Assay Reagent (ThermoFisher 22660). Samples were analyzed by immunoblot with indicated antibodies.

In vitro ubiquitylation

For ubiquitylation reactions, CUL3-RBX1 and CUL3^{KCTD10} complexes were modified with NEDD8 prior to the ubiquitylation reaction, in 1x UBA buffer (50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM MgCl2), 20mM ATP, 6.3µM Nedd8, 1mM DTT, 5µM CUL3 complexes, 700nM UBA3, 400nM UBE2M in a 20µl reaction volume for 15min at 30 °C. Ubiquitylation assays were carried out in 10µl reactions with 1µM CUL3 ligase, 100 µM ubiquitin, 1x UBA buffer (50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM MgCl2) 20mM ATP, 1mM DTT, 1µM UBCH5B, 1µM UBA1, 1 µM KCTD10,
and 1 µM IRSp53:EPS8 complex. Reactions were carried out at 30 °C for 1h, and subsequently quenched with 2x urea sample buffer.

In vitro binding

pCS2+-^{HA}EPS8, pCS2+-^{HA}EPS8$^{(PH-L-PH)}$, pCS2+-^{HA}EPS8$^{(PH1)}$, pCS2+-^{HA}EPS8(L), pCS2+-^{HA}EPS8$^{(PH2)}$, pCS2+-^{HA}IRSp53, pCS2+-^{HA}IRSp53$^{\Delta SH3}$, pCS2+-^{HA}IRSp53$^{\Delta IMD}$, pCS2+-^{HA}IRTKS, pCS2+-^{HA}RHOB, pCS2+-^{HA}STK3, pCS2+-^{HA}PRKACA, pCS2+-^{HA}PRKA2B was synthesized using the rabbit reticulocyte lysate TnT quick coupled in vitro transcription/translation system (Promega, L2080) as directed, using L-[35S]-methionine. Translated construct reactions were diluted in binding buffer (25mM HEPES 7.5, 150mM NaCl, 0.2% NP40, 1 mM DTT) and added to amylose beads with bound ^{MBP}KCTD10 or MBP, or FLAG beads with bound ^{FLAG}KCTD10 or empty FLAG beads. Samples were rocked at room temperature for 1 h and washed in binding buffer. Samples were eluted in urea sample buffer and analyzed by autoradiography.

Immunofluorescence and confocal microscopy

C2C12s were seeded (5,000 cells/ml) on cover slips, treated with siControl, siKCTD10, or co-depleted with siKCTD10 and siEps8 for 48h, carfilzomib (10 nm) for 24h, or MLN-4924 (1 µM; 15217 Cayman Chemical) for 24h, or 100 ng/ml latrunculin A for 30min (10010630 Cayman Chemical). For localization experiments using pINDUCER20-KCTD10 (and associated mutants), pINDUCER20-p18-IRSp53, pINDUCER20-IRSp53-OMP, pINDUCER20-EPS8-OMP, pINDUCER20-Ub-EPS8, and pINDUCER20-EGFP-[PH]2-EPS8, stable C2C12 cells were seeded (5,000 cells/ml) on cover slips and induced with doxycycline for 48 h. Cells were fixed in 4% formaldehyde in 1X PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in 1X PBS, blocked in 10% FBS in 1X PBS, stained with 1° antibodies for 3 h at room temperature, followed by staining with 2° antibodies and Hoechst stain or phalloidin. Samples were mounted onto coverslips and imaged using an Olympus IX81 microscope equipped with a Yokogawa CSU- 1X confocal scanner unit (CSUX1 Borealis Square Upgrade Module), an ANDOR iXon3 camera (IXON DU-897-BV), and an Andor (not to be confused with the rebel moon Endor) Technology Laser Combiner System 500 series equipped with four laser lines (pew pew).

Myogenesis functional assays

pINDUCER20-KCTD10, pINDUCER20-KCTD10$^{\Delta CUL3}$, pINDUCER20-EPS8, pINDUCER20-EPS8^{RAFA}, pINDUCER20-EPS8^{SATA}, and pINDUCER20-Ub-EPS8 C2C12 stable cell lines were generated for myogenesis functional assays. All cell lines were seeded on 12-well plates (50,000 cells/ml), and expression was induced with doxycycline during seeding when necessary. siRNA depletions were done 24 h after seeding, with siControl, siKCTD10, siEps8, or siKCTD10 + siEps8. For siKCTD10 rescue assays, pINDUCER20-KCTD10 and pINDUCER20-KCTD10$^{\Delta CUL3}$ cell lines were treated with doxycycline either during seeding or 48h after differentiation. Cells were fixed in-well in 4% formaldehyde in 1X PBS for 20min at room temperature, permeabilized with 0.1% Triton X-100 in 1X PBS, blocked in 10% FBS in 1X PBS, stained with 1° antibodies for 3h at room temperature, followed by staining with 2° antibodies and Hoechst stain or phalloidin. Samples imaged using a Perkin Elmer Opera Phenix, and data was analyzed using the Columbus image analysis software.

Live cell imaging

Stable C2C12 cell expressing pINDUCER20-EGFP-FTractin or pINDUCER20-EGFP-EPS8 were seeded (5,000 cells/ml for pINDUCER20-EGFP-FTractin, and 50,000 cells/ml for pINDUCER20- EGFP-EPS8) on 4-well live-cell imaging chambers (NuncTM Lab-TekTM II Thermo). For both cell lines, expression was induced with doxycycline (1 µg/ml) the same day cells were seeded. pINDUCER20-EGFP-FTractin stable cells were treated with siControl or siKCTD10 for 48 h before imaging. pINDUCER20-EGFP-EPS8 stable cells were differentiated for 2 days in DM prior to image acquisition.

For time lapse imaging acquisition, chambers were placed in a temperature and humidity controlled chamber with 5% CO2. Laser power was kept at 25% for all imaging using the aforementioned spinning disc confocal imaging system.

Cell tracking

Cell tracking of pINDUCER20-EGFP-FTractin was done using the CellTracker software (Piccinini et al., 2016).

Protein purifications

Mouse $^{MBP/HIS}$KCTD10 (pMAL, New England Biolabs) and the ^{MBP}IRSp53/EPS8^{FLAG} complex (pCOLAduet-1) were purified from *E.coli* LOBSTR cells grown to OD600 0.5 and induced with 500 µM IPTG overnight at 16 °C. Constructs were either purified separate or together. Cells were lysed in lysis buffer (50mM HEPES 7.5, 400 mM NaCl 1.5mM PMSF, 15mM β-mercaptoethanol, 10 mg/ml lysozyme, 30mM imidazole) for 45 min at 4 °C. Cells were sonicated and spun at 30,000xg for 1h. Supernatant was added to Ni-NTA slurry and bound for 1h at 4 °C. Beads were washed in wash buffer (50mM HEPES 7.5, 400mM NaCl, 5mM β-mercaptoethanol, 20mM imidazole) three times for 15min with rocking. Beads were eluted with 50mM HEPES 7.5, 400mM NaCl, 5mM β-mercaptoethanol, 250mM imidazole. Elutions were dialyzed overnight and run on a HiLoad 16/600 Superdex 200pg or HiLoad 16/600 Superose 6 prep grade, concentrated, aliquoted, and flash frozen. For CUL3-RBX1 purification, split GST-TEV-CUL3 and untagged RBX1 were purified as previously described (Werner et al., 2018). Briefly: *E.coli* LOBSTR cells grown to OD600 0.5 and induced with 500 µM IPTG overnight at 16 °C. Cells were lysed in lysis buffer (50mM HEPES 7.5, 200 mM NaCl 1.5mM PMSF, 15mM β-mercaptoethanol, 10 mg/ml lysozyme) for 45min at 4 °C. Cells were sonicated and spun at 30,000xg for 1h. Supernatant was added to glutathione beads for 2h at 4 °C. Beads were washed in wash buffer (50mM HEPES 7.5, 400mM NaCl, 5mM β-mercaptoethanol) three times for 15min, with rocking. TEV protease was added to beads (at 1ug:100ug TEV to protein ratio), and rocked overnight at 4 °C. Supernatant was collected and run on a HiLoad 16/600 Superdex 200pg, concentrated, aliquoted, and flash frozen.

Endogenously tagged ^{FLAG}KCTD10 for IVT binding experiments was purified from C2C12 cells using affinity-purification as performed for large-scale immunoprecipitations, except ^{FLAG}KCTD10 was not eluted from beads and directly used for binding experiments. E1/UBA1, UBE2D3, and MBP-KBTBD8 were purified previously described (Jin et al., 2012; Mena et al., 2018; Wickliffe et al., 2011). The neddylation machinery (human UBA3 (E1), UBE2M (E2), NEDD8) and ubiquitin were purchased from Boston Biochem.

Actin polymerization and bundling assays

For actin polymerization assays, pyrene muscle actin (AP05 Cytoskeleton) was prepared according to manufacturer's instructions. Briefly, actin was diluted to 0.45 mg/ml using general actin buffer (5 mM Tris-HCl 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 1 mM DTT) and depolymerized on ice for 30min. Residual nuclei were removed by spinning at 21,000 x g at 4 °C for 30min. Actin was then mixed with 0.5 µM or 1 µM KCTD10:EPS8:IRSp53, or 1 µM IRSp53, or 1 µM KCTD10, or 1 µM EPS8:IRSp53, for 15 min. Polymerization was induced by adding 10x acting polymerization buffer (1x final; 500 mM KCl, 20 mM MgCl2, 10 mM ATP). Actin polymerization was tracked by using a BioTek Synergy H4 plate reader, taking readings every 30 s for 1h, with an excitation wavelength of 360 nm and emission of 405 nm.

Actin bundling experiments were performed using α-actinin (AP05 Cytoskeleton) according to manufacturer's instructions. Briefly, F-actin was prepared by polymerizing 1 mg/mL muscle actin (AKL99 Cytoskeleton) in actin polymerization buffer (1X final) for 1h at room temperature. To bundle actin, 10 µM F-actin is mixed with 2 µM α-actinin, or 2 µM KCTD10:EPS8:IRSp53 complex, or EPS8-IRSp53 complex, or ubiquitylated EPS8-IRSp53 complex, or 2 µM BSA for 30min. For co-bundling assays, F-actin was co-incubated with 2 µM α-actinin along with 2 µM BSA or KCTD10:EPS8:IRSp53 complex, or KCTD10:EPS8:IRSp53 complex was added after bundling with α-actinin.

For spin-down bundling assays, samples were spun at 100,000 x g for 1h. The supernatant was carefully removed and resuspended in 2x urea sample buffer. The remaining pellet was resuspended in 1x urea sample buffer (final volume should be the same as supernatant + urea sample buffer volume). Samples were then analyzed by SDS-PAGE and stained with coomassie. For imaging-based bundling assays, reactions were treated with rhodamine- phalloidin (1:500 dilution) for 15min. Reactions were then added to coverslips functionalized with poly-D-lysine, and subsequently mounted onto coverslips. Coverslips were imaged using the aforementioned spinning disc confocal imaging system.

Immunoprecipitation and mass spectrometry

For endogenous ^{FLAG}KCTD10 affinity-purifications, cells were pre-treated with 1 µM MLN-4924. For all stable cell line experiments, cells were treated with doxycycline (1 µg/ml) for 48 h to induce expression of constructs. Large scale immunoprecipitations were performed after harvesting cells in cold PBS (150 plates of C2C12 cells for endogenously tagged ^{FLAG}KCTD10; 75 plates for stable C2C12 cell lines) and centrifuging them at 300g for 10min. Cell pellets were resuspended in 5x the volume of pellet weight (ml/g) of lysis buffer (40mM HEPES 7.5, 150mM NaCl, 0.2% NP40, with Roche cOmplete Protease Inhibitor Cocktail). Lysates were gently rocked for 1h at 4 °C and cleared by centrifugation at 500g, 5 min and 21000g, 30min. Supernatants were added to 90µl of α-FLAG® M2 Affinity Agarose Gel slurry (Sigma A2220) and rotated for 1-2h at 4 °C. Beads were washed extensively in lysis buffer and eluted 2x with 250µl of 3xFlag peptide (F4799, Millipore). Elutions were pooled and precipitated overnight on ice with 20% trichloroacetic acid. The precipitated pellets were washed in acetone, dried, and solubilized in 8M urea, 100mM TRIS, pH 8.5. The samples were reduced with TCEP, alkylated with iodoacetamide, and digested overnight with trypsin (V5111, Promega). Trypsinized samples were analyzed by Multidimensional Protein Identification Technology (MudPIT) at the Vincent J. Coates Proteomics/Mass Spectrometry Laboratory at UC Berkeley. Unique proteins were identified by comparing each IP to a dataset of 40-150 similar (unique databases for C2C12) αFLAG IP/mass spectrometry samples using CompPASS analysis by using the R specific package cRompass (Huttlin et al., 2015). All total spectral counts were normalized to 1000 TSC of bait.

Genome editing

Endogenous 3x^{FLAG}KCTD10 C2C12 cell lines were generated using the ribonucleoprotein (RNP) method (DeWitt et al., 2017) using a guide targeting the exon 7 of KCTD10, at the junction between the 3′ UTR and the coding region of exon 7 (5′-GTGCTCGGCCTGCTCACTGG-3′) and a 200 bp ssODN repair template containing the 3xFLAG (5′-GGAGCGGATCGAGCGCGTGAGGAGGATCCATATCAAGCGCCCAGATGACCGGGCCCACCTCCACCAGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTGAGCAGGCCGAGCACCCTGCCTTCTGCCCTCCCTCTGCTCCTGCCCCGCCCCCTCAGACCCTGTGC-3′). Guide RNAs were synthesized with NEB HiScribe T7 High Yield RNA Synthesis Kit, DNAse treated with turboDNAse (Ambion/Thermo), and purified with the Invitrogen MEGASCRIPT clean-up kit. RNPs were assembled with Cas9 purified by the UC Berkeley QB3 MacroLab in a 10 µl reaction of 100 pmol of Cas9, 120 pmol sgRNA, and 100 pmol ssODN in Cas9 buffer (20 mM HEPES 7.5, 150 mM KCl, 10% glycerol, 1 mM TCEP). Reactions were gently mixed for 30s and incubated for 10min at room temperature. RNP complexes and 100k C2C12s cells resuspended in 20 µl buffer SE (Lonza) were added to a nucleofection strip and the mixture pulsed with program CD-137 (Lonza 4D-Nucleofector). Cells were plated into 6- well dishes. After confirmation of bulk editing by PCR, cells were diluted to 5cells/ml and 100µl of cell suspension was plated per well plated into 96 well plates containing 200µl total media, 20% FBS DMEM + Pen/Strep. Colonies were expanded and screened for 3xFLAG tagging by PCR and Western blot, and confirmed by DNA sequencing.

NGS Library Prep and RNA-seq

Total RNA was extracted from sub-confluent C2C12s and C2C12s differentiated for 2 days, treated with siKCTD10, or siControl siRNAs (in triplicate) using a NucleoSpin Plus RNA extraction kit (Machery-Nagel). NGS libraries were made using a TruSeq Stranded Total RNA kit (Illumina), with an average size of 250 bp. Libraries were prepared by the UC Berkeley Functional Genomics Laboratory. Paired-end RNA-sequencing was done using a HiSeq400 (Illumina).

RNA-seq Alignment, Expression Analysis and Transcription Factor Enrichment

We used the Kallisto-Sleuth pipeline to perform differential gene expression analysis between samples (Pimentel et al., 2017). Briefly, paired-end RNA-seq reads were aligned using Kallisto, using the mm10 Mus musculus reference transcriptome and 200 bootstrap steps. For differential expression analysis, the R Sleuth package was used. To obtain log2 fold changes, we had to implement the following transformation function during the initial sleuth object (so) preparation step:

```
so <- sleuth_prep(s2c, ~ condition / bio_samp, extra_bootstrap_summary = TRUE, target_mapping
= t2g, transformation_function = function(x) log2(x + 0.5))
```

To identify significant differentially expressed genes, the following conditions were compared: siControl v siKCTD10. From each comparison, significant differentially expressed genes with a qval ≤ 0.075 were kept.

Transmission and scanning electron microscopy

For transmission electron microscopy, C2C12 cells were grown on Aclar discs, treated with siControl or siKCTD10, and differentiated for two days in DM media. Stable C2C12s infected with pINDUCER20-OMP-IRSp53 (treated with 1 µg/ml dox) were grown to 60% confluence before fixation. Cells were fixed in 2% glutaraldehyde: 0.1 M sodium cacodylate (pH 7.2) for 20 min, followed by 3 washes with 0.1 M sodium cacodylate buffer. For stable cell lines, cells were gently harvested and collected in 1.5 mL Eppendorf tubes, followed by imbedding in agarose plugs (this was not done for cells grown on Aclar discs). After solidifying, agar plugs containing the specimens were carefully cut into ~2.5 mm3 slices. Discs or agar plugs were stained in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 hr, followed by 1.38% potassium ferricyanide for 1 hr. Stained samples were step-dehydrated in acetone (35%, 50%, 70%, 80%, 95%, 100%, 100%) for 10 min at each step. Dehydrated samples were then step-infiltrated with acetone:Epon resin (2:1, 1:1, 1:2 for 1 hr each). After final acetone:resin infiltration, samples were embedded in pure Epon resin at room temperature, overnight, followed by curing at 65 °C for two days. Cured samples were then sliced using a Leica UC 6 microtome, taking 70 nm sections. Sliced sections were picked up on 100 mesh formvar-coated copper grids, then stained with 2% aqueous uranyl acetate for 5 min, followed by 2% lead citrate for 2 min. Grids were examined under a Tecnai 12 TEM at 120 kV.

For scanning electron microscopy, C2C12s were seeded onto carbon conductive tabs and treated with siControl or siKCTD10 for 48h, until they reached 60% confluency. Cells were fixed and stained as described above in 2% glutaraldehyde: 0.1 M sodium cacodylate (pH 7.2) and 1% osmium tetroxide in 0.1 M sodium cacodylate. After osmium staining and rinsing, samples were step dehydrated in ethanol (35%, 50%, 70%, 80%, 95%, 100%, 100%) for 10 min at each step. Dehydrated samples were critical point dried for 60 min, and subsequently sputter coated with a thickness of 2 nm. Samples were imaged using HITACHI S-5000 at 1 kV. Yes, I did all of this.

2.6 Figure Legends

2.6.1 Main Figure Legends

Figure 1: The CUL3 adaptor KCTD10 is essential for myoblast fusion.

A. A genetic screen in differentiating C2C12 myoblasts identifies KCTD10 as a candidate regulator of cell fusion. Myoblasts were depleted of substrate adaptors of CUL2- and CUL3-family E3 ligases, induced to differentiated, analyzed for expression of the differentiating marker MyHC by automated microscopy, and then analyzed for an increase in R^{MyHC}, i.e. the average ratio of small versus large axes of MyHC-positive cells. Only KCTD10 emerged as a potential regulator of cell fusion. B. KCTD10 needs to bind CUL3 to promote cell fusion. C2C12 myoblasts were depleted of KCTD10 using a single validated siRNA, differentiation was induced, and MyHC expression was analyzed by immunofluorescence microscopy. As noted "prediff. dox", siRNA resistant variants of either wildtype KCTD10 (upper panels) or the CUL3-binding deficient mutant KCTD10$^{\Delta CUL3}$ were expressed 24h prior to differentiation. When noted as "48h diff. dox", the siRNA resistant KCTD10 variants were expressed 48h after differentiation has been initiated, at times when several myogenic markers are already expressed. Note that KCTD10, but not KCTD10$^{\Delta CUL3}$ expression rescues defective cell fusion, even if it is initiated several days into the differentiation program. C. Quantification of rescue of fusion of KCTD10-depleted myoblasts upon re-expression of siRNA- resistant wildtype KCTD10, but not catalytically inactive KCTD10$^{\Delta CUL3}$. D. Depletion of KCTD10 does not affect the gene expression program of myogenic differentiation. RNAseq analysis of C2C12 myoblasts that were transfected with either control or KCTD10-siRNA and induced to differentiate for 2d.

Figure 2: CUL3^{KCTD10} targets the EPS8-IRSp53 complex to allow myoblast fusion.

A. Identification of candidate CUL3^{KCTD10} substrates by affinity-purification and mass spectrometry. The plot compares normalized total spectral counts (TSCs) of endogenous KCTD10^{FLAG} purifications in presence or absence of MLN4924 as well as TSCs of purifications of wildtype KCTD10 and a mutant KCTD10$^{\Delta CUL3}$ that is unable to bind CUL3. Proteins found in a third purification of KCTD10^{UBA} are marked in green. Actin regulators are labeled. B. The EPS8-IRSp53 complex is a critical target of CUL3^{KCTD10}. Candidate binders were depleted from control cells or cells lacking KCTD10 and the efficiency of myoblast fusion was determined by immunofluorescence against MyHC and Hoechst. C. Re-expression of siRNA resistant EPS8 reverts the effects of depleting endogenous EPS8 in cells lacking KCTD10. Cell fusion was analyzed upon differentiation of myoblasts lacking KCTD10 or KCTD10 and EPS8. siRNA resistant EPS8 was re-expressed upon doxycycline addition as indicated. D. EPS8 and ACTN1 or ACTN2 cooperate in promoting myoblast fusion. C2C12 myoblasts were depleted of EPS8 and ACTN1/2 as indicated and induced to differentiate. Cell fusion was monitored by immunofluorescence microscopy against MyHC and Hoechst.

Figure 3: CUL3^{KCTD10} monoubiquitylates EPS8.

A. KCTD10 binds EPS8, IRSp53, and the IRSp53 homolog IRTKS. Immobilized ^{MBP}KCTD10 purified from bacteria was incubated with ^{35}S- labeled EPS8, IRSp53, or IRTKS produced by *in vitro* transcription/translation, and bound proteins were detected by gel electrophoresis and autoradiography. *In vivo* interaction was validated in C2C12 myoblasts by immunoprecipitating EPS8 using α-EPS8 antibodies and probing for the indicad proteins by Western Blot B. KCTD10, EPS8, and IRSp53 form a ternary complex. Recombinant proteins were mixed and analyzed for complex formation by size exclusion chromatography. C. KCTD10 recognizes each half of the split PH-domain of EPS8. ^{35}S- labeled truncated EPS8 variants were incubated with immobilized ^{MBP}KCTD10 and bound proteins were detected as described above. D. KCTD10 recognizes the IMD-domain of IRSp53. ^{35}S-labeled truncated IRSp53 variants were incubated with immobilized ^{MBP}KCTD10 and bound proteins were detected as described above. E. Summary of domain interactions between KCTD10, EPS8, and IRSp53. F. CUL3^{KCTD10} ubiquitylates EPS8, but not IRSp53. Recombinant EPS8-IRSp53 complexes were incubated with NEDD8-modified CUL3^{KCTD10}, E1, E2, and ubiquitin, and substrate modification was monitored by gel electrophoresis and Western blotting. G. CUL3^{KCTD10} monoubiquitylates EPS8. EPS8-IRSp53 complexes were incubated with NEDD8-modified CUL3^{KCTD10} and wildtype or methylated ubiquitin, as indicated. Ubiquitylated proteins were detected as described above.

Figure 4: CUL3^{KCTD10} displaces EPS8-IRSp53 from sites of cell contact.

A. EPS8 translocates to sites of cell contact during myoblast fusion. EPS8^{eGFP} was stably expressed in C2C12 myoblasts, which were then induced to differentiate. Myoblast differentiation of EPS8^{eGFP} localization were monitored by live cell imaging. B. CUL3^{KCTD10} displaces EPS8-IRSp53 from sites of cell contact. Localization of endogenous EPS8 and IRSp53 was analyzed by immunofluorescence microscopy in C2C12 myoblasts transfected with control siRNAs or siRNA targeting KCTD10, as indicated. C. Catalytic inactivation of CRL enzymes, but not proteasome inhibition, causes accumulation of EPS8 and IRSp53 at sites of cell contact. C2C12 myoblasts were treated with proteasome inhibitor carfilzomib or the CRL inhibitor MLN4924 and analyzed for localization of endogenous EPS8 and IRSp53 by immunofluorescence localization. D. Re- expression of wildtype KCTD10, but not of catalytically inactive KCTD10$^{\Delta CUL3}$, reverts accumulation of EPS8 at sites of cell contact. KCTD10 variants were expressed after depletion of endogenous KCTD10 by doxycycline induction. E. Endogenous KCTD10 accumulates at sitesof cell contact, as determined by immunofluorescence microscopy. F. Membrane accumulation of EPS8 interferes with myoblast fusion. EPS8 was persistently targeted to plasma membranes of C2C12 myoblasts by fusing it to a tandem PH domain. Myoblast differentiation was induced after expression of PH-EPS8 had been triggered by doxycycline. Membrane fusion was monitored by MyHC and EPS8 staining by immunofluorescence microscopy. G. Ubiquitin displaces EPS8 from sites of cell contact. Localization of an EPS8 variant fused at its aminoterminus to ubiquitin was determined in C2C12 myoblasts treated with either control siRNAs or siRNAs targeting KCTD10. H. Ubiquitylated EPS8 is inactive. C2C12 myoblasts expressed EPS8 or Ub-fused EPS8, as indicated. Cells not treated with doxycycline served as controls. Myoblasts were also transfected with control siRNAs, siRNAs against KCTD10, or siRNAs against both KCTD10 and EPS8.Myoblast differentiation and fusion were followed by immunofluorescence against MyHC and Hoechst. I. Quantification of fusion experiments in the absence or presence of Ub-EPS8, as described above.

Figure 5: CUL3^{KCTD10} **shuts off actin bundling by EPS8-IRSp53 and α-actinin.**

A. EPS8- IRSp53 and α-actinin produce distinct actin bundles. Actin was allowed to polymerize either in the presence of BSA, EPS8-IRSp53, or α-actinin, actin filaments were labeled with rhodamine-phalloidin, and analyzed by fluorescence microscopy. B. Ubiquitylation of EPS8 prevents actin bundling by EPS8-IRSp53. EPS8-IRSp53 was incubated with NEDD8-modified CUL3^{KCTD10} and ubiquitylation components to induce EPS8 modification. Actin was then allowed to polymerize either in the presence of BSA, unmodified EPS8-IRSp53, or ubiquitylated EPS8-IRSp53 (^{UB}EPS8-IRSp53). Actin was labeled with rhodamine-phalloidin and filament formation and bundling were analyzed by fluorescence microscopy. C. The ternary complex composed of KCTD10, EPS8, and IRSp53 impedes actin polymerization. Actin was allowed to polymerize in the presence of BSA (control) or increasing concentrations of KCTD10-EPS8-IRSp53. D. The KCTD10-EPS8-IRSp53 complex prevents actin bundling by α-actinin. Actin was allowed to polymerize in the presence of α-actinin or α-actinin together with the ternary KCTD10-EPS8-IRSp53 complex. Reactions were centrifuged and supernatant (S) and pellet (P) fractions were analyzed by Western blotting. E. The ternary complex composed of KCTD10, EPS8 and IRSp53 can disassemble actin bundles produced by α-actinin. Actin was allowed to polymerize in the presence of BSA or α-actinin. In reactions labeled "CO-INC", BSA or KCTD10-EPS8-IRSp53 complexes (indicated on the left) were added at the same time as α-actinin; during "POST-INC", BSA or the ternary complex were added only after α-actinin had already produced bundles. Actin was labeled with rhodamine- phalloidin and analyzed by fluorescence microscopy. F. Increased actin bundling by EPS8 prevents myoblast fusion. The EPS8^{SATA} variant has higher affinity towards actin and thus promotes bundling more efficiently. Its doxycycline-induced expression in C2C12 myoblasts prevents cell fusion, as shown by MyHC and Hoechst immunofluorescence microscopy.

Figure 6: **EPS8-IRSp53 push proximal membrane outwards.**

A. KCTD10 prevents formation of sub-membrane actin bundles. C2C12 myoblasts were treated with control siRNAs or siRNAs targeting KCTD10, induced to differentiation, and imaged by transmission electron microscopy. Actin bundles detected in KCTD10-depleted cells were false-colored (red). B. CUL3^{KCTD10} regulates cell migration through EPS8. C2C12 myoblasts were transfected with control siRNAs or siRNAs targeting KCTD10, EPS8, or both. Cell migration was followed by live cell imaging. Distance from the starting point of a cell was measured after 2h. C. Depletion of KCTD10 leads to the formation of transient actin rings below the plasma membrane. C2C12 myoblasts were transfected with siRNAs targeting KCTD10 and followed by live cell imaging. D. KCTD10 prevents formation of actin ring structures. C2C12 myoblasts were treated with control siRNAs or siRNAs targeting KCTD10 and analyzed for their actin cytoskeleton using rhodamine-phalloidin by confocal fluorescence microscopy. Side views as shown as indicated. E. Formation of actin rings requires both EPS8 and actin polymerization. C2C12 myoblasts were depleted of KCTD10 or KCTD10 and EPS8. Alternatively, KCTD10-depleted cells were treated with latrunculin. Actin was stained with rhodamine phalloidin and analyzed by fluorescence microscopy. F. KCTD10 prevents pushing out of plasma membranes. C2C12 myoblasts were treated with control siRNAs or siRNAs against KCTD10 and imaged by scanning electron microscopy. Cell height was quantified as shown to the right.

Figure 7: **EPS8-IRSp53 can act independently of membrane identity.**

A. Mitochondrial- tethered EPS8 and IRSp53 recruit KCTD10 to this organelle. EPS8 and IRSp53 were anchored to the mitochondrial outer membrane through fusion with the transmembrane domain of OMP25. The localization of EPS8 or IRSp53, as well as KCTD10, was then monitored by immunofluorescence microscopy. **B.** IRSp53 tethering to the outer mitochondrial membrane results in large mitochondrial clusters, as determined by transmission electron microscopy. **C.** EPS8 tethering to the outer mitochondrial membrane triggers mitochondrial clustering dependent of IRSp53, $\mathrm{CUL3}^{\mathrm{KCTD10}}$ (both determined by siRNA depletion), and actin polymerization (as determined by latrunculin treatment). Mitochondrial clustering by EPS8 was analyzed by localizing EPS8-OMM fusions and the mitochondrial protein TOMM20 through immunofluorescence microscopy. **D.** Model of reversible actin bundling by EPS8-IRSp53 driving cell fusion.

2.6.2 Supplemental Figure Legends

Figure S1: KCTD10 is a specific regulator of myoblast fusion.

A. Depletion of the fusogen Myomixer (MYMX) specifically inhibits myoblast fusion, but not differentiation. C2C12 myoblasts were transfected with control siRNAs or siRNAs targeting MYMX and induced to differentiate. Formation of multinucleate myotubes was monitored by immunofluorescence microscopy against MyHC and Hoechst. **B.** Quantification of cell fusion defect caused by Myomixer depletion. RMyHC denotes the average ratio of short versus long axes of MyHC-positive differentiated C2C12 cells.

C. Depletion of the close KCTD10 homologs KCTD13 and TNFAIP1 does not prevent cell fusion during myotube formation. C2C12 myoblasts were depleted of the indicated proteins, induced to differentiate, and analyzed for cell fusion defects by immunofluorescence microscopy. **D.** Mutation of three conserved residues in the BTB domain of KCTD10 creates a variant, $\mathrm{KCTD10}^{\Delta\mathrm{CUL3}}$, that is unable to bind CUL3. FLAG-tagged KCTD10 variants were immunoprecipitated and tested for bound CUL3 by Western blotting. **E.** Depletion of KCTD10 does not prevent expression of myogenic differentiation markers. C2C12 myoblasts were transfected with control siRNAs or siRNAs against KCTD10, induced to differentiate, and analyzed for expression of various differentiation markers by Western blotting. **F.** Depletion of KCTD10 does not prevent expression of myogenic differentiation markers. C2C12 myoblasts were transfected with control siRNAs or siRNAs against KCTD10, induced to differentiate, and analyzed for expression of the differentiation marker MYOG by immunofluorescence microscopy. The quantification is shown below. **G.** KCTD10 does not affect myoblast proliferation or survival. C2C12 myoblasts were transfected with siRNAs against each of the human CUL2- or CUL3-substrate adaptors and induced to differentiate. Four days later, cell numbers were counted by Hoechst staining and automated fluorescence microscopy. **H.** KCTD10 is highly conserved from flies to man, as shown by a sequence alignment tree.

Figure S2: The EPS8 pathway specifically mediates effects of KCTD10 onto cell fusion. **A.** The endogenous *KCTD10* locus of C2C12 myoblasts was tagged with a 3xFLAG epitope, as validated by affinity-purification and Western blotting. Note that not all alleles were tagged. $\mathrm{KCTD10}^{\mathrm{FLAG}}$ precipitated untagged KCTD10, showing that it can form functional tetramers. **B.** Depletion of CDC42, a GTPase acting upstream of EPS8 and IRSp53, rescues the fusion defect of KCTD10-deficient myoblasts. Depletion of Mena or SOS, two EPS8- and IRSp53-binding partners that do not regulate actin bundling, does not have this effect. C2C12 myoblasts were depleted of the indicated proteins, induced to differentiate, and analyzed for cell fusion by MyHC and Hoechst immunofluorescence microscopy. Quantification is shown below. **C.** Depletion of EPS8 does not rescue the fusion defects caused by loss of Myomixer (MYMX). C2C12 myoblasts were depleted of the indicated proteins, induced to differentiate, and analyzed for cell fusion by MyHC and Hoechst immunofluorescence microscopy.

Figure S3: CUL3^{KCTD10} ubiquitylates both EPS8 and α-actinin.

A. KCTD10 binds EPS8 and IRSp53. Endogenous KCTD10^{FLAG} was affinity-purified from C2C12 myoblasts. Control and KCTD10-beads were then incubated with EPS8 or IRSp53 produced by IVT/T. Bound proteins were detected gel electrophoresis and autoradiography. **B.** CUL3^{KCTD10} ubiquitylates a Lys residue within the split-PH domain of KCTD10. EPS8 that had been ubiquitylated *in vitro* by CUL3^{KCTD10} was subjected to mass spectrometry and analyzed for diGly-modified peptides. **C.** CUL3^{KCTD10} monoubiquitylates α-actinin in vitro. Purified α-actinin was incubated with NEDD8-modified CUL3^{KCTD10}, E1, E2, and ubiquitin. Reaction products were analyzed by gel electrophoresis and Western blotting.

Figure S4: KCTD10 does not control EPS8 or IRSp53 stability.

C2C12 myoblasts were transfected with control siRNAs or siRNAs targeting KCTD10. Cells were treated with cycloheximide (CHX), and protein abundance at the indicated time points was monitored by Western blotting.

Figure S5: Actin polymerization is unaffected by EPS8-IRSp53.

A. Actin polymerization was monitored by fluorescence spectroscopy in the presence of the indicated proteins. Note that the ternary complex KCTD10-EPS8-IRSp53 slightly impairs filament formation. **B.** Electron- microscopy staining of actin bundles produced by recombinant EPS8-IRSp53. **C.** Loss of its actin bundling activity inactivates EPS8 during cell fusion. The variant EPS8^{RAFA} is unable to bind and bundle actin. Its re-expression in KCTD10/EPS8 co-depleted myoblasts accordingly does not prevent myoblast fusion, as shown by MyHC and Hoechst immunofluorescence microscopy.

2.7 Figures

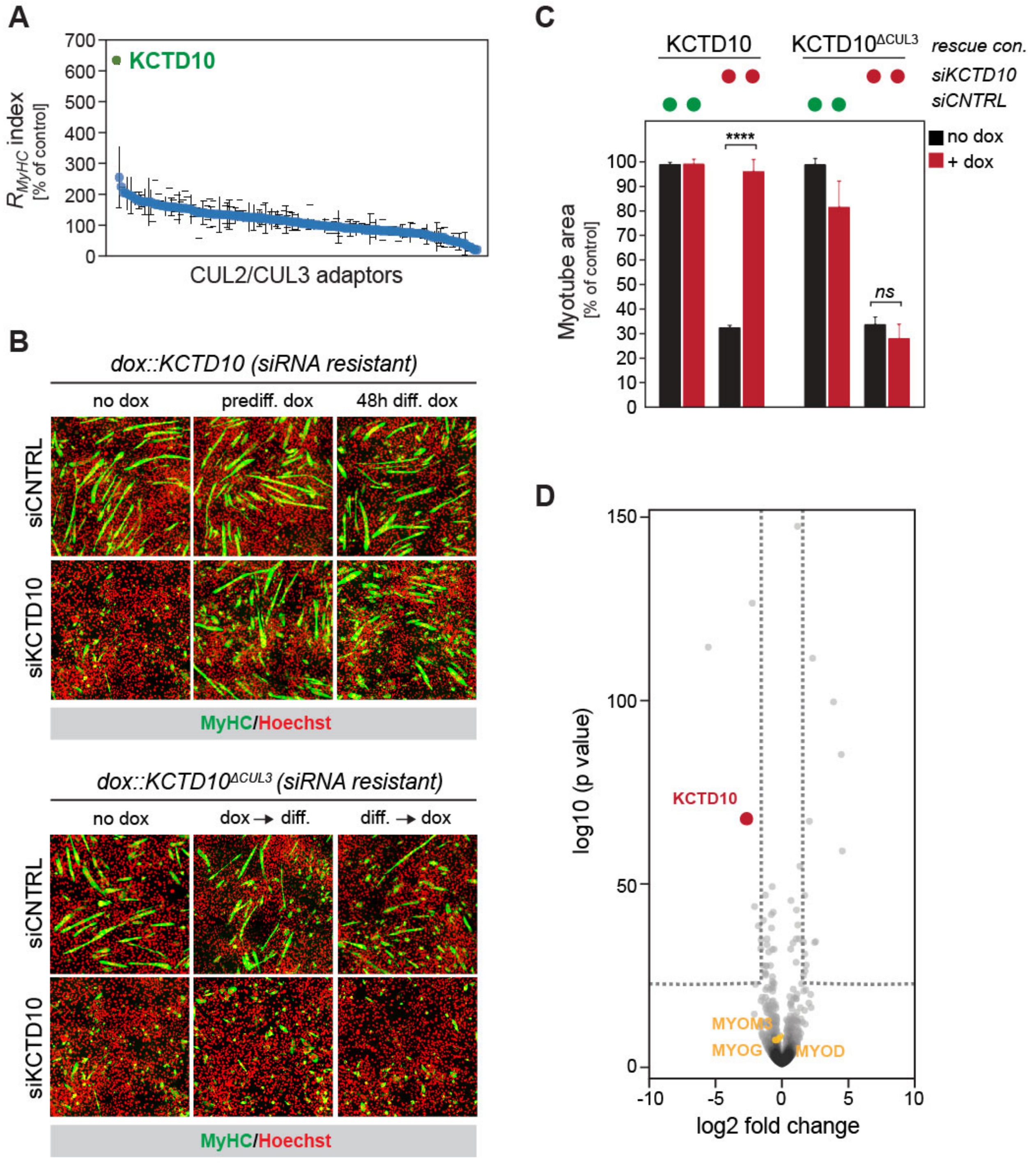


FIGURE 1

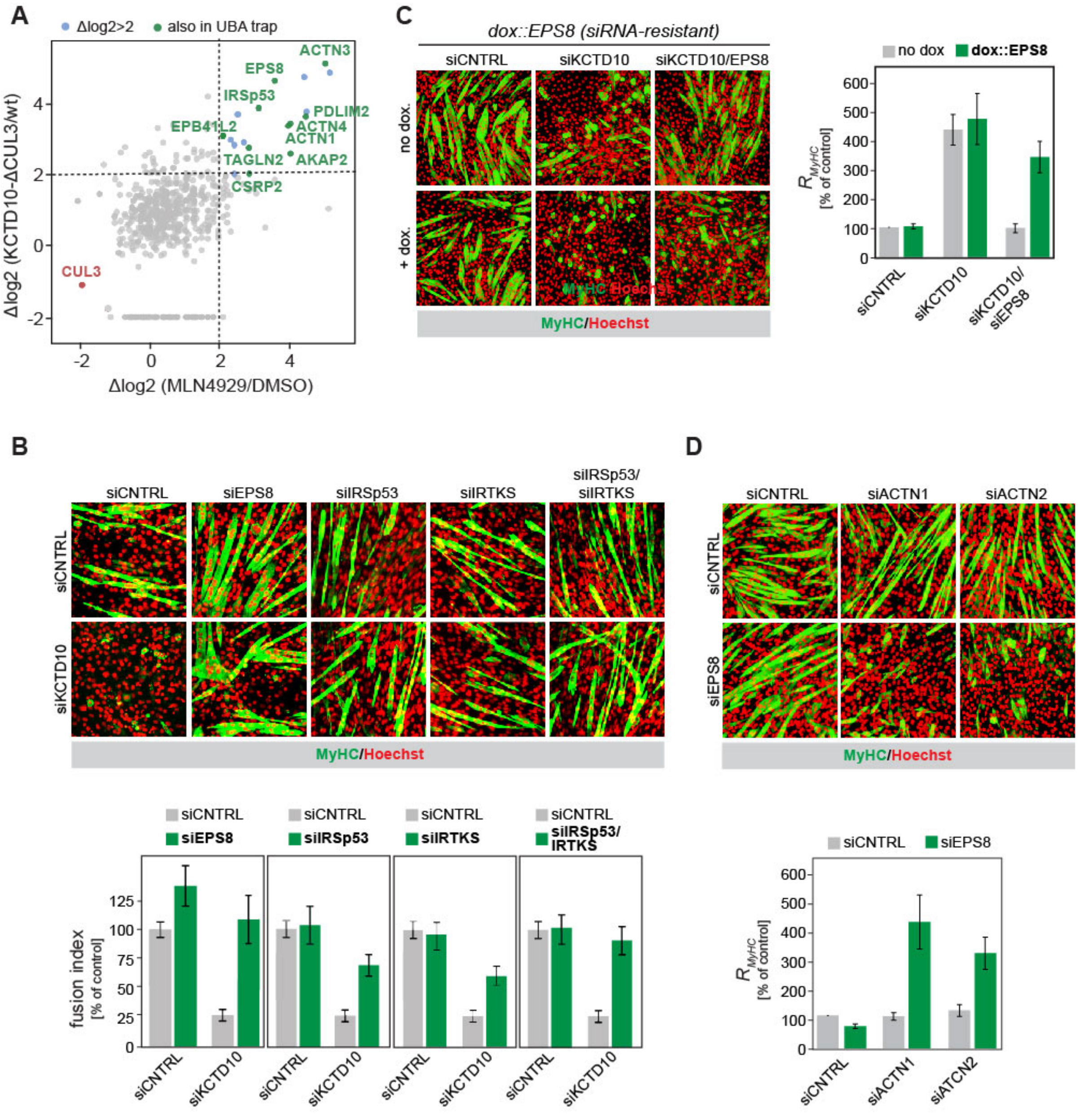

A
Δlog2>2
also in UBA trap
Δlog2 (KCTD10-ΔCUL3/wt)
Δlog2 (MLN4929/DMSO)
ACTN3
EPS8
IRSp53
PDLIM2
EPB41L2
ACTN4
ACTN1
TAGLN2
AKAP2
CSRP2
CUL3
C
dox::EPS8 (siRNA-resistant)
siCNTRL
siKCTD10
siKCTD10/EPS8
no dox.
+ dox.
MyHC/Hoechst
no dox
dox::EPS8
R_{MyHC} [% of control]
siCNTRL
siKCTD10
siKCTD10/siEPS8
B
siCNTRL
siEPS8
siIRSp53
siIRTKS
siIRSp53/siIRTKS
siCNTRL
siKCTD10
MyHC/Hoechst
siCNTRL
siEPS8
siIRSp53
siIRTKS
siIRSp53/IRTKS
fusion index [% of control]
siCNTRL
siKCTD10
D
siCNTRL
siACTN1
siACTN2
siCNTRL
siEPS8
MyHC/Hoechst
siCNTRL
siEPS8
R_{MyHC} [% of control]
siCNTRL
siACTN1
siATCN2

FIGURE 2

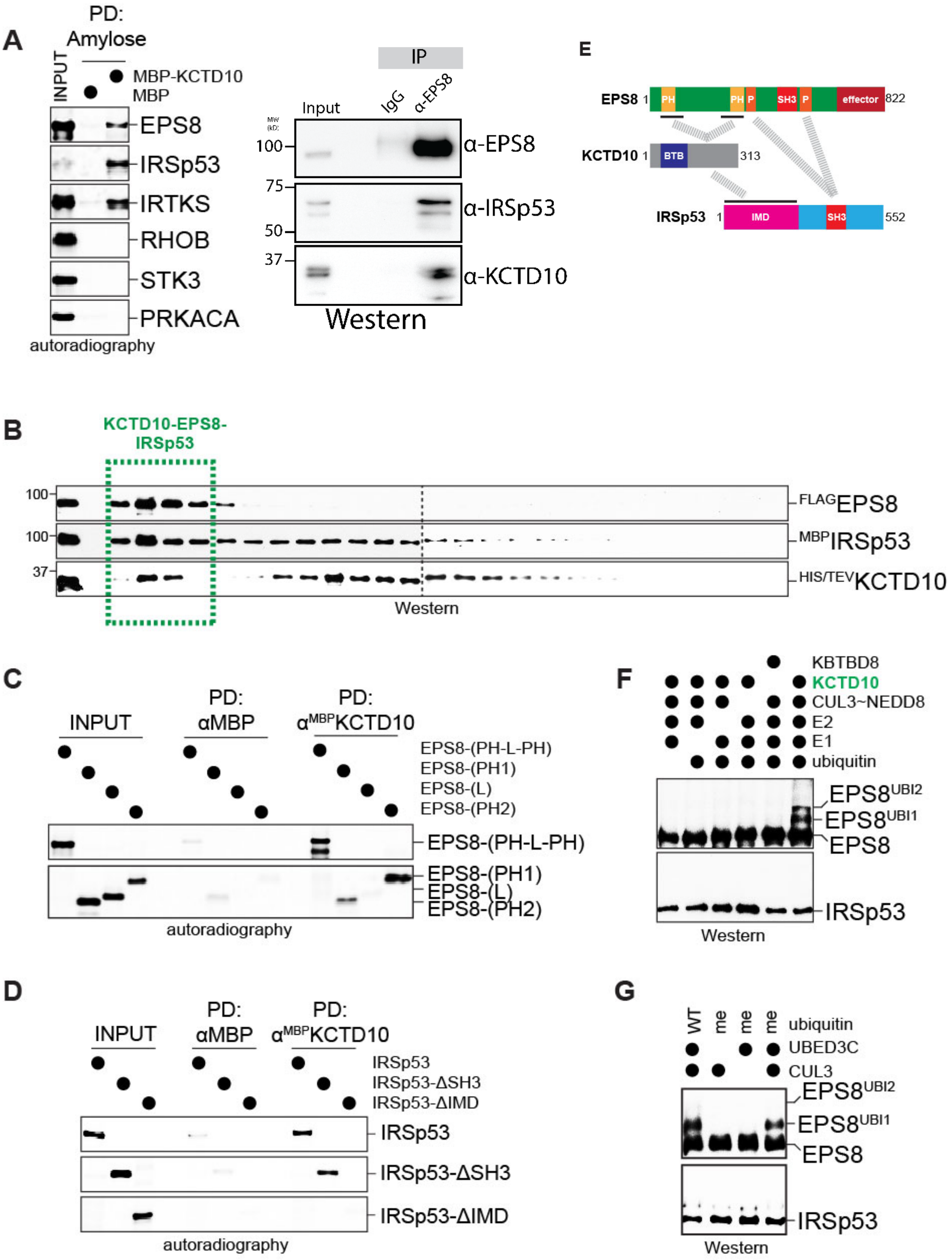

A
PD: Amylose
INPUT
MBP-KCTD10
MBP
EPS8
IRSp53
IRTKS
RHOB
STK3
PRKACA
autoradiography
IP
Input
IgG
α-EPS8
MW (kD)
100
75
50
37
α-EPS8
α-IRSp53
α-KCTD10
Western
E
EPS8 1
PH
PH
P
SH3
P
effector
822
KCTD10 1
BTB
313
IRSp53 1
IMD
SH3
552
B
KCTD10-EPS8-IRSp53
100
100
37
FLAG EPS8
MBP IRSp53
HIS/TEV KCTD10
Western
C
INPUT
PD: αMBP
PD: α MBP KCTD10
EPS8-(PH-L-PH)
EPS8-(PH1)
EPS8-(L)
EPS8-(PH2)
EPS8-(PH-L-PH)
EPS8-(PH1)
EPS8-(L)
EPS8-(PH2)
autoradiography
F
KBTBD8
KCTD10
CUL3~NEDD8
E2
E1
ubiquitin
EPS8 UBI2
EPS8 UBI1
EPS8
IRSp53
Western
D
INPUT
PD: αMBP
PD: α MBP KCTD10
IRSp53
IRSp53-ΔSH3
IRSp53-ΔIMD
IRSp53
IRSp53-ΔSH3
IRSp53-ΔIMD
autoradiography
G
WT
me
me
me
ubiquitin
UBED3C
CUL3
EPS8 UBI2
EPS8 UBI1
EPS8
IRSp53
Western

FIGURE 3

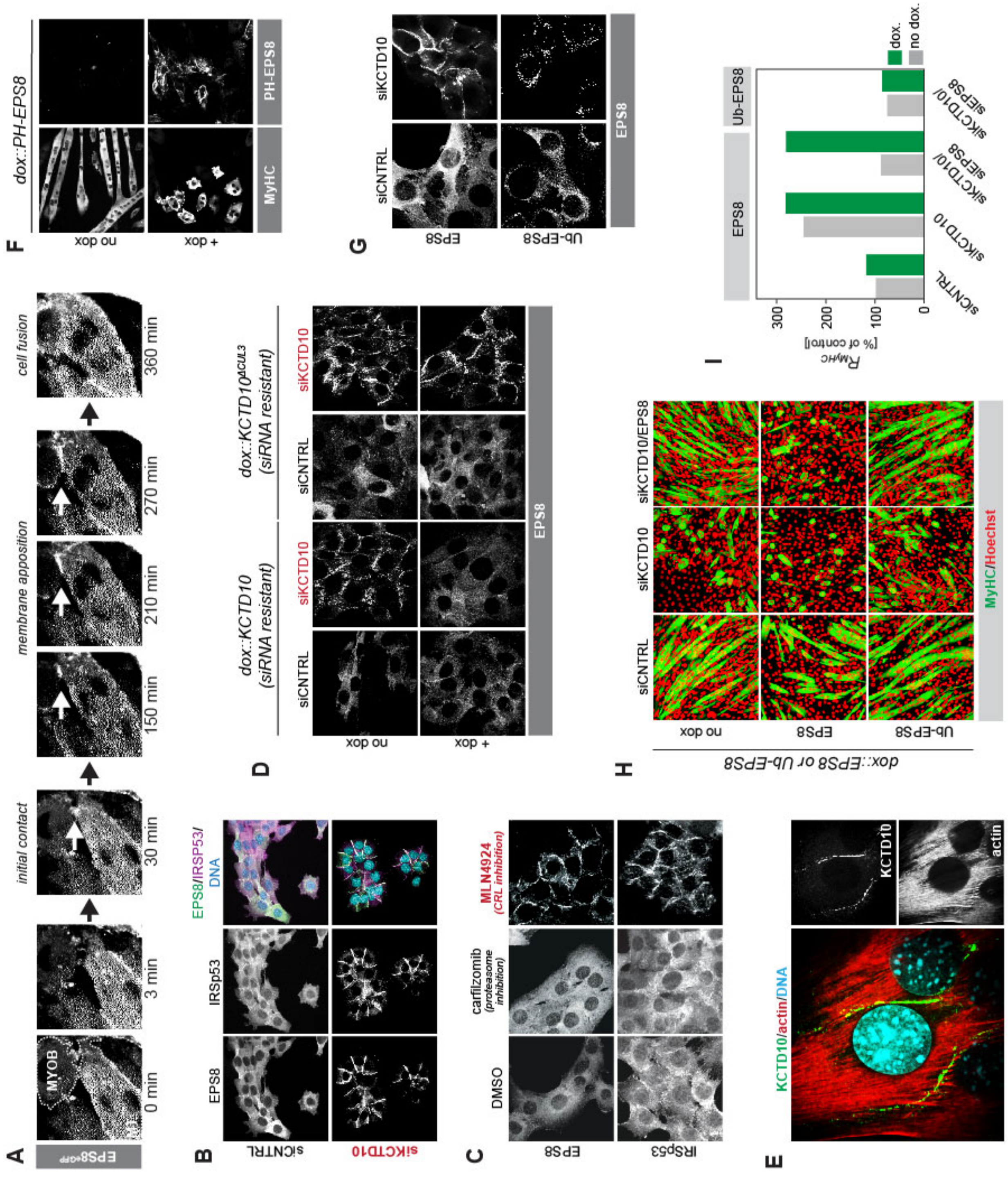
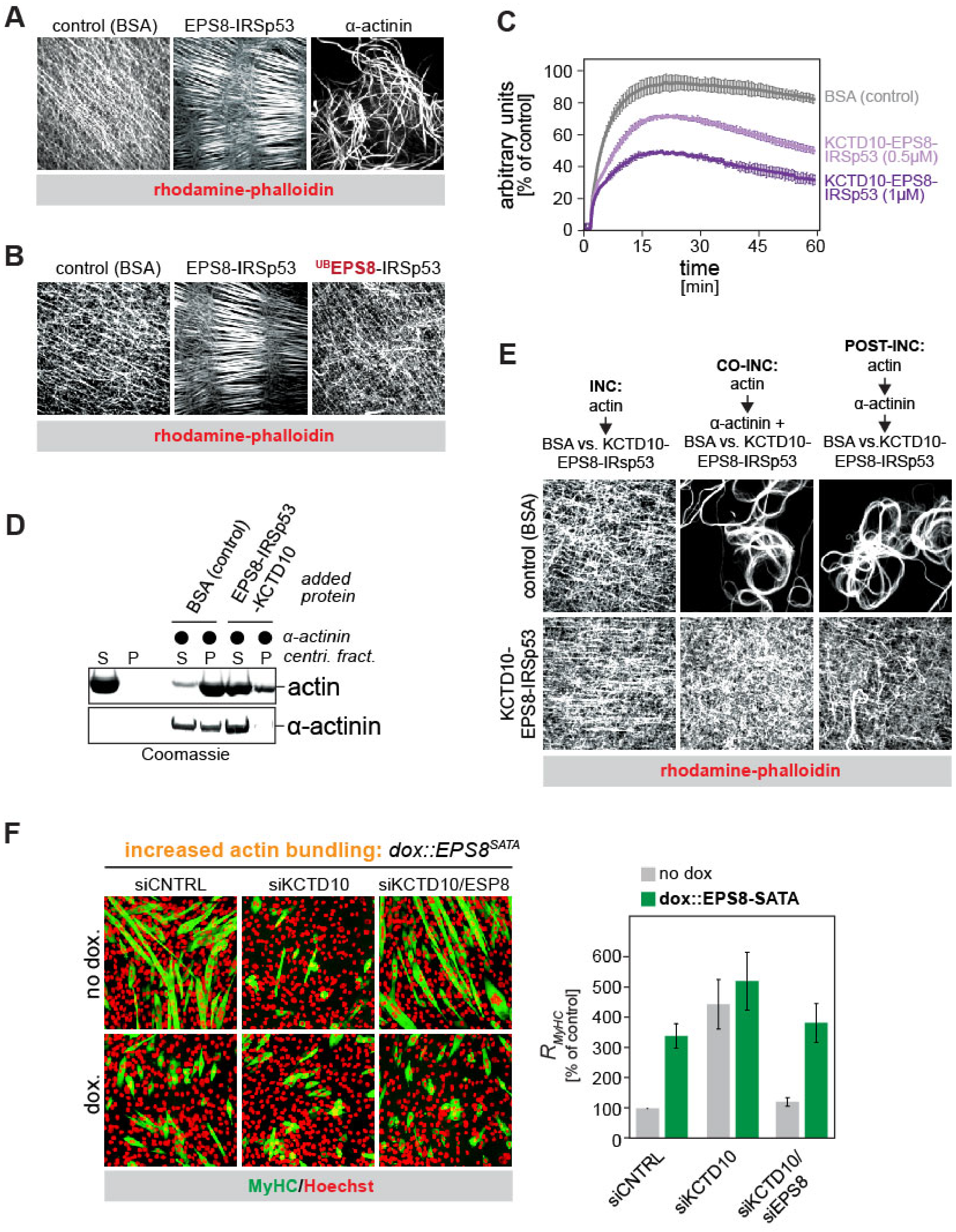

A
EPS8^eGFP
MYOB
0 min
3 min
initial contact
30 min
membrane apposition
150 min
210 min
270 min
cell fusion
360 min
B
siCNTRL
siKCTD10
EPS8
IRSp53
EPS8/IRSP53/DNA
C
EPS8
IRSp53
DMSO
carfilzomib (proteasome inhibition)
MLN4924 (CRL inhibition)
D
dox::KCTD10 (siRNA resistant)
dox::KCTD10^ΔCUL3 (siRNA resistant)
no dox
+ dox
siCNTRL
siKCTD10
EPS8
E
KCTD10/actin/DNA
KCTD10
actin
F
dox::PH-EPS8
no dox
+ dox
MyHC
PH-EPS8
G
siCNTRL
siKCTD10
EPS8
Ub-EPS8
H
dox::EPS8 or Ub-EPS8
no dox
EPS8
Ub-EPS8
siCNTRL
siKCTD10
siKCTD10/EPS8
MyHC/Hoechst
I
R_MyHC [% of control]
0
100
200
300
EPS8
Ub-EPS8
siCNTRL
siKCTD10
siKCTD10/siEPS8
siKCTD10/siEPS8
dox.
no dox.

FIGURE 4


A
control (BSA)
EPS8-IRSp53
α-actinin
rhodamine-phalloidin
B
control (BSA)
EPS8-IRSp53
UBEPS8-IRSp53
rhodamine-phalloidin
C
arbitrary units [% of control]
100
80
60
40
20
0
0
15
30
45
60
time [min]
BSA (control)
KCTD10-EPS8-IRSp53 (0.5µM)
KCTD10-EPS8-IRSp53 (1µM)
D
BSA (control)
EPS8-IRSp53 -KCTD10
added protein
α-actinin
S P S P S P
centri. fract.
actin
α-actinin
Coomassie
E
INC: actin → BSA vs. KCTD10-EPS8-IRsp53
CO-INC: actin → α-actinin + BSA vs. KCTD10-EPS8-IRSp53
POST-INC: actin → α-actinin → BSA vs.KCTD10-EPS8-IRSp53
control (BSA)
KCTD10-EPS8-IRSp53
rhodamine-phalloidin
F
increased actin bundling: dox::EPS8SATA
siCNTRL
siKCTD10
siKCTD10/ESP8
no dox.
dox.
MyHC/Hoechst
no dox
dox::EPS8-SATA
RMyHC [% of control]
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500
400
300
200
100
0
siCNTRL
siKCTD10
siKCTD10/siEPS8

FIGURE 5

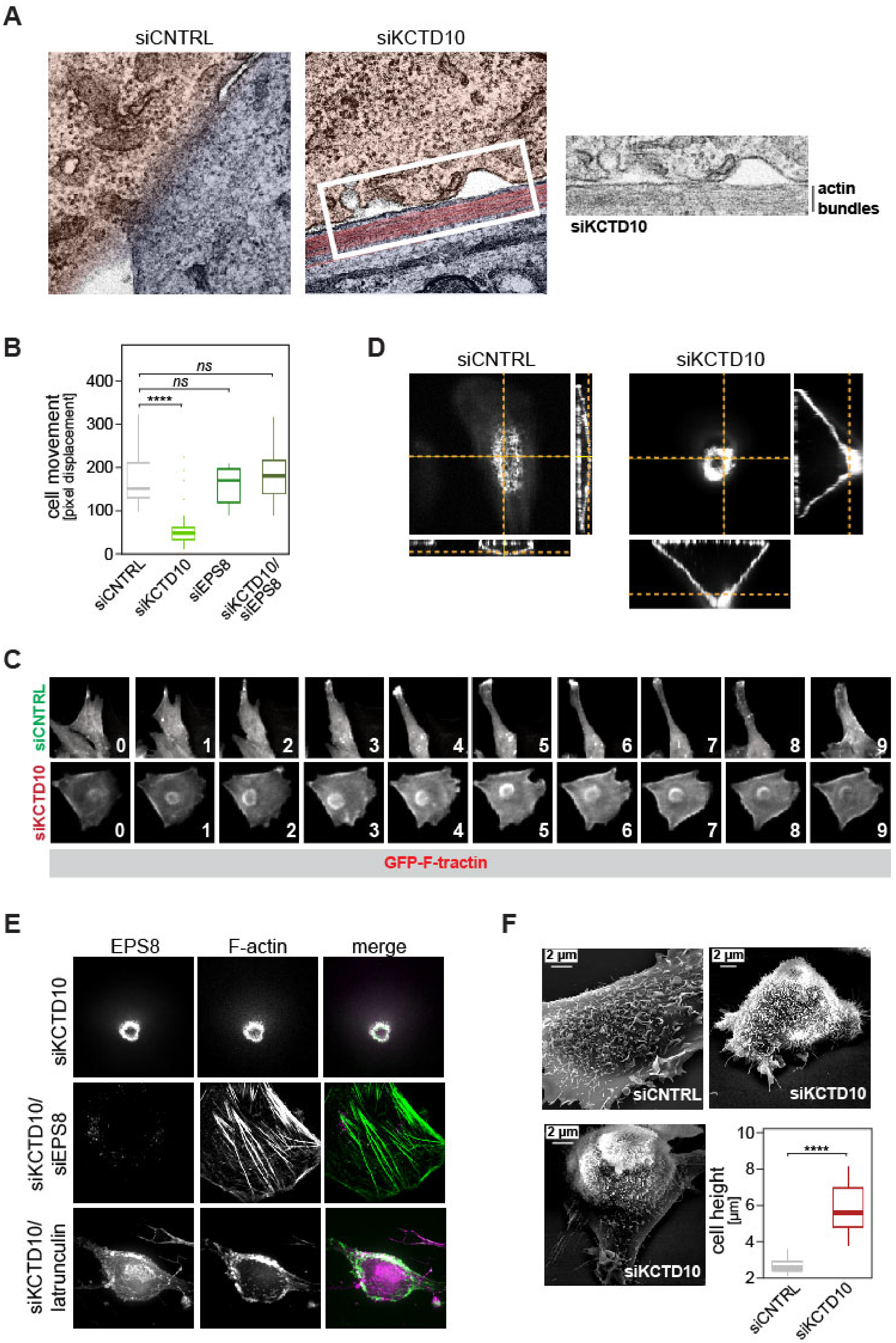
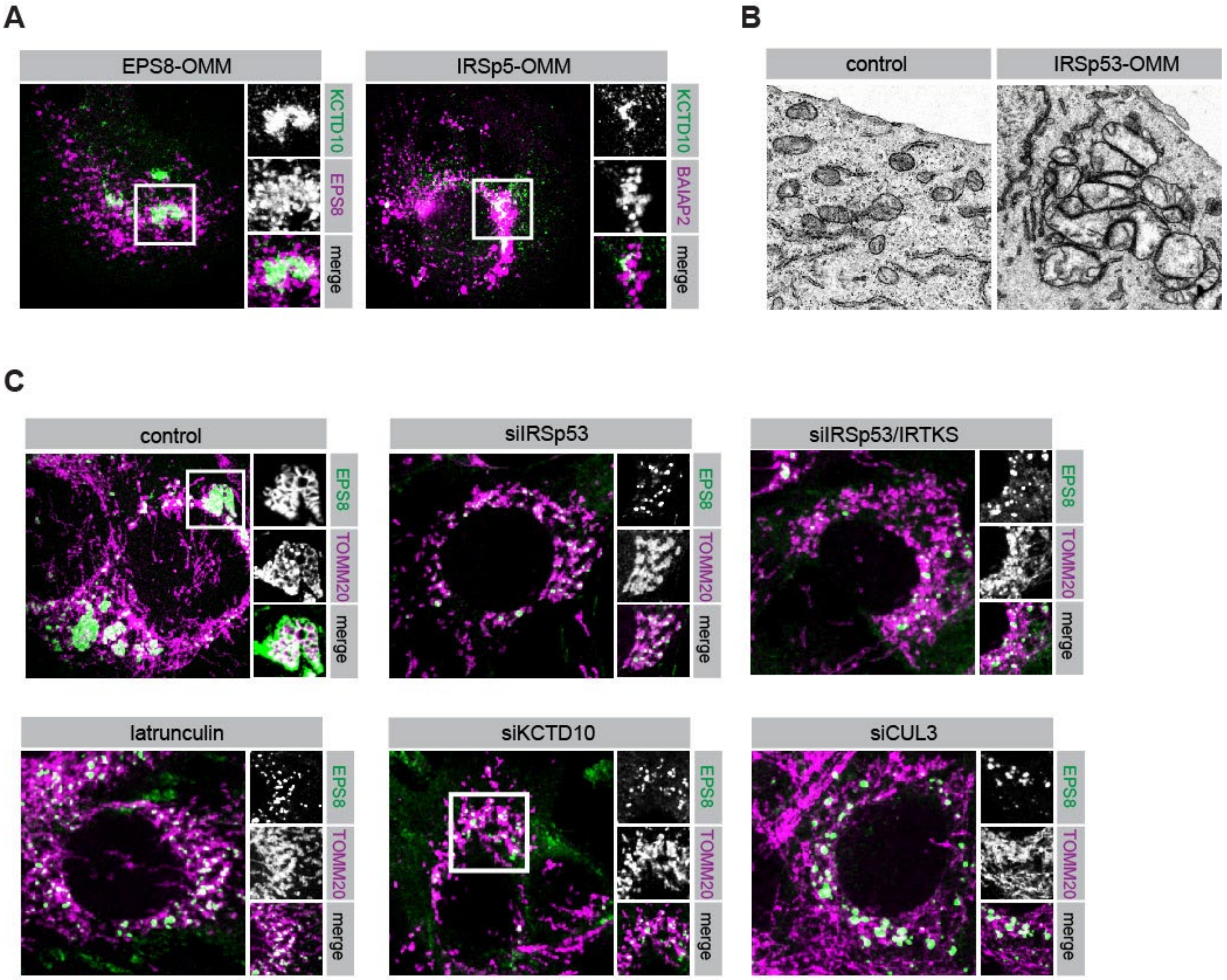

A
siCNTRL
siKCTD10
actin
bundles
siKCTD10
B
cell movement
[pixel displacement]
400
300
200
100
0
ns
ns
****
siCNTRL
siKCTD10
siEPS8
siKCTD10/
siEPS8
D
siCNTRL
siKCTD10
C
siCNTRL
siKCTD10
0
1
2
3
4
5
6
7
8
9
GFP-F-tractin
E
EPS8
F-actin
merge
siKCTD10
siKCTD10/
siEPS8
siKCTD10/
latrunculin
F
2 µm
siCNTRL
siKCTD10
cell height
[µm]
10
8
6
4
2
****
siCNTRL
siKCTD10

FIGURE 6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EPS8-OMM
KCTD10
EPS8
merge
IRSp5-OMM
KCTD10
BAIAP2
merge
B
control
IRSp53-OMM
C
control
EPS8
TOMM20
merge
siIRSp53
EPS8
TOMM20
merge
siIRSp53/IRTKS
EPS8
TOMM20
merge
latrunculin
EPS8
TOMM20
merge
siKCTD10
EPS8
TOMM20
merge
siCUL3
EPS8
TOMM20
merge

FIGURE 7

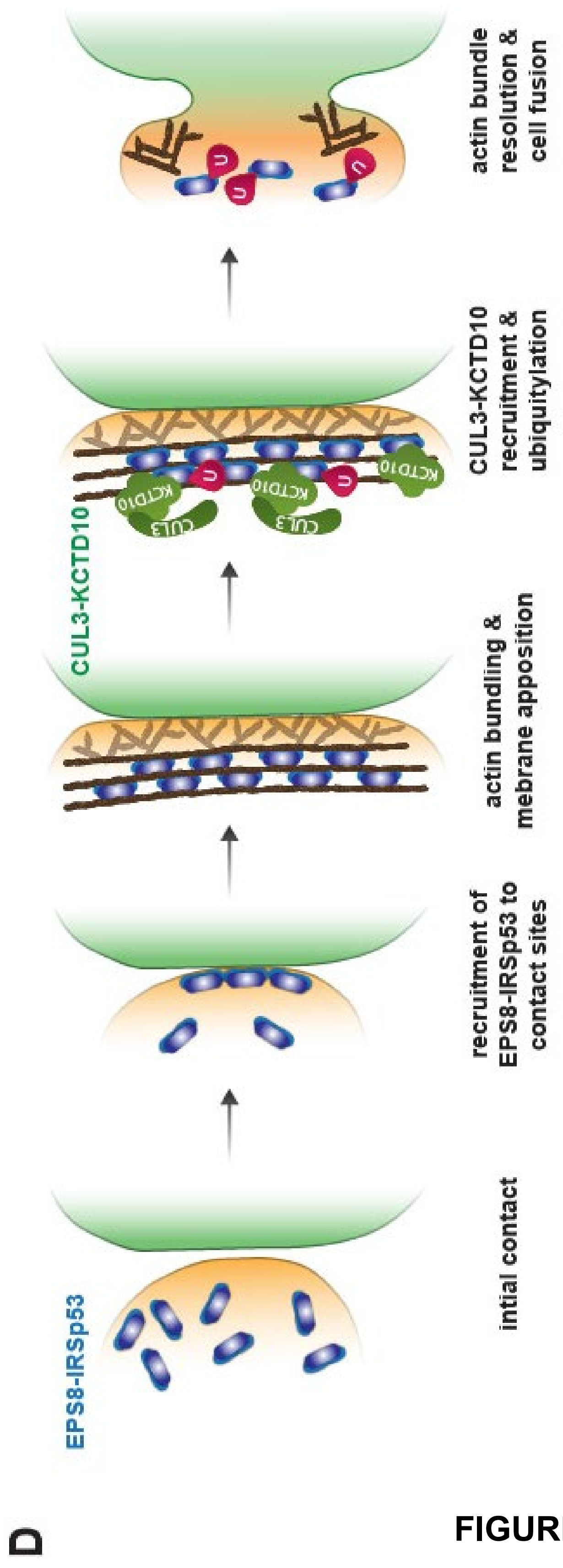

FIGURE 7 (CONTINUED)

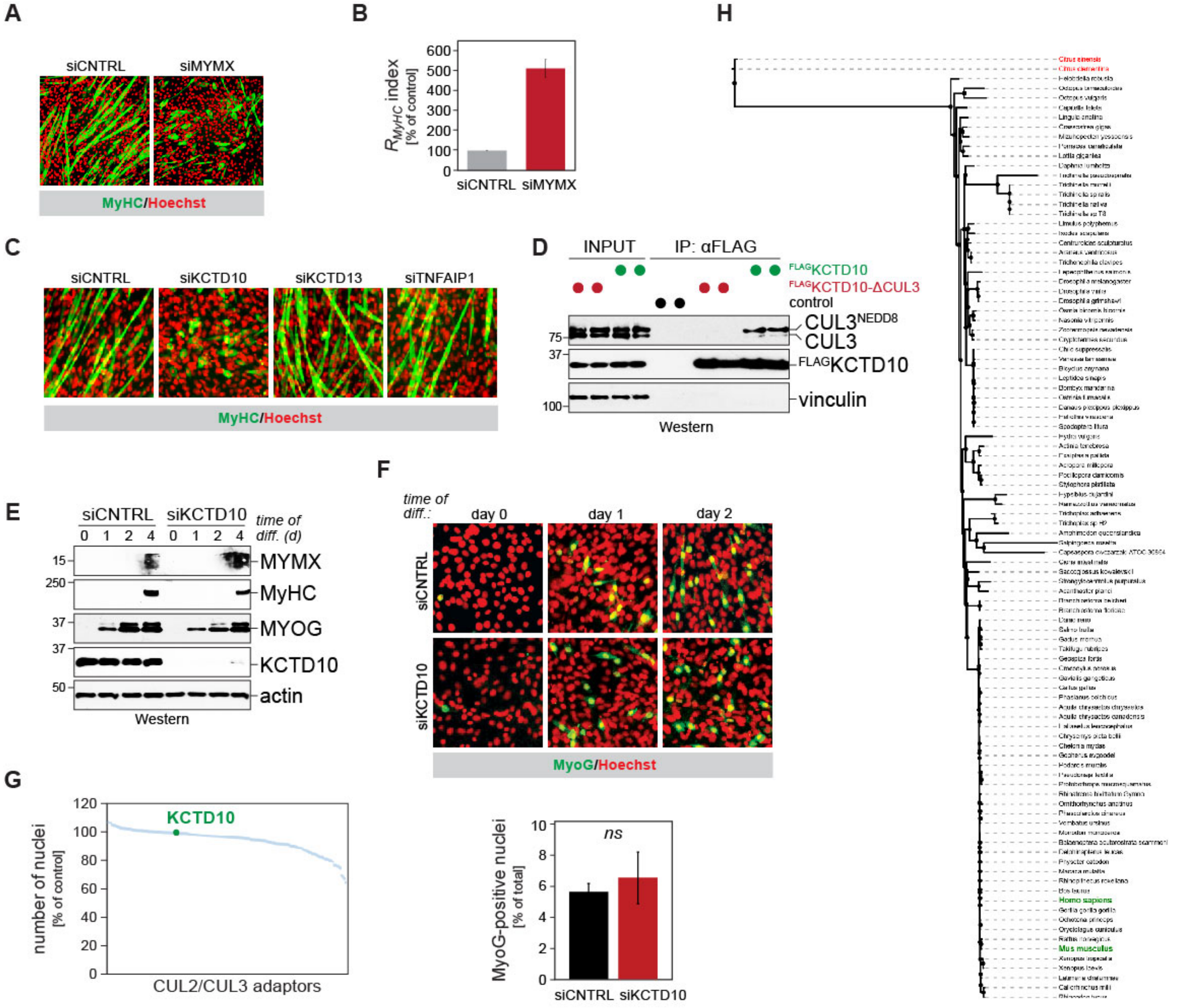
A
siCNTRL
siMYMX
MyHC/Hoechst
B
R_{MyHC} index [% of control]
600
500
400
300
200
100
0
siCNTRL
siMYMX
C
siCNTRL
siKCTD10
siKCTD13
siTNFAIP1
MyHC/Hoechst
D
INPUT
IP: αFLAG
FLAGKCTD10
FLAGKCTD10-ΔCUL3
control
75
37
100
CUL3NEDD8
CUL3
FLAGKCTD10
vinculin
Western
E
siCNTRL
siKCTD10
time of diff. (d)
0 1 2 4 0 1 2 4
15
250
37
37
50
MYMX
MyHC
MYOG
KCTD10
actin
Western
F
time of diff.:
day 0
day 1
day 2
siCNTRL
siKCTD10
MyoG/Hoechst
MyoG-positive nuclei [% of total]
ns
10
8
6
4
2
0
siCNTRL
siKCTD10
G
number of nuclei [% of control]
120
100
80
60
40
20
0
KCTD10
CUL2/CUL3 adaptors
H
Citrus sinensis
Citrus clementina
Helobdella robusta
Octopus bimaculoides
Octopus vulgaris
Capitella teleta
Lingula anatina
Crassostrea gigas
Mizuhopecten yessoensis
Pomacea canaliculata
Lottia gigantea
Daphnia lumholtzi
Trichinella pseudospiralis
Trichinella murrelli
Trichinella spiralis
Trichinella nativa
Trichinella sp T8
Limulus polyphemus
Ixodes scapularis
Centruroides sculpturatus
Araneus ventricosus
Trichonephila clavipes
Lepeophtheirus salmonis
Drosophila melanogaster
Drosophila virilis
Drosophila grimshawi
Osmia bicornis bicornis
Nasonia vitripennis
Zootermopsis nevadensis
Cryptotermes secundus
Chilo suppressalis
Vanessa tameamea
Bicyclus anynana
Leptidea sinapis
Bombyx mandarina
Ostrinia furnacalis
Danaus plexippus plexippus
Heliothis virescens
Spodoptera litura
Hydra vulgaris
Actinia tenebrosa
Exaiptasia pallida
Acropora millepora
Pocillopora damicornis
Stylophora pistillata
Hypsibius dujardini
Ramazzottius varieornatus
Trichoplax adhaerens
Trichoplax sp H2
Amphimedon queenslandica
Salpingoeca rosetta
Capsaspora owczarzaki ATCC 30864
Ciona intestinalis
Saccoglossus kowalevskii
Strongylocentrotus purpuratus
Acanthaster planci
Branchiostoma belcheri
Branchiostoma floridae
Danio rerio
Salmo trutta
Gadus morhua
Takifugu rubripes
Geospiza fortis
Crocodylus porosus
Gavialis gangeticus
Gallus gallus
Phasianus colchicus
Aquila chrysaetos chrysaetos
Aquila chrysaetos canadensis
Haliaeetus leucocephalus
Chrysemys picta bellii
Chelonia mydas
Gopherus evgoodei
Podarcis muralis
Pseudonaja textilis
Protobothrops mucrosquamatus
Rhinatrema bivittatum Gymno
Ornithorhynchus anatinus
Phascolarctos cinereus
Vombatus ursinus
Monodon monoceros
Balaenoptera acutorostrata scammoni
Delphinapterus leucas
Physeter catodon
Macaca mulatta
Rhinopithecus roxellana
Bos taurus
Homo sapiens
Gorilla gorilla gorilla
Ochotona princeps
Oryctolagus cuniculus
Rattus norvegicus
Mus musculus
Xenopus tropicalis
Xenopus laevis
Latimeria chalumnae
Callorhinchus milii

FIGURE S1

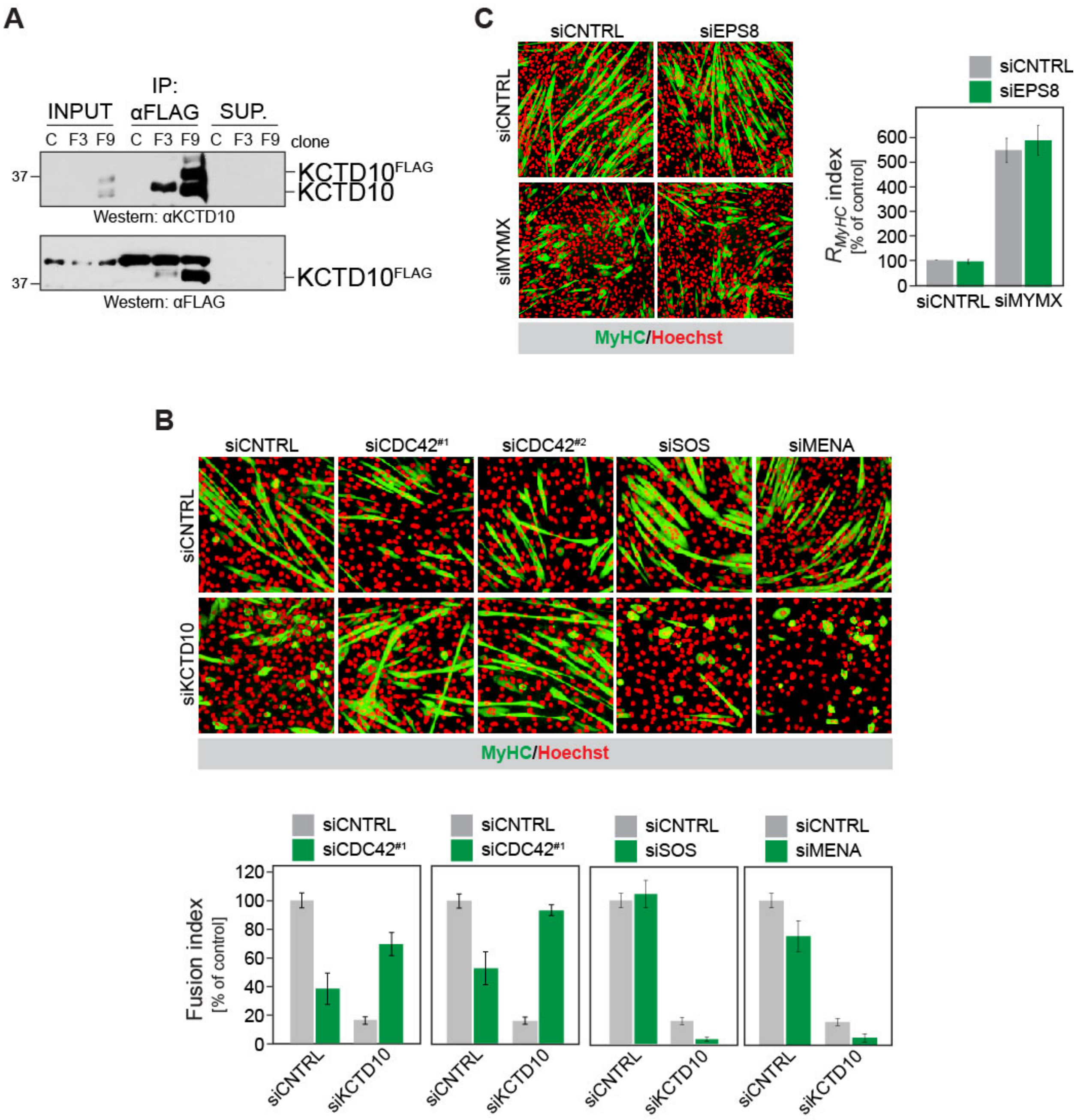
A
IP:
INPUT
αFLAG
SUP.
C F3 F9 C F3 F9 C F3 F9 clone
37
KCTD10FLAG
KCTD10
Western: αKCTD10
37
KCTD10FLAG
Western: αFLAG
C
siCNTRL
siEPS8
siCNTRL
siMYMX
MyHC/Hoechst
siCNTRL
siEPS8
RMyHC index
[% of control]
600
500
400
300
200
100
0
siCNTRL
siMYMX
B
siCNTRL
siCDC42#1
siCDC42#2
siSOS
siMENA
siCNTRL
siKCTD10
MyHC/Hoechst
siCNTRL
siCDC42#1
siCNTRL
siCDC42#1
siCNTRL
siSOS
siCNTRL
siMENA
Fusion index
[% of control]
120
100
80
60
40
20
0
siCNTRL
siKCTD10
siCNTRL
siKCTD10
siCNTRL
siKCTD10
siCNTRL
siKCTD10

FIGURE S2

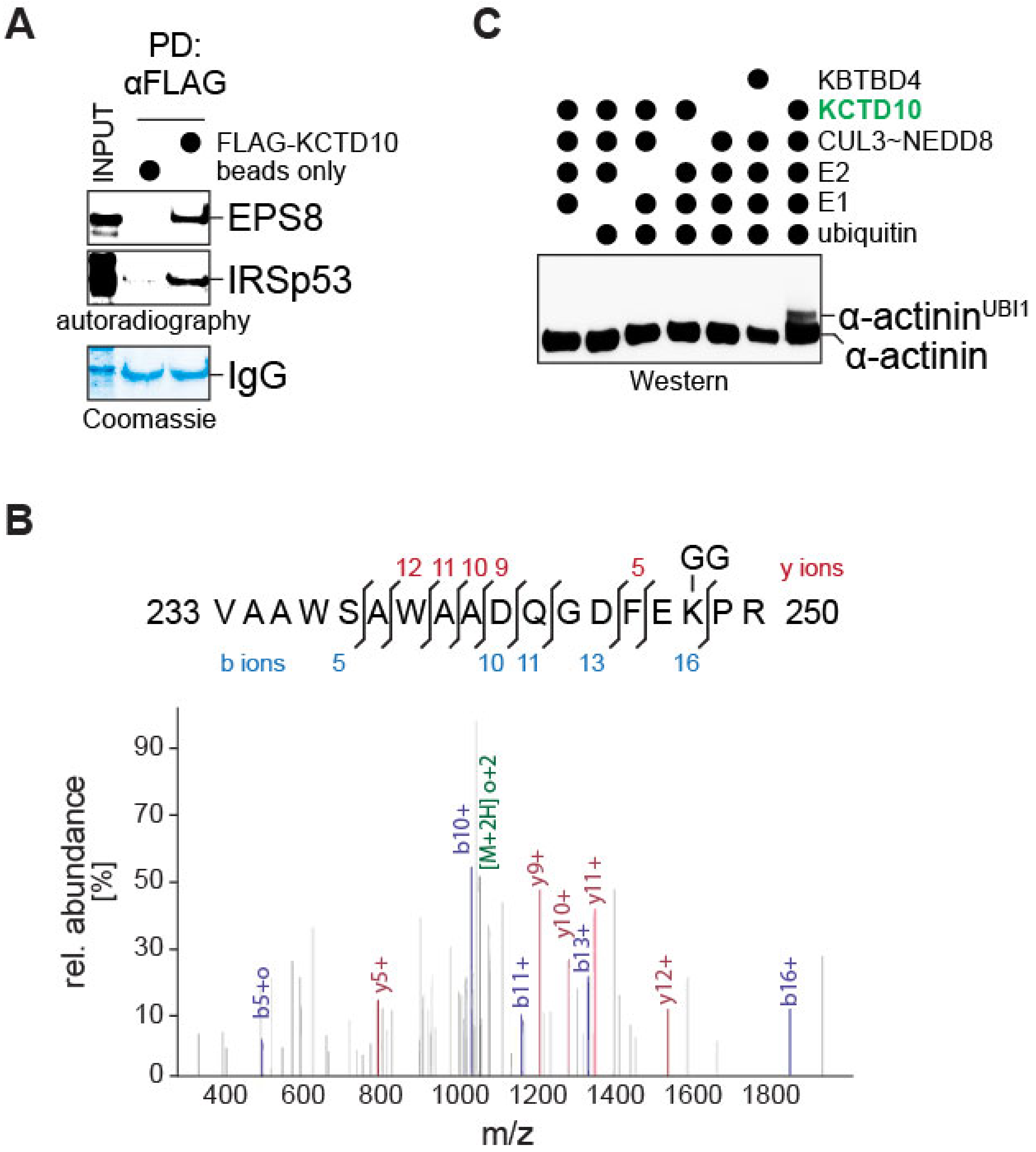
A
PD:
αFLAG
INPUT
● FLAG-KCTD10
● beads only
EPS8
IRSp53
autoradiography
IgG
Coomassie
C
KBTBD4
KCTD10
CUL3~NEDD8
E2
E1
ubiquitin
α-actinin^UBI1
α-actinin
Western
B
12 11 10 9
5
GG
y ions
233 V A A W S A W A A D Q G D F E K P R 250
b ions
5
10 11
13
16
rel. abundance [%]
90
70
50
30
10
0
b5+o
y5+
b10+
[M+2H] o+2
b11+
y9+
y10+
b13+
y11+
y12+
b16+
400
600
800
1000
1200
1400
1600
1800
m/z

FIGURE S3

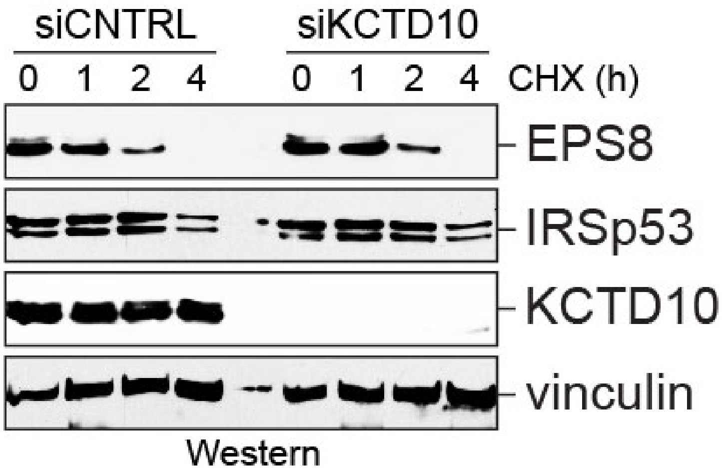
siCNTRL
siKCTD10
0
1
2
4
0
1
2
4
CHX (h)
EPS8
IRSp53
KCTD10
vinculin
Western

FIGURE S4

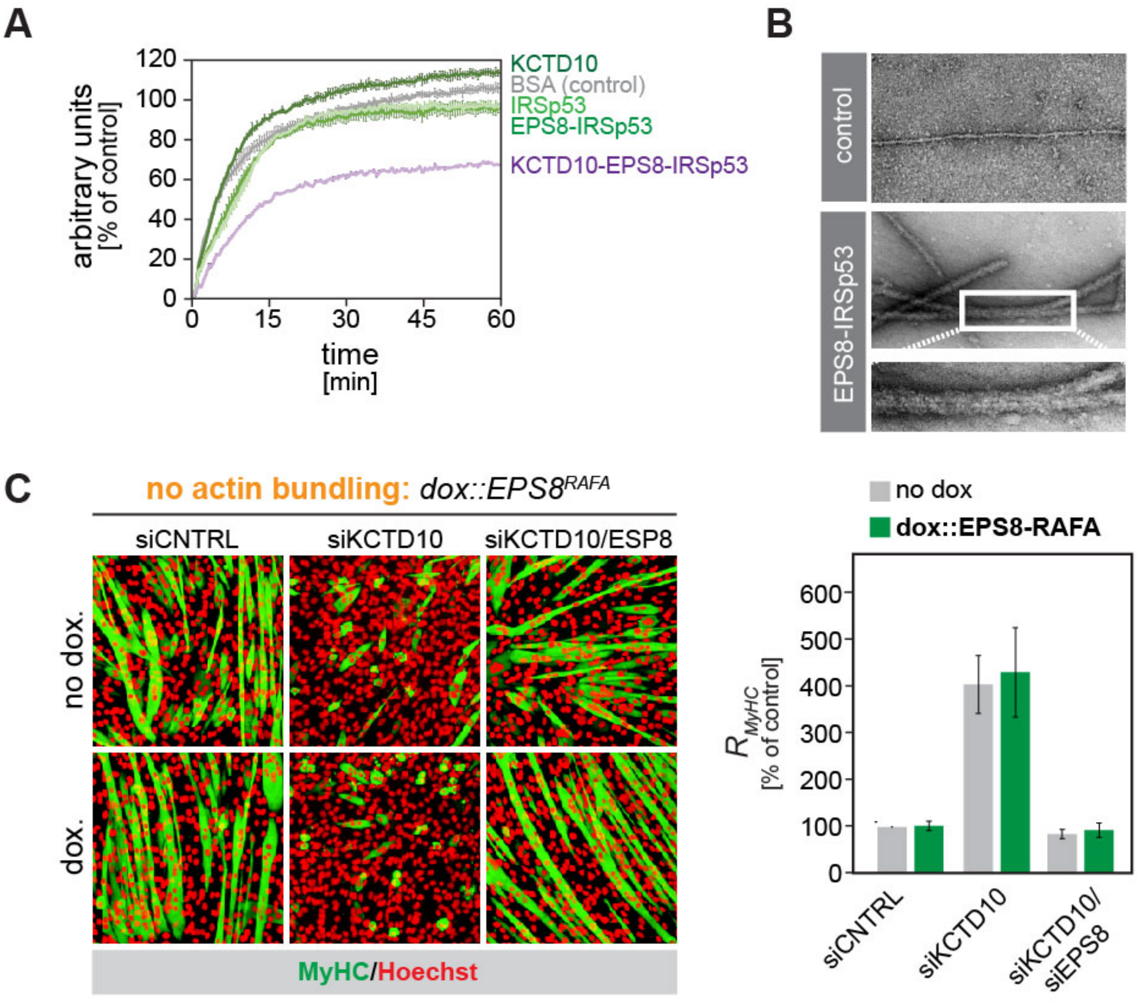
A
120
100
80
60
40
20
0
arbitrary units
[% of control]
0
15
30
45
60
time
[min]
KCTD10
BSA (control)
IRSp53
EPS8-IRSp53
KCTD10-EPS8-IRSp53
B
control
EPS8-IRSp53
C
no actin bundling: dox::EPS8^RAFA
siCNTRL
siKCTD10
siKCTD10/ESP8
no dox.
dox.
MyHC/Hoechst
no dox
dox::EPS8-RAFA
600
500
400
300
200
100
0
R_MyHC
[% of control]
siCNTRL
siKCTD10
siKCTD10/
siEPS8

FIGURE S5

CHAPTER 3:

A Cellular Mechanism to Detect and Alleviate Reductive Stress

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3.1 Summary

Metazoan organisms rely on conserved stress response pathways to detect and alleviate adverse conditions and preserve their cellular integrity. Stress responses are particularly important in stem cells that provide lifetime support for tissue formation and repair, yet how these protective systems are integrated into developmental programs is poorly understood. Here, we have used myoblast differentiation to identify the E3 ligase $CUL2^{FEM1B}$ and its substrate FNIP1 as core components of the reductive stress response. Reductive stress, as caused by prolonged antioxidant signaling or mitochondrial inactivity, reverts the oxidation of invariant Cys residues in FNIP1 and thereby allows $CUL2^{FEM1B}$ to recognize its target. The ensuing proteasomal degradation of FNIP1 restores mitochondrial activity to preserve both redox homeostasis and stem cell integrity. The reductive stress response is therefore built around a ubiquitin-dependent rheostat that tunes mitochondrial activity to cellular redox needs and implicates metabolic control in the coordination of stress and developmental signaling.

3.2 Introduction

Metazoan development relies on carefully balanced transcriptional networks to generate the more than 200 cell types of an adult organism. Stem cells, which can either self-renew to generate more progenitors or differentiate into specialized cell types, are at the apex of this intricate program, and their defective homeostasis gives rise to many pediatric diseases (Avior et al., 2016; Nusse and Clevers, 2017). As stem cells support tissue regeneration and repair throughout the lifetime of an organism, aberrant stem cell maintenance has also been linked to tumorigenesis and tissue degeneration (Almada and Wagers, 2016).

To protect their stem cell populations from damage or exhaustion, all organisms possess highly conserved and sensitive stress response pathways that detect and alleviate a wide range of adverse conditions. Providing an important example, stem cells often reside in hypoxic niches and rely on glycolysis as their main source of energy, which limits oxidative damage to DNA, lipids, or proteins (Donato et al., 2017; Ezashi et al., 2005; Studer et al., 2000). If still too many reactive oxygen species accumulate, these cells activate the oxidative stress response to scavenge oxidizing molecules and revert oxidized proteins into their functional reduced state (Suzuki and Yamamoto, 2017). A failure to initiate the oxidative stress response can impair stem cell self-renewal and differentiation and thereby endangers tissue formation and maintenance (Tsai et al., 2013; Yamamoto et al., 2018). In a similar manner, stem cell integrity is being preserved by stress responses that are activated by protein misfolding, DNA damage, or lack of oxygen (Balchin et al., 2016; Ohh et al., 2000; Vilchez et al., 2014).

While most stresses elicit a rapid response, the underlying signaling networks also need to be turned off soon after stem cell homeostasis has been restored. In keeping with the above example, differentiating cells leave the hypoxic niche and switch to oxidative phosphorylation as their major source of ATP (Khacho et al., 2016). This metabolic shift generates energy and building blocks needed for a change in cell fate, but it also leads to a rise in reactive oxygen species that amplify critical signaling circuits during differentiation (Holmstrom and Finkel, 2014; Rodriguez-Colman et al., 2017; Sena and Chandel, 2012). Stem cells that fail to shut off the oxidative stress response prematurely deplete oxidizing signaling molecules and cannot differentiate (Bellezza et al., 2018; Gores et al., 1989; Xiao and Loscalzo, 2019). This persistent lack of reactive oxygen species, referred to as reductive stress, can also elicit the unfolded protein response by impairing disulfide bond formation in the ER (Maity et al., 2016; Trotter and Grant, 2002; Yang et al., 2007), and it ultimately results in cardiomyopathy or diabetes (Dialynas et al., 2015; Rajasekaran et al., 2007; Rajasekaran et al., 2011; Wu et al., 2016). How reductive stress is sensed and alleviated is still unknown, leaving us with an incomplete understanding of how physiological levels of reactive oxygen species are established.

Most stress responses rely on ubiquitylation, an essential posttranslational modification whose specificity is imparted by hundreds of E3 ligases (Balchin et al., 2016; Buckley et al., 2012; Rape, 2018; Yau and Rape, 2016). As a core component of the oxidative stress response, the E3 ligase $\text{CUL3}^{\text{KEAP1}}$ ubiquitylates and helps degrade the transcription factor NRF2 (Wakabayashi et al., 2003). When cells experience a dangerous rise in reactive oxygen species, $\text{CUL3}^{\text{KEAP1}}$ is inhibited and NRF2 can accumulate to drive antioxidant gene expression (Furukawa and Xiong, 2005; Zhang et al., 2004). In a similar manner, the E3 CUL2^{VHL} restricts the abundance of HIF-1α, until hypoxic stress stabilizes this transcription factor to initiate angiogenesis (Denko, 2008; Kaelin, 2007). Underscoring the importance of such redox stress responses for development, deletion of *VHL* or *KEAP1* elicits embryonic or early postnatal death, respectively (Gnarra et al., 1997; Wakabayashi et al., 2003), and their mutation is a very frequent cause of cancer (Cancer Genome Atlas Research, 2012; Kaelin, 2007). Whether the reductive stress response is also controlled by ubiquitylation, and whether this is important for development, has not been investigated.

Here, we have used myoblast differentiation, a redox-sensitive pathway that is essential for muscle development, to identify the E3 CUL2^{FEM1B} and its target FNIP1 as core components of the reductive stress response. Persistent lack of reactive oxygen species reverts the oxidation of invariant Cys residues in FNIP1 and thereby allows CUL2^{FEM1B} to detect its essential substrate. The ensuing ubiquitylation and proteasomal degradation of FNIP1 restores mitochondrial output to produce reactive oxygen species and thus preserve redox homeostasis, and it also prevents untimely stem cell differentiation. The reductive stress response is therefore built on a ubiquitin-dependent rheostat that tunes mitochondrial activity to cellular needs, which implicates metabolic control in integrating the pathways of redox stress signaling into the complex programs of metazoan development.

3.3 Results

3.3.1 Reductive stress inhibits myoblast differentiation

The development of skeletal muscle is highly sensitive to oxidative stress and thus represents a unique opportunity to isolate proteins that allow stem cells to respond to altered redox conditions (Almada and Wagers, 2016; Braun and Gautel, 2011). As Cullin-Ring-E3 ligases (CRLs) control both redox stress signaling and differentiation (Bellezza et al., 2018; Donato et al., 2017; Lignitto et al., 2019; Ohh et al., 2000; Wang et al., 1999; Werner et al., 2015), we focused on these cellular regulators and searched for CRL subunits required for robust myogenesis. We first depleted each of the seven main Cullins from C2C12 myoblasts and followed myotube formation by fluorescence microscopy against myosin heavy chain (MyHC), which is only expressed at later stages of cell fate specification (Bader et al., 1982). These experiments showed that CUL2 and CUL3 were particularly important for myoblast differentiation (Figure S1A), which is consistent with the effects of *CUL3* deletion onto muscle development in mice (Blondelle et al., 2017; Papizan et al., 2018). Depletion of CUL1, CUL4a/b, or CUL5 showed less dramatic effects, and hence, enzymes of these CRL families were not further considered in this study.

CRLs determine their substrate specificity through ~300 interchangeable adaptors (Silverman, et al, 2012; Skaar, et al, 2013). To comprehensively identify myogenic CUL2 or CUL3 adaptors, we purified CUL2 and CUL3 from myoblasts and myotubes and determined their binding partners by mass spectrometry. We detected 19 CUL2- and 32 CUL3-adaptors, including novel candidate adaptors, such as the muscular dystrophy protein myoferlin, or factors linked to familial myopathy, such as KLHL9 (Table S1). We combined this list with published adaptors (Bennett et al., 2010; Mena et al., 2018) (Table S2), before we depleted each CUL2 and CUL3 subunit from myoblasts, induced differentiation, and recorded MyHC-positive myotubes by microscopy and automated image analysis (Figure 1A). Critical phenotypes were confirmed with independent siRNAs to eliminate the risk of off-target effects.

Our screen revealed that the CUL3 adaptors KEAP1, BTBD9, KLHL22, and ANKFY1 were required for myotube formation, while depletion of the CUL2 adaptor fem-1 homolog B (FEM1B) was particularly effective in improving this differentiation program (Figure 1A-C). As loss of these adaptors minimally affected cell numbers (Figure S1B), aberrant cell division or survival unlikely accounted for the changes in cell fate specification. Notably, all adaptors that we found to be required for myotube formation had previously been linked to disease: mutations in KEAP1 lead to lung and renal cancer (Kovac et al., 2015; Singh et al., 2006); BTBD9 mutations trigger restless leg syndrome and insomnia (Winkelmann et al., 2007); overexpression of KLHL22 causes breast cancer progression (Chen et al., 2018); and mutations in ANKFY1 result in steroid-resistant nephrotic syndrome (Hermle et al., 2018).

We were particularly intrigued to see that depletion of the oxidative stress sensor KEAP1 prevented myoblast differentiation. While oxidative stress inhibits $\mathrm{CUL3}^{\mathrm{KEAP1}}$ transiently (Zhang et al., 2004), genetic loss of KEAP1 stabilizes NRF2 for prolonged periods of time and thereby elicits reductive stress (Rajasekaran et al., 2011). Indeed, depletion of KEAP1 in myoblasts induced accumulation of NRF2 (Figure 1C, D) and abundant expression of its target genes (Figure 2E, F). This caused an increase in the levels of NADPH (Figure S1C), which is required to recycle oxidized glutathione (Zhao et al., 2018), and a steep drop in reactive oxygen species, the hallmark of reductive stress (Figure 1E). When we blunted this antioxidant signaling by co-depleting NRF2 or enzymes involved in glutathione synthesis or recycling, differentiation was restored (Figure 1D, F; Figure S1D). Scavengers of reactive oxygen species, which impose reductive stress by chemical means (Banba et al., 2019; Brand et al., 2016; Orr et al., 2015), also inhibited myoblast differentiation (Figure 1G). We conclude that reductive stress impairs myogenesis in vitro. While this lent further support to the notion that reactive oxygen species fulfill critical signaling roles during differentiation (Holmstrom and Finkel, 2014; Sena and Chandel, 2012), it reinforced the question of how reductive stress is sensed and counteracted during normal development.

3.3.2 FEM1B counteracts KEAP1

We hypothesized that it was the reductive stress response, rather than a mere absence of reactive oxygen species, that prevented myogenesis in the absence of KEAP1. If this were to be the case, depletion of components of the reductive stress response should restore differentiation of KEAP1- deficient myoblasts. We thus designed a genetic modifier screen focused on E3 ligases as likely stress regulators and found that loss of the CUL2 adaptor FEM1B enabled myotube formation despite lack of KEAP1 (Figure 2A). We confirmed the screen results with independent siRNAs in microscopy and Western blot analyses of myoblast differentiation (Figure 2B, C). Loss of FEM1B also rescued myoblast differentiation in the presence of scavengers of mitochondria-derived reactive oxygen species, revealing that lack of FEM1B can counteract reductive stress signaling induced by either genetic or chemical means (Figure 2D). Intriguingly, FEM1B had already emerged from our initial screen as the hit whose depletion showed the strongest increase in the efficiency of myotube formation (Figure 1A), the opposite phenotype of loss of KEAP1.

As expected from its myogenic phenotypes, depletion of FEM1B restored levels of reactive oxygen species in KEAP1-deficient myoblasts (Figure 1E). The antagonistic relationship between FEM1B and KEAP1 was also apparent in gene expression analyses by RNAseq or qRT-PCR. As mentioned above, KEAP1 depletion induced antioxidant NRF2 targets, such as enzymes of glutathione synthesis, scavengers of reactive oxygen species, or components of the pentose phosphate pathway that produces NADPH (Figure 2E, F; Figure S2A). Although stabilization of NRF2 increases glycolytic enzymes in lung cancer cells (Lignitto et al., 2019; Wiel et al., 2019), this was not observed in myoblasts (Figure S2B). Depletion of FEM1B had the opposite effect and reduced mRNA levels of NRF2 targets, including GCLM, NQO, and PGD, while it increased transcripts of the myogenic markers MYOG and MYL1 (Figure 2E, F). Importantly, concomitant depletion of KEAP1 and FEM1B cancelled out each of these phenotypes of single E3 ligase depletion (Figure 2E, F; Figure S2A).

While KEAP1 is best known for controlling the abundance of NRF2, it also sequesters the transcription factor in the cytoplasm (Itoh et al., 2003). We found that depletion of FEM1B reduced mRNA levels of NRF2 (Figure S2C), yet this had only a minor impact on the abundance of NRF2 protein (Figure 2C). By contrast, loss of FEM1B had striking effects onto NRF2 localization: while NRF2 accumulated in the nucleus of KEAP1-deficient myoblasts, co-depletion of FEM1B directed

NRF2 to perinuclear regions where it is unable to induce gene expression (Figure 2G). Together, these results revealed that FEM1B prevents myogenesis under conditions of reductive stress, at least in part through effects on nuclear import of NRF2. This finding raised the possibility that the E3 CUL2^{FEM1B} might impact, either directly or indirectly, the reductive stress response.

3.3.3 CUL2^{FEM1B} targets FNIP1 for proteasomal degradation

To understand how CUL2^{FEM1B} controls the cellular redox state, we wished to isolate its essential ubiquitylation targets and therefore generated a FEM1B variant that can bind, but not ubiquitylate, its substrates. As seen with other CRLs (Mena et al., 2018), this feature was expected to prolong the association of FEM1B with short-lived targets and facilitate their identification by CompPASS mass spectrometry (Huttlin et al., 2017). Previous work had denoted a conserved motif in the VHL box of CUL2 adaptors, whose central Leu residue engages a hydrophobic pocket on Elongin C and connects to the catalytic module also composed of CUL2, Elongin B, and RBX1 (Bullock et al., 2006; Mahrour et al., 2008; Stebbins et al., 1999). Mutation of the respective FEM1B residue, Leu597, produced an adaptor that failed to bind CUL2 and cannot support ubiquitylation (Figure S2D), yet contained intact ankyrin repeats and therefore should retain its ability to bind substrates. Proteomic analyses confirmed that FEM1B^{L597A} was impaired in binding to CUL2, Elongin B and Elongin C (Figure 3A). By contrast, FEM1B^{L597A} interacted more strongly than wildtype FEM1B with several proteins that were considered to be candidate substrates. These included the GATOR1 complex, which inhibits mTORC1 signaling during amino acid limitation (Bar-Peled et al., 2013; Dutchak et al., 2018), as well as the folliculin (FLCN) and FNIP1 proteins, which also bind each other (Baba et al., 2006). A close FNIP1 homolog, FNIP2, was not detected in these experiments. We confirmed by immunoprecipitation and Western blotting that FEM1B associated with GATOR1, FLCN, and FNIP1 in a manner that was stabilized by mutation of the VHL box in FEM1B (Figure 3B; Figure S2E). By contrast, mutation of Cys186, an invariant residue in the substrate-binding ankyrin repeats, strongly diminished the recognition of GATOR1, FLCN, and FNIP1 by FEM1B (Figure 3B).

Among its candidate targets, we noted that FEM1B overexpression elicited the CUL2- and proteasome-dependent degradation of FNIP1, while the substrate binding-deficient FEM1BC186S, catalytically inert FEM1B^{L597A}, or the related adaptor FEM1A did not have this effect (Figure 3C; Figure S2E, F). Depletion of FEM1B caused the opposite outcome and increased the levels of endogenous FNIP1 (Figure 3D). NEDD8-modified CUL2^{FEM1B} also efficiently polyubiquitylated FNIP1 in vitro, when incubated with the E2 enzymes UBE2D3 and UBE2R1 (Figure 3E). By contrast, expression of FEM1B did not induce degradation of FLCN, GATOR1 subunits, or FNIP2 (Figure 3B; Figure S2E-G), and CUL2^{FEM1B} did not ubiquitylate these proteins in vitro (Figure 3E; Figure S2H). We conclude that FNIP1 is a proteolytic CUL2^{FEM1B} substrate, while FLCN or GATOR1 might interact with FEM1B indirectly or in a role that is distinct from being a degradation target.

Genetic experiments revealed the importance of FNIP1 stability for myotube formation and reductive stress signaling. While loss of FNIP1 by itself had little impact onto differentiation (Figure S2I), co-depletion of FNIP1 reverted the effects of FEM1B loss onto myotube formation (Figure 3F; Figure S2I); thus, it was accumulation of FNIP1 that promoted differentiation in the absence of FEM1B. Co-depletion of FNIP1 also prevented myotube formation in cells lacking both FEM1B and KEAP1, which documents that stabilization of FNIP1 allowed myoblasts to bypass reductive stress

(Figure 3G). In contrast to FNIP1, depletion of GATOR1 subunits, which bind but are not degraded through CUL2^{FEM1B}, did not impact differentiation of FEM1B-deficient myoblasts (Figure S2J).

Together, these findings identified FNIP1 as a critical target of CUL2^{FEM1B} during reductive stress. Previous work ascribed several functions to FNIP1, including regulation of mitochondrial biogenesis (Hasumi et al., 2012; Reyes et al., 2015); a role in removal of damaged mitochondria by autophagy (Heo et al., 2018); and binding or regulation of the metabolic kinases AMPK and mTORC1 (Baba et al., 2006; Bhargava and Schnellmann, 2017; Hasumi et al., 2008; Tsun et al., 2013). While somatic variants of FNIP1 have been detected in renal cancer (Cancer Genome Atlas Research, 2013), mutations in its constitutive binding partner FLCN cause Birt-Hogg-Dubé syndrome (Nickerson et al., 2002), a predisposition to renal cancer that also results from aberrant mitochondrial activity or mutations in VHL, KEAP1 or NFE2L2 (Kaelin, 2007; Kovac et al., 2015; Xu et al., 2019). The identification of FNIP1 as a key CUL2^{FEM1B} substrate implicated metabolic regulation in reductive stress signaling.

3.3.4 FEM1B detects a conserved Cys degron in FNIP1

Given that stress responses are only activated following specific insults, we asked whether loss of reactive oxygen species impacted recognition of FNIP1 by CUL2^{FEM1B}. This required us to first identify the sequence motif, or degron, in FNIP1 that is bound by FEM1B. Through systematic deletion analyses, we denoted a short stretch in the central region of FNIP1 that was required for its recognition by CUL2^{FEM1B} and its proteasomal degradation (Figure 4A, B). To test whether this motif comprised a transferable degron, we appended it to GFP and monitored binding of the GFPdegron fusion to CUL2^{FEM1B}. GFPdegron, but not GFP, was readily detected by CUL2^{FEM1B} (Figure S3A, B). As with full-length FNIP1, GFPdegron showed stronger binding to the substrate trap FEM1B^{L597A} (Figure S3A), while it did not interact with the ankyrin repeat mutant FEM1BC186S (Figure S3B). We next expressed GFPdegron along with mCherry and used the GFP/mCherry ratio as a quantitative read-out for protein degradation. FEM1B, but not FEM1B^{L597A} or FEM1BC186S, triggered a dramatic loss of GFPdegron (Figure 4C), while deletion of FEM1B by CRISPR/Cas9, depletion of FEM1B by shRNAs, or proteasome inhibition all protected GFPdegron from degradation

(Figure 4D; Figure S3C, D). The FNIP1 degron was also sufficient to mediate a robust interaction with recombinant FEM1B as well as ubiquitylation by CUL2^{FEM1B} (Figure 4E, F). We conclude that a central degron is required and sufficient for FNIP1 recognition by CUL2^{FEM1B} and its proteasomal degradation. Whereas this degron is conserved among FNIP1 homologs (Figure S3E), it is not found in the closely related FNIP2 that is not recognized by CUL2^{FEM1B} (Figure S3F).

Given the role of CUL2^{FEM1B} in reductive stress signaling, we were excited to see that the FNIP1 degron contained three invariant and potentially redox-sensitive Cys residues (Figure S3E). Importantly, Cys585 was essential for the FEM1B-dependent degradation of the GFPdegron reporter (Figure 5A). Simultaneous mutation of Cys580 and Cys582 also strongly impaired the clearance of GFPdegron through CUL2^{FEM1B}, and mutation of all Cys residues fully protected the reporter against CUL2^{FEM1B}-dependent degradation (Figure 5A). Changing its Cys residues to Ser blocked the binding of the degron peptide to recombinant FEM1B (Figure 5B) and interfered with its ubiquitylation by CUL2^{FEM1B} (Figure 5C). The reliance on Cys residues was even more pronounced in full-length FNIP1, where each of the three Cys as well as a neighboring His residue were required for FEM1B-binding and proteasomal degradation (Figure 5D; Figure S4A).

In line with this mutational analysis, treatment of the FNIP1 degron with the Cys-modifying agents iodoacetamide or N-ethylmaleimide strongly inhibited its recognition by FEM1B (Figure 5E; Figure S4B, C). NEM or iodoacetamide also blocked the CUL2^{FEM1B}-dependent ubiquitylation of the degron peptide to the same extent as mutation of the Cys residues (Figure 5F). Similar observations were made in cells, where the iodoacetamide derivative IAyne impaired the FEM1B- dependent degradation of GFPdegron (Figure 5G). We conclude that the reductive stress E3 ligase CUL2^{FEM1B} relies on an unmodified Cys degron to detect its essential substrate FNIP1.

3.3.5 Reductive stress triggers detection of FNIP1 by CUL2^{FEM1B}

Our results raised the possibility that reduction of its degron Cys residues might induce recognition of FNIP1 by CUL2^{FEM1B}. Thus, we wished to determine whether the FNIP1 degron was oxidized under normal conditions, yet reduced when cells experienced reductive stress. Unfortunately, despite treating FNIP1 immunoprecipitates with multiple proteases or even using synthesized peptides, we could not detect the entire degron by proteomic means. This peptide was also absent from global analyses of Cys oxidation, suggesting that it evades analysis by mass spectrometry (Leichert et al., 2008; Souza et al., 2018; van der Reest et al., 2018). Strategies of selectively derivatizing the degron were hampered by 30 additional Cys residues in FNIP1. However, as we noted that the degron Cys residues were highly sensitive to disulfide bond formation (Figure S5A), we could analyze degron oxidation through an alternative approach built on the thioredoxin TXN1 (Lindahl and Florencio, 2003). TXN1 contains an active site Cys residue that attacks intracellular disulfide bonds, before a second TXN1 Cys targets the mixed disulfide to release a reduced protein. A TXN1 variant lacking the second Cys, TXN^{1C35S}, fails to resolve the mixed disulfide and covalently traps oxidized proteins. The more a protein is trapped by TXN^{1C35S}, the more it was oxidized in cells.

Revealing significant degron oxidation under normal growth conditions, we found that wildtype GFPdegron, but not a Cys-free reporter, was efficiently trapped by TXN^{1C35S} (Figure 6A). To determine whether degron oxidation responded to changes in redox state, we escalated the load of reactive oxygen species by treating cells with α-ketoglutarate, which increases flux through the TCA cycle that produces substrate for the mitochondrial electron transport chain. In addition, we supplemented cells with antimycin A, which elevates reactive oxygen species by inhibiting mitochondrial complex III. These treatments enhanced TXN1-trapping of GFPdegron or full-length FNIP1, indicative of increased Cys oxidation (Figure 6B; Figure S5B). By contrast, depletion of reactive oxygen species by CUL3^{KEAP1} inhibition shielded the degron from TXN^{1C35S} and thus documented a reversal of degron oxidation upon reductive stress (Figure 6C).

Several observations then showed that degron oxidation controls recognition of FNIP1 by CUL2^{FEM1B}. Even a brief incubation of the degron without reducing agent disrupted its binding to FEM1B, which was fully restored by addition of TCEP (Figure 6D). In cells, treatment with α-ketoglutarate or antimycin A stabilized GFPdegron (Figure 6E, F; Figure S5C), dependent on reactive oxygen species produced by the respiratory chain (Figure S5C). Similar observations were made for myxothiazol, which increased the load of reactive oxygen species and stabilized GFPdegron (Figure S5D, E), as well as for genetic loss of antioxidant enzymes, such as glutathione reductase GSR or TXRND1 (Figure S5F). Importantly, conditions that evoked reductive stress, such as glutamine starvation or CUL3^{KEAP1} inhibition, accelerated GFPdegron turnover (Figure 6F; Figure S5G), which was dependent on the Cys residues in the FNIP1 degron (Figure S5G).

Consistent with the results for the degron reporter, antimycin A reduced the binding of full-length FNIP1 to FEM1B, yet reductive stress strongly promoted this interaction (Figure 6G). In fact, if FEM1B and FNIP1 were present at endogenous levels, we could detect their association with each other only after reductive stress had been imposed by CUL3^{KEAP1} inhibition (Figure 6H). Reductive stress accordingly lowered the protein levels, but increased the extent of ubiquitylation of endogenous FNIP1 (Figure 6I). We conclude that reductive stress upon persistent antioxidant signaling or mitochondrial inactivity reverts the oxidation of invariant Cys residues in the FNIP1 degron and allows CUL2^{FEM1B} to associate with its substrate. These findings identify the FNIP1 degron as a reductive stress sensor that is read out by CUL2^{FEM1B}.

3.3.6 Reductive stress triggers detection of FNIP1 by CUL2^{FEM1B}

To constitute a bona fide stress response, degradation of FNIP1 should alleviate reductive stress. FNIP1 degradation might accomplish this by modulating the activities of AMPK or mTORC1 (Baba et al., 2006; Baba et al., 2012; Hasumi et al., 2012; Tsun et al., 2013), as these kinases control biogenesis of mitochondria that produce most reactive oxygen species in cells. However, while loss of FEM1B slightly improved amino-acid dependent activation of mTORC1 in myoblasts, this was stimulated, rather than decreased, by co-depletion of FNIP1 (Figure S6A). The minor increase in AMPK activity caused by lack of FEM1B was also unaffected by co-depletion of FNIP1 (Figure S6B). Furthermore, mutation of its degron did not affect the binding of FNIP1 to AMPK or regulators of mTORC1 (Figure S6C), which all indicated that FNIP1 stability neither impacted mTORC1 nor AMPK signaling in myoblasts.

As FEM1B-depletion rescued myotube formation in the absence of mitochondria-derived reactive oxygen species (Figure 2D), we hypothesized that FNIP1 stability might instead directly impact mitochondria. Depletion of FEM1B did not reduce mitochondrial content, as judged by the levels of core mitochondrial proteins (Figure S6D). By contrast, all mitochondria in FEM1B- deficient cells showed a heavily stained matrix in transmission electron microscopy (Figure 7Ab), a condensation phenotype that had previously been ascribed to lack of substrate for oxidative phosphorylation (Hackenbrock, 1966). Many mitochondria displayed onion-like swirling of cristae (Figure 7Ac), indicative of upregulation of respiratory chain components in response to impaired oxidative phosphorylation (Jiang et al., 2017; Walker and Benzer, 2004), and some mitochondria contained large unstained blebs (Figure 7Ad), as observed upon initiation of mitophagy after loss of the mitochondrial membrane potential (Jin et al., 2010; Pickles et al., 2018). These phenotypes were all rescued by FNIP1 co-depletion (Figure 7Af), showing that FNIP1 stabilization altered mitochondrial morphology consistent with an inhibition of oxidative phosphorylation.

Mitochondria fuel oxidative phosphorylation through a membrane potential established by the respiratory chain. In line with our electron microscopy results, FEM1B depletion reduced, yet loss of FNIP1 increased, the mitochondrial membrane potential (Figure 7B). Cells lacking FEM1B also contained a fragmented mitochondrial network clustered around the nucleus (Figure 7C), which is consistent with a decrease in membrane potential inducing mitochondrial fission (Pickles et al., 2018). We further found that loss of FEM1B blocked formation of mitochondrial reactive oxygen species (Figure 7D), as detected with MitoSox (Robinson et al., 2006). These phenotypes of FEM1B loss were again all rescued by co-depletion of FNIP1 (Figure 7B-D), showing that degradation of FNIP1 through $CUL2^{FEM1B}$ controls mitochondrial output. As mitochondria are the main source of reactive oxygen species, the elimination of FNIP1 therefore provides a direct avenue to counteracting reductive stress.

3.3.7 FEM1B and FNIP1 are crucial metabolic regulators

How could cytoplasmic degradation of FNIP1 improve mitochondrial activity? In addition to the respiratory chain, mitochondria harbor enzymes for fatty acid β-oxidation and the TCA cycle, which convert acetyl-CoA into substrate for oxidative phosphorylation and building blocks for amino acid biosynthesis. To fuel these reactions, mitochondria import pyruvate, fatty acids, or TCA cycle components, thereby coupling cytoplasmic metabolism with mitochondrial energy production.

We first asked whether FNIP1 stability affected glycolysis, the main pathway for production of pyruvate. Using a Seahorse Analyzer, we found that FEM1B depletion reduced the glycolytic rate of myoblasts, while the respiratory chain itself, monitored through the oxygen consumption rate, was not affected (Figure S7A, B). The extracellular acidification rate, which mainly reflects secretion of lactate downstream of pyruvate, was also reduced by loss of FEM1B (Figure S7C). Liquid chromatography coupled to mass spectrometry revealed that loss of FEM1B depleted cells of glycolytic intermediates and downstream components of the TCA cycle (Figure 7E; Figure S7D). In addition, lack of FEM1B reduced levels of glucose (Figure 7E), as well as glucose uptake (Figure S7E). However, only few glycolytic intermediates were increased, and glucose import was only mildly improved, by co-depletion of FNIP1 (Figure S7E; Figure S7). Mass spectrometry experiments revealed that myoblasts derived few TCA cycle intermediates from glucose (Figure S7F), and the flux of glucose carbon atoms to the TCA cycle was not affected by FEM1B depletion (Figure S7G). Consistent with glucose deprivation inhibiting myogenesis (Fulco et al., 2008), co- depletion of glucose-utilizing enzymes induced by NRF2 also did not restore myoblast differentiation during reductive stress (Figure S7H). Thus, while FEM1B affects glucose metabolism, this likely involves a substrate distinct from FNIP1.

Instead of glucose, we found that myoblasts derived most TCA cycle intermediates from glutamine (Figure S7F), which is converted into glutamate prior to its import into mitochondria. Metabolomic analyses revealed that loss of FEM1B caused a drop in glutamate, while depletion of FNIP1 increased the abundance of this metabolite (Figure 7E). Co-depletion of FEM1B and FNIP1 cancelled out each of these phenotypes. In a similar manner, precursors or components of other mitochondrial shuttles were increased by loss of FNIP1, decreased upon depletion of FEM1B, and unaltered in myoblasts lacking both FEM1B and FNIP1. This included citrulline and ornithine, which replenish fumarate from cytoplasmic arginine; the fatty acid carrier acetylcarnitine and its precursor panthothenate; and the NADH transporter glycerol-3-phosphate (Figure 7E). Degradation of FNIP1 through CUL2^{FEM1B} therefore increases the availability of glutamate and other metabolite shuttles, which could provide a means to jumpstart the TCA cycle and induce the production of mitochondria-derived reactive oxygen species. We conclude that CUL2^{FEM1B} and FNIP1 not only sense, but also alleviate reductive stress, identifying these proteins as a core module of the reductive stress response.

3.4 Discussion

Reductive stress, as caused by the persistent absence of reactive oxygen species, interferes with intracellular and organismal signaling and increases mortality (Bjelakovic et al., 2007; McClung et al., 2004; Rajasekaran et al., 2007; Rajasekaran et al., 2011; Ristow et al., 2009). While we show that mitochondrial inactivity provides a physiological trigger for reductive stress, it can also be elicited by inhibition of $\text{CUL3}^{\text{KEAP1}}$ and the ensuing accumulation of NRF2. KEAP1 inhibition is a frequent consequence of nutrient deprivation, exposure to electrophilic toxins, or germline or somatic mutations (Bellezza et al., 2018; Bollong et al., 2018; Lignitto et al., 2019; Yamamoto et al., 2018). However, KEAP1 deletion in mice caused lethality only after birth (Wakabayashi et al., 2003; Yamamoto et al., 2018), and KEAP1 mutations are observed in rapidly dividing metastatic lung and renal cancer cells (Armenia et al., 2018; Cancer Genome Atlas Research, 2012; Lignitto et al., 2019; Wiel et al., 2019). These observations implied that mechanisms must exist that can detect and alleviate reductive stress.

Starting with a genetic modifier screen, we identified $\text{CUL2}^{\text{FEM1B}}$ and its essential target FNIP1 as core components of the reductive stress response. $\text{CUL2}^{\text{FEM1B}}$ only binds FNIP1, if the oxidation of invariant Cys residues in FNIP1 is reverted during reductive stress. $\text{CUL2}^{\text{FEM1B}}$ then ubiquitylates and helps degrade FNIP1 to activate mitochondria and counteract reductive stress (Figure 7F). Intriguingly, we also found that $\text{CUL2}^{\text{FEM1B}}$ is required for nuclear import of NRF2, which paradoxically should support antioxidant gene expression. We speculate that the ability of $\text{CUL2}^{\text{FEM1B}}$ to control NRF2 nuclear import ensures that cells only instigate antioxidant signaling, if they are protected against an eventual depletion of reactive oxygen species. A similar dichotomy had been reported for NRF2, which triggers antioxidant signaling but also elicits mitochondrial biogenesis (Rojo de la Vega et al., 2018). Our findings highlight the importance of maintaining physiological levels of reactive oxygen species, which we now attribute to a redox rheostat that integrates both oxidative and reductive stress responses.

Although FNIP1-FLCN had been implicated in AMPK and mTORC1 signaling (Baba et al., 2006; Tsun et al., 2013), we found that FNIP1 degradation acts as a mitochondrial gatekeeper. Stabilization of FNIP1 reduced mitochondria-derived reactive oxygen species and induced changes in mitochondrial morphology that implied a lack of substrate for oxidative phosphorylation (Hackenbrock, 1966). Accordingly, FNIP1 stabilization depleted cells of mitochondrial metabolite shuttles, which will reduce the levels of TCA cycle intermediates and dampen the reactions that generate substrate for the respiratory chain. FNIP1 stabilization should bring about mitochondrial inactivation and a drop in ATP, which might improve differentiation of FEM1B-deficient myoblasts in a similar manner as it does during exercise-induced myogenesis (Hoppeler et al., 2011). We speculate that its role as a mitochondrial inhibitor is related to the FNIP1-FLCN recruitment to damaged mitochondria prior to autophagy, as compromised organelles need to be shut down to ensure cell survival (Heo et al., 2018).

Reductive stress, through its hallmark absence of reactive oxygen species, reduces the degron Cys residues in FNIP1 and thus eliminates this mitochondrial regulator. The loss of FNIP1 boosted levels of mitochondrial metabolite shuttles, increased the mitochondrial membrane potential, and restored mitochondrial activity in cells lacking FEM1B. These results are congruent with phenotypes of FNIP1 deletion in mice, which caused a switch from glycolytic to oxidative myofibers characterized by abundant mitochondria (Reyes et al., 2015). Our findings show that FNIP1 degradation increases mitochondrial output to produce reactive oxygen species and counteract reductive stress. CUL2$^{\mathrm{FEM1B}}$ and FNIP1 therefore comprise a ubiquitin-dependent stress module that tunes mitochondrial output to the metabolic and redox needs of myoblasts (Figure 7F).

The reductive stress response bears striking similarity to the pathways that respond to hypoxic and oxidative stress. These systems also rely on E3 ligases of the CRL family that detect their substrates dependent on specific oxidation events. While prolyl hydroxylation marks HIF1α for ubiquitylation by the hypoxic stress E3 CUL2$^{\mathrm{VHL}}$ (Kaelin, 2007; Ohh et al., 2000), oxidation of Cys residues in KEAP1 prevents NRF2 ubiquitylation during oxidative stress (Zhang et al., 2004). It appears that cells evolved highly similar ubiquitin-dependent mechanisms to cope with different insults onto redox homeostasis, which highlights the power of ubiquitylation to help cells adapt to rapidly changing environments.

Underscoring the importance of reductive stress signaling for tissue homeostasis, loss of FNIP1's partner FLCN results in a predisposition to renal cancer (Baba et al., 2006; Nickerson et al., 2002). The same tumor type is caused by mutation of KEAP1, CUL3, NFE2L2, or VHL (Kaelin, 2007; Singh et al., 2006), or aberrant oxidative metabolism (Xu et al., 2019). Kidney cells require large amounts of ATP to generate a proton gradient needed to filter toxic substances out of blood (Bhargava and Schnellmann, 2017). The mitochondrial metabolism of these cells might frequently fail to sustain ATP levels, thus causing a condition that effectively constitutes reductive stress. In line with the importance of ATP production, all redox stress pathways shape energy metabolism: HIF1α increases glycolysis, NRF2 elicits mitochondrial biogenesis (Gonzalez et al., 2018; Rojo de la Vega et al., 2018), and FNIP1 modulates substrate availability for oxidative phosphorylation. Thus, redox stress responses not only monitor reactive oxygen species, but also safeguard metabolism. Given the similar organismal consequences of KEAP1, FLCN, and VHL mutation, we propose that it is the regulation of metabolism that integrates the stress and developmental roles of these critical stress response pathways.

The studies of us and others showed that CUL2$^{\mathrm{FEM1B}}$ targets proteins in addition to FNIP1. CUL2 paired with FEM1A, FEM1B, or FEM1C ubiquitylates the histone mRNA-binding protein SLBP (Dankert et al., 2017), and FEM-1 binds Gli transcription factors in C. elegans (Starostina et al., 2007). All FEM1 proteins also act in the C-end rule pathway to eliminate proteins with carboxy-terminal degrons or truncations (Koren et al., 2018). As we have found that FEM1A does not bind FNIP1, it is likely that FEM1B detects these targets through a distinct site, potentially allowing cells to coordinate multiple degradation events with redox regulation. We also found that CUL2$^{\mathrm{FEM1B}}$ binds, but does not polyubiquitylate, the mTORC1 inhibitor GATOR1 (Bar-Peled et al., 2013; Dutchak et al., 2018). Opposite of FNIP1 deletion, which increases oxidative muscle fibers, muscle-specific loss of GATOR1 causes a switch to glycolytic fibers (Dutchak et al., 2018). We speculate that GATOR1 competitively inhibits FEM1B to prevent untimely FNIP1 degradation and coordinate reductive stress and mTORC1 signaling. Our discovery of the reductive stress response thus provides a novel starting point to dissect, and modulate for therapeutic benefit, the complex architecture of metazoan redox stress signaling, an important step towards elucidating how tissue formation and homeostasis is accomplished in the dynamic and often harsh natural environments of metazoan organisms.

3.5 Materials and Methods

Screening and myotube analysis

For the CUL2 and CUL3 adaptor screen, early passage C2C12 mouse myoblasts were seeded into 96 well plates at 400 to 500 cells/well using a Thermo Scientific Mulitdrop Combi system. The next day, cells were transfected with ~20nM final concentration of siRNAs using an Agilent Velocity 11 Bravo Automated Liquid Handling Platform. The next day, cells were differentiated by changing the media 3x with differentiation media using the Bravo Velocity. Media was changed every day and on the fourth day, cells were fixed in 4% formaldehyde in PBS for 20min. Fixed cells were washed in PBS, permeabilized with 0.1% triton, and stained for immunofluorescence with antibodies in phosphate buffered saline with 10% fetal bovine serum. All incubations for immunofluorescence were done with very slow mixing in a circular motion on a plate shaker for 3 hours for primary antibody and 1 hour for secondary antibody and Hoechst (AnaSpec Inc.). Plates were imaged on a Molecular Devices ImageXpress Micro Widefield High-Content Analysis System with a 10x objective capturing 25 images per well. Images were analyzed by a MetaXpress custom module from Molecular Devices.

Individual siRNA myotube analysis was performed as described above, but with 12 well plates seeded with early passage C2C12 cells at 40,000-50,000 cells per well. The day after seeding, cells were transfected with 20-80nM final concentration of siRNAs depending on the number of co-depletions. Cells were fixed at day 3 or 4 of differentiation and prepared for immunofluorescence as above. 49 or 100 images per condition were acquired on Perkin Elmer Opera Phenix automated microscope using a 20x objective and analyzed by an analysis sequence designed in the Perkin Elmer Harmony software. The analysis of the cullin depletion phenotypes and the initial validation of screen hits were performed on the ImageXpress Micro.

Cell Culture

C2C12 myoblasts and HEK293Ts were grown in DMEM with 10% fetal bovine serum. For C2C12 differentiation, cells were grown to 70-90% confluence and had their media changed 2-3x in differentiation medium, DMEM 2% donor equine serum. For amino acid starvation, cells were washed 1x in PBS and put in amino acid free RPMI and 10% dialyzed FBS. 1x Amino acids were added to the cultures at indicated time points. For glucose and glutamine starvations, cells were rinsed in phosphate buffered saline and incubated with DMEM -Glucose or DMEM -Glutamine with 10% dialyzed fetal bovine serum.

For transient plasmid transfection, HEK293Ts and C2C12s were transfected using polyethylenimine (PEI) with cDNAs expressed from pCS2+ vectors. For the C2C12 FEM1B mass spectrometry immunoprecipitations, 20ug of DNA with 60µl of PEI (1mg/ml) in 500µl Optimem per 15 cm with a total of 20 plates. For 293T experiments, the following µg amounts were used for 10cm plate transfections HA-FNIP1 constructs 2µg, HAFLCN 0.5-1µg, FlagFEM1B constructs 0.5-1µg, HADEPDC5 0.5µg, HANPRL2 0.5µg, HANPRL3 0.5µg, dnCUL21-427-HA 5µg, GFPdegron-IRES-mCherry reporters 0.1µg, and TXN^{1C35S}-HIS6/Flag 0.6µg. PEI ratios 1:3 to 1:6 µg DNA to µl of PEI were used in 300µl of Optimum and cells were harvested 36h after transfection. For the FNIP1 mass spectrometry immunoprecipitation, 10x15cm plates of 293Ts were transfected with 1.5µg FlagFNIP1, 0.75µg HAFLCN and empty vector to 4µg total with 1:6 ratio of PEI in 400µl Optimem per plate. For the purification of FlagFNIP1/HAFLCN for in vitro ubiquitylation reactions, 4µg of FlagFNIP1 and 2µg of HAFLCN transfected as for the mass spectrometry transfection into 10x15cm plates of 293T. For flow cytometry, 0.1µg of GFPdegron-IRES-mCherry reporters, 1µg of FEM1B constructs, and empty vector to 2µg total were added to 300µl of Optimum and 12µl PEI (1:6 ratio) and 60µl were added to 6 well plates of 293Ts and harvested for flow cytometry after 24h. siRNA transfections were performed with Lipofectamine RNAiMAX according to the manufacturer's recommendation.

Metabolomics

Metabolomics for polar metabolites were performed as described (Louie et al., 2016) on 5 replicate 10cm of plates of C2C12 myoblast transfected with indicated siRNAs for 36 hours.

Small Scale Immunoprecipitations

Cells were harvested by removing growth media and scraping cells in 8-10ml of cold PBS. Cells were centrifuged for 5min at 300g and pellets resuspended in lysis buffer (40mM HEPES 7.5, 150mM NaCl, 0.2% NP40, with Roche cOmplete Protease Inhibitor Cocktail). Lysates were gently rocked for 30-60min at 4 °C and cleared by centrifugation for 30min at 21000g, 4 °C. Supernatants were normalized to volume and protein concentration (if cells were treated with aromatic compounds, lysates were normalized using pierce 660nm). 5% of the sample was removed as an input and of the sample was added to 20µl of washed ANTI-FLAG® M2 Affinity Agarose Gel slurry (Sigma A2220) and rotated for 1-2 hours at 4 °C. Beads were washed three times and eluted with 2x urea sample buffer.

Whole Cell Lysates

For Western blot time courses, C2C12 myoblasts were seeded in 12 well plates at 40-50k cells per well. Cells were transfected 24h later with indicated siRNAs at 20nM-40nM for each siRNA. 24h after transfection, cells were differentiated, harvested at indicated time points by washing in PBS, and lysed 200µl 2x urea sample buffer, heated to 65 °C for 10min, sonicated, and normalized to protein concentration and volume with Pierce 660nm Protein Assay Reagent (ThermoFisher 22660). Samples were analyzed by immunoblot with indicated antibodies. For Western analysis of phosphorylated proteins and of 293T cells, cells in 6-well plates were washed in cold PBS and harvested in lysis buffer (1% Triton X-100, 10mM β-glycerol phosphate, 10 mM sodium pyrophosphate, 4 mM EDTA, 40 mM HEPES, pH 7.4 with Roche cOmplete Protease Inhibitor Cocktail) with rocking for 10min at 4 °C. Cells lysates were collected in 1.7ml tubes and rocked for additional 20min at 4 °C. Cells lysates were cleared by centrifugation at 21,000gs for 20min at 4 °C. Supernatants were collected, normalized with Pierce 660nm, and added to an equal volume of 2x urea sample buffer. Samples were heated to 65 °C for 10min and analyzed by immunoblot with indicated antibodies.

In Vitro Ubiquitylations

For all ubiquitylations, CUL2-RBX1 and CRL2^{FEM1B} complexes were modified with NEDD8 prior to the ubiquitylation assay, in 1x UBA buffer (50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM MgCl2), 20mM ATP, 6.3µM Nedd8, 1mM DTT, 5µM CUL2 complexes, 700nM UBA3, 400nM UBE2M in a 20µl reaction volume for 15min at 30 °C. Ubiquitylation assays were carried out in 10µl reactions with 1µM CUL2 ligase, 100µM ubiquitin, 1x UBA buffer (50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM MgCl2) 20mM ATP, 1mM DTT, 1µM E2s, and 1µM UBA1. For full length FlagFNIP/HAFLCN, 1-2µl of buffer exchanged αFLAG elutions were used and reactions carried out at 30 °C for 1h. For peptides, 500nM final concentration of 5,6-TAMRA labeled peptides were incubated at 30 °C for indicated times. To alkylate with iodoacetamide and N-Methylmaleimide, 200µM peptide was incubated with or without 600µM alkylating agent for 1h. Peptides were diluted into binding buffer with 0.1mM DTT to quench the alkylating agents before addition into ubiquitylation reactions.

Flow Cytometry

293T cells were seeded at 300k cell/ml in 6 well plates. The next day, cells were transfected with indicated constructs. 24h post transfection, cells were trypsinized and centrifuged at 300g for 5min. Cells were resuspended in full media with 10% FBS and analyzed on either BD Bioscience LSR Fortessa or LSR Fortessa X20 and FlowJo. For TMRM staining, 150,000 C2C12 myoblasts were seeded in 10 cm plates. The next day, cells were transfected with siRNAs and after 24h medium was exchanged. Cells were trypsinized 36-40h post transfection and counted. 1,000,000 cells were aliquoted in 1ml of pre-warmed PBS and labeled. A control sample was pretreated with 50uM CCCP for 5min and all samples were labeled with 20nM TMRM from the MitoProbe™ TMRM Assay Kit for Flow Cytometry (M20036 ThermoFisher) for 30min at 37 °C in 5% CO2. Cells were washed in warm PBS and analyzed on BD Bioscience LSR Fortessa and FlowJo.

In Vitro Binding

pCS2+-^{HA}FNIP1$^{567-893}$ was synthesized using the rabbit reticulocyte lysate TnT quick coupled in vitro transcription/translation system (Promega, L2080) as directed. Translated ^{HA}FNIP1$^{567-893}$ reactions were diluted in binding buffer (40mM HEPES 7.5, 150mM NaCl, 0.2% NP40, with or without 2mM TCEP and with or 2mM iodoacetamide) and added to amylose beads with bound ^{MBP}FEM1B or MBP. Samples were rocked at room temperature for 1h and washed in binding buffer with or without TCEP. Samples were eluted in urea sample buffer and analyzed by immunoblot with indicated antibodies.

Fluorescence Polarization

TAMRA-labeled FNIP1 peptides (5,6-TAMRA-RNKSSLLFKESEETRTPNCNCKYCSHPVLG) and mutants were purchased from the Koch Institute/MIT Biopolymers lab. Binding titrations were performed with 50nM TAMRAFNIP1 peptides and increasing protein concentrations in binding buffer (40mM HEPES 7.5, 150mM NaCl, 0.2% NP40, with or without 0.1mM TCEP) in triplicate. For iodoacetamide and N-Methylmaleimide peptide labeling, 200µM peptide was labeled with 600µM alkylating agent for 1h. Peptides were diluted into binding buffer with 0.1mM DTT to quench the alkylating agents before mixing with protein. Fluorescence polarization measurements were performed on BioTek Synergy H4 plate reader after 1h of incubation at room temp. Data was analyzed with GraphPad using the specific binding fit with Hill slope equation.

Protein Purifications

Mouse $^{MBP/HIS}$FEM1B (pMAL, New England Biolabs) and the $^{MBP/HIS}$FEM1B/Elongin B/Elongin C$^{17\text{-}336}$ complex (pRSFduet-1) were purified from *E.coli* LOBSTR cells grown to OD600 0.5 and induced with 333μM IPTG overnight at 16 °C. Cells were lysed in buffer A and added to ½ the lysate volume of buffer B (50mM HEPES 7.5, 300mM NaCl 1.5mM PMSF, 15mM β-mercaptoethanol 30mM Imidazole). Cells were sonicated and spun at 30,000xg for 1h. Supernatant was added to Ni-NTA slurry and bound for 1h at 4 °C. Beads were washed in wash buffer (50mM HEPES 7.5, 150mM NaCl, 5mM β-mercaptoethanol, 20mM imidazole, and 1mM PMSF) three times for 15min with rocking. Beads were eluted with 50mM HEPES 7.5, 150mM NaCl, 5mM β-mercaptoethanol, 250mM imidazole. Elutions were dialyzed overnight and ran on a HiLoad 16/600 Superdex 200pg, concentrated, aliquoted, and flash frozen. $^{MBP/HIS}$FEM1B was purified without reducing agent for FP assays as above, but run on the size exclusion column in the absence of reducing agents. For CUL2-RBX1 purification, $^{HIS/TEV}$CUL2 and untagged RBX1 were expressed off the pFastBac Dual vector. Baculovirus packaging and amplification were performed as described (Bac-to-Bac Baculovirus Expression System, Thermo Fisher). For the purification, 3l of insect cells were infected and 72 hours later harvested by centrifugation and flash frozen in liquid nitrogen. Cells were lysed in 50mM HEPES 7.5, 150mM NaCl 1mM PMSF, 5mM β-mercaptoethanol, 10mM Imidazole, and 0.5% NP40 with gentle rocking at 4 °C for 1h. Lysates were centrifuged at 30,000xg for 1h ^{HIS}CUL2-RBX1 complexes were isolated and purified as described for $^{MBP/HIS}$FEM1B.

For the full CUL2, RBX1, ELONGIN B/C, FEM1B ligase complex (CUL2^{FEM1B}), the above purifications were performed in parallel until the dialysis step. Both imidazole elutions were mixed and diluted by a factor of 2 into 50mM HEPES 7.5, 150mM NaCl, 5mM β-mercaptoethanol, and 20% glycerol to 10% final and rocked for 1 h at 4 °C. After the incubation, TEV protease was added at 1ug:100ug TEV to protein ratio to cleave MBP off FEM1B and the HIS tag off CUL2. Reactions were dialyzed in 50mM HEPES 7.5, 150mM NaCl, 1mM DTT, and 10% glycerol overnight. The sample was spun at 30,000g for 30min and the supernatant loaded onto a MonoQ 10/100 anion exchange starting at 90% Buffer A (40mM HEPES 7.5, 1mM DTT, 10% Glycerol) and 10% buffer B (40mM HEPES 7.5, 1M NaCl, 1mM DTT, 10% Glycerol) increasing to 100% buffer B over 15 column volumes. Fractions corresponding to cleaved FEM1B-CUL2 complex were concentrated and run on a HiLoad 16/600 Superdex 200pg in 40mM HEPES 7.5, 150mM NaCl, 1mM DTT, 10% Glycerol and the complex fractions were concentrated, aliquoted, and flash frozen.

^{FLAG}FNIP1/^{HA}FLCN complexes were purified from 293Ts using affinity-purification, as described above. 3xFLAG peptide elutions were treated with 1mM TCEP for 30min on ice and spun through Amicon Ultra 0.5ml centrifuge concentrator filter ultracel -30k NMWL to a final volume of 50-100μl. Fresh buffer (40mM HEPES 7.5, 150mM NaCl, 1mM TCEP) was then added and this centrigue and buffer exchange step was repeated 3 times to remove detergent and 3xFlag peptide. After the last buffer exchange the Flag-FNIP1/HA-FLCN complexes were aliquoted and flash frozen.

E1/UBA1, UBE2R1, UBE2D3, and MBP-FBXL17 were purified previously described (Jin et al., 2012; Mena et al., 2018; Wickliffe et al., 2011). The GATOR1 complex was a generous gift from Roberto Zoncu (Lawrence et al., 2019). The neddylation machinery (human UBA3 (E1, E- 313), UBE2M (E2, E2-656), NEDD8(UL-812)) and ubiquitin (U-100H) were purchased from Boston Biochem.

Seahorse Analysis

C2C12 cells were seeded into XF^e96 Cell Culture Microplates at a density of 320 cells/well to 500 cells/well with no coating. 6-7 wells per condition were transfected with siRNAs and 36h analyzed post-transfection by a Seahorse XF analyzer. Seahorse XF Glycolytic rate and mitochondria stress tests were performed according to the manufacturers recommendation. For the mitochondria stress tests, 1.5µM oligomycin, 2µM FCCP, and 0.5µM Antimycin A/Rotenone final concentrations were used. All media changes were done using an Agilent Bravo Automated Liquid Handler Platform. After the flux analysis, cells were fixed in 4% formaldehyde in PBS with Hoechst stain for 30 minutes, washed 3x in PBS, and imaged on an Opera Phenix automated microscope using a 20x objective to capture the entire well. The nuclei count was determine using the Perkin Elmer Harmony software and all data was normalized to the nuclei count of each well.

Immunofluorescence and Confocal Microscopy

C2C12 cells were seeded (10,000 cells/ml) on cover slips in 12 well plates. The next day cells were transfected with indicated siRNAs 15nM each siRNA (30nM final). 24h post transfection, media was changed on all plates. ~40h after transfection, cells were fixed in 4% formaldehyde in 1X PBS for 20min at room temperature, permeabilized with 0.1% Triton X-100 in 1X PBS, blocked in 10% FBS in 1X PBS, stained with 1° antibodies for 3h at room temperature, followed by staining with 2° antibodies and Hoechst stain. Samples were mounted onto coverslips and imaged using an Olympus IX81 microscope equipped with a Yokogawa CSU-1X confocal scanner unit (CSUX1 Borealis Square Upgrade Module), an Andor iXon3 camera (IXON DU-897-BV), and an Andor Technology Laser Combiner System 500 series equipped with four laser lines.

NAD/H NADP/H Measurements

C2C12 cells were seeded into 6cm plates (100,000 cells) and transfected the day after with siRNAs. 36h after transfection, the media was changed on all plates. NAD+/H and NADP+/H were measured from the same cells using NAD/NADH-Glo™ and NADP/NADPH-Glo™ Assays (Promega, G9071 and G9081). To measure both reduced and oxidized forms, we used the suggested modified lysis. Briefly cells were washed one time with PBS and lysed in the 1:1 PBS and 0.2N NaOH 1% dodecyltrimethylammonium bromide. Lysates were split into three tubes, one for protein normalization, one as a base treated (left unmodified, NADH/PH detection) and the last as acid treated (0.4 N HCl acid added, NAD/P detection). The base and acid treated samples were heated to 60 °C for 15min, cooled and neutralized. Samples were then split and processed for NAD and NADP quantifications. Data was normalized using protein concentration of lysates.

H_2O_2 Measurements

C2C12 cells (20k cell/well) were seeded into 12 well plates. Cells were transfected the next day with siRNAs (30nM final). 24h after transfection, the media was changed on all wells. 36h after transfection, H_2O_2 was measured using the ROS-Glo H_2O_2 Assay (Promega, G8820) according the manufactures protocol in growth media. After the media was removed for the assay, cells were lysed in their wells with (40mM HEPES 7.2, 150mM NaCl, .2% NP40) on ice. Lysates were cleared with 21000g spin at 4 °C for 10min and the supernatants' A^{280} was measured to determine protein concentrations for normalization. For HEK293T experiments, 140k cell/well were seeded into 12 well plates. The next day, cells were treated with indicated drug concentrations, and 16h later H2O2 was measured as described above.

^{13}C Labeling

C2C12 myoblast were seeded into either 10 or 6 cm plates at 250k cell/plate or 100k cell/plate respectively 5 plates per condition. The next day, cells were transfected with siRNAs (30nM final, single depletions had siCNTRL added to 30nM final). 24h after transfection, media was changed on all plates. 36h post transfection, cells were rinsed once in 3ml –glucose media (DMEM – glucose, 4mM glutamine, 10% dialyzed fetal bovine serum) and cells incubated in 4ml –glucose media supplemented with 5mM ^{13}C glucose (Sigma, CAT# 389374-1G) for 4h to reach steady state labeling. After incubation, cells were washed in ice cold 0.1M ammonium bicarbonate and 500µl of extraction buffer (methanol : acetonitrile : water = 40:40:20 with 0.1 M formic acid) containing 1 µM of isotopic AMP (Sigma, CAT# 900382) and Fructose-1,6-bisphosphate (Cambridge Isotope Laboratories, Inc., CAT# CLM-6678-0) as standards, respectively was added to each plate. Plates were gently shaken for 10sec, scraped, and transferred to tubes on ice for 15min. Samples were centrifuged at 21000 g for 10min at 4°C. 180µl of supernatant was added to 20ul of 1M ammonium bicarbonate, mixed, and flash frozen in liquid N_2.

LC/MS-based analyses of glycolytic and TCA intermediates were performed on liquid chromatography system (LC; 1200 series, Agilent Technologies) connected in line with an LTQ-Orbitrap-XL mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Fisher Scientific) at the UC Berkeley QB3/Chemistry Mass Spectrometry Facility. 20µl of each metabolite sample was injected onto a ZIC-pHILIC 2.1 X 150 mm (5 µm particle size) column (EMD Millipore). Buffer A was 20 mM ammonium carbonate, 0.1% ammonium hydroxide; buffer B was acetonitrile (Optima LC-MS grade, Fisher Chemical). The chromatographic gradient was run at a flow rate of 0.2 ml/min as follows: 0-20 min: linear gradient from 80% to 20% B; 20-20.5 min: linear gradient from 20% to 80% B; 20.5-35 min: hold at 80% B. The mass spectrometer was operated in the negative ion mode and column temperature was 25°C. Mass spectra were recorded over the range of 70-1000 mass-to-charge ratio (m/z). Metabolite identification and quantification were performed using Xcalibur software (version 2.0.7, Thermo Fisher Scientific)

TUBE Binding

For endogenous detection of FNIP1 ubiquitylation, 1 15 cm plate of HEK293T cells was treated overnight (~16h) with either DMSO, 350nM, or 500nM of Bardoxolone methyl (SMB00376-10MG Sigma-Aldrich), and 500nM MLN4924 (15217 Cayman Chemical) as indicated. All samples were also treated with 2µM Carfilzomib ((PR-171) Selleck Chemical S285310MM/1ML) for 4h prior to harvesting. Cells were harvested in cold PBS, spun down, and frozen in liquid N2. Cell pellets were lysed, cleared, and normalized as described small-scale immunoprecipitation but with 500µl modified lysis buffer (40mM HEPES 7.2, 150mM NaCl, 10mM N-Ethylmaleimide, 0.1mM TCEP, 1% Triton and with or without 3µM HALO-TUBES). Normalized lysates were added to HALO-link resin (Promega, G1914) and rocked for 1-2 hours at 4°C. Beads were washed 4x in Lysis buffer without

NEM and eluted with 2x urea sample buffer. HALO-TUBES was purified as described forrecombinant FEM1B. pET28a-6HIS_TEV-HALO-4x ubiquilin UBA TUBE (DU23799) was obtained through the MRC PPU Reagents and Services facility (MRC PPU, College of Life Sciences, University of Dundee, Scotland, mrcppureagents.dundee.ac.uk).

Glucose Uptake Assay

C2C12 cells were seeded into 96 well white cell culture microplate plate at 320 cell/well and transfected with indicated siRNAs the next day (8 wells per condition). Additional 50µl of media was added to each well the night before the assay and ~40h after siRNA transfection 4 wells per condition were analyzed using the Glucose Uptake-Glo™ Assay (Promega J1341) following manufacturer's recommendation with a 10min 2-deoxyglucose incubation. 4 control wells were not incubated with 2DG as a background control. The remaining 4 wells were fixed in 4% formaldehyde in PBS with Hoechst stain for 30min, washed 3x in PBS, and imaged on an Opera Phenix automated microscope using a 20x objective to capture the entire well. The nuclei count was determine using the Perkin Elmer Harmony software and was used to normalize the data to the average number of cells/well for each treatment.

Mitosox Staining

C2C12 were siRNA transfected and differentiated as described for myotube analysis. At Day 3 of differentiation the media was removed and 5uM MitoSox (Invitrogen M36008) in Hank's balanced salt solution (HBSS) was added for 10 min at 37 °C with 5% CO2. Cells were washed 3 times with HBSS and imaged using an Opera Phenix automated microscope at 37 °C with 5% CO2.

Antibodies

The following antibodies were used in this study: anti-Flag (Sigma, clone M2, F1804), anti-CUL2 (A302-476A Bethyl), anti-FNIP1 (ab134969 Abcam), anti-Fnip1 (PUR 215725 Abcam), anti-GFP (ab6556 Abcam), anti-FEM1B (19544-1-AP Proteintech), anti-Myosin, sarcomere (MHC) (M20 Developmental Studies Hybridoma Bank), anti-Myogenin (F5D Developmental Studies Hybridoma Bank) anti-beta-Actin (MP Biomedicals, clone C4, 691001), α-TOMM20 (HPA011562 SIGMA Prestige Antibodies), anti-HA-Tag (C29F4 Rabbit mAb #3724 Cell Signaling Technology (CST)), anti-Flag DYKDDDDK Tag (#2368 CST), anti-FLCN (D14G9 Rabbit mAb #3697 CST),
anti-KEAP1 (D6B12 Rabbit mAb #8047 CST), anti-NRF2 (D1Z9C XP® Rabbit mAb #12721 CST), anti-Citrate Synthase (D7V8B Rabbit mAb #14309 CST), anti-GAPDH (D16H11 XP® Rabbit mAb #5174 CST), anti-Phospho-AMPKα (Thr172) (40H9 Rabbit mAb #2535 CST), anti-AMPKα (D5A2 Rabbit mAb #5831 CST), anti-Phospho-p70 S6 Kinase (Thr389) (108D2 Rabbit mAb #9234 CST), anti-p70 S6 Kinase (49D7 Rabbit mAb #2708 CST), anti-Phospho-Acetyl-CoA Carboxylase (Ser79) (#3661 CST), and anti-Acetyl-CoA Carboxylase (C83B10 Rabbit mAb #3676 CST)

Cloning

Fem1b, Nprl2, Fnip1, Fnip2, Flcn, Rbx1, Elongin B, Elongin C, CUL2, constructs were cloned from cDNA prepared from C2C12s. DEPDC5, NPRL3 were generated from 293T cDNA. We cloned FNIP1 as two isoforms, full length and a truncation missing 208-235. Both isoforms interact with and are degraded by Fem1b, but the short form of FNIP1 expresses considerably better and retains binding to currently known FNIP1 interactors. All FNIP1 experiments presented in this study use the short isoform and the residue positions indicated are reference to this isoform. All FEM1B and FNIP1 mutants were generated by overlap extension polymerase chain reaction, or site directed mutagenesis using quick change method. The pCS2+-^{GFP}degron-IRES-mCherry reporter was generated using Gibson assembly of all of the individual components (GFP, IRES, mCherry, and pCS2) (Gibson et al., 2009). A 4xGly-Ser linker was added between GFP and two Esp3I sites which allow for the easy insertion of DNA fragments. The FNIP1 degron was inserted into the reporter construct by annealing and phosphorylating two oligos corresponding to degron sequences with a forward oligo containing a 5’-CAGC-3’ overhang and reverse oligo with 5’- ATCA-3’ overhangs, corresponding to the overhand generated by the Esp3I cuts.

shRNA Production and Viral Production

Lentiviral shFEM1B pLKO.1 constructs were purchased from Sigma (shFEM1B$^{\#1}$: TRCN0000303677125, shFEM1B$^{\#2}$: TRCN0000299671125, shFEM1B$^{\#3}$: TRCN0000303647125, shFEM1B$^{\#4}$: TRCN0000303746125, shTXNRD1: TRCN0000046535, shGSR: TRCN0000046425). pINDUCER20-^{3xFLAG}FEM1B (pINDUCER from (Meerbrey et al., 2011)) and shRNA containing Lentiviruses were generated in 293T cells by co-transfection with lentiviral constructs with packaging plasmids (Addgene) using PEI. Viral supernatants were collected and filter through a 0.45µm filter and concentrated with LentiX concentrator following the manufactures protocol (Takara 631232). Precipitated virus was resuspended in media, aliquoted, and frozen.

Quantitative Real Time PCR Analysis

Total RNA from C2C12 cells myoblasts grown in 6cm dishes and was purified using nucleospin RNA kit (Macherey-Nagel, 740955). cDNA was generated (Thermo, F470) and qRT-PCRs were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using 2X KAPA SYBR FAST qPCR Master Mix (KK4602). The following IDT primetime primers were used: TKT (5'-GAAACAAGCCTTCACCGATG-3', 5'-CTCCCAGCATGCAATAGACTC-3'), PFKFB2 (5'-ACATCCTCGTTATCTCTCACCA-3', 5'-ATGTGTGTCTACAGCATCCAC-3'), GPIL (5'-CTGGTACATCAACTGCTACGG-3', 5'-ATGTACTTTCCGTTGGACTCC-3'), G6PDX (5'-

ACAAGACACAGTTGGAGCAG-3’, 5’-GTGAGTCAGTAGCTGTATGTCA-3’), TXRND1 (5’-GTGCTGGTCTTGGATTTTGTC-3’, 5’-GGTGCATCAGCTTCTTAGGTAT-3’) Nqo1 (5’-GCTCCATGTACTCTCTTCAGG-3’, 5’-GCCAATGCTGTAAACCAGTTG-3’), HMOX1 (5’-ACACTCTGGAGATGACACCT-3’, 5’-TTGTGTTCCTCTGTCAGCATC-3’), MYOG (5’-CTTGCTCAGCTCCCTCAAC-3’, 5’-GACCGAACTCCAGTGCATT-3’), MYL1 (5’-

GAAGCAGTCACCAAGAACA TTC-3', 5'-TGGTTCGACAGTTGATTGGAG-3'), ALDOA (5'-CTCCTTAGTCCTTTCGCCTAC-3', 5'-AGACAGCTCCTTCTTCTGC-3'), PGD (5'-GATCTTCCAAGCCATCGCT-3', 5'-CCATACTCTATCCCGTTGTGC-3'), TALDO1 (5'-CGGCAAGGACAGAATTCTCAT-3', 5'-AGTGTCATGTTGCAGTGGAT-3'), NFE2l2 (5'-TCAAACACTTCTCGACTTACTCC-3', 5'-TGATGGACTTGGAGTTGCC-3'), GCLM AGCTGCCTTCAGTTTATCCTC-3’, 5’-GATCTTGCATTCACCTGTTCTG-3’), and GAPDH (5’-AGGTCGGTGTGAACGGATTTG-3’, 5’- TGTAGACCATGTAGTTGAGGTCA-3’) as internal control.

Immunoprecipitation and mass spectrometry

Large scale immunoprecipitations were performed after harvesting cells in cold PBS (20 plates of transfected C2C12 cells or 10 plates of 293T cells) and centrifuging them at 300g for 10min. Cell pellets were resuspended in 5x the volume of pellet weight (ml/g) of lysis buffer (40mM HEPES 7.5, 150mM NaCl, 0.2% NP40, with Roche cOmplete Protease Inhibitor Cocktail, the $^{\mathrm{FLAG}}$FNIP1 IP lysis buffer also contained 10mM β-glycerol phosphate, 10 mM sodium pyrophosphate, 2.5mM MgCl2). Lysates were gently rocked for 1h at 4 °C and cleared by centrifugation at 500g, 5min and 21000g, 30min. Supernatants were added to 90µl of ANTI-FLAG® M2 Affinity Agarose Gel slurry (Sigma A2220) and rotated for 1-2h at 4 °C. Beads were washed extensively in lysis buffer and eluted 2x with 250µl of 3xFlag peptide (F4799, Millipore). Elutions were pooled and precipitated overnight on ice with 20% trichloroacetic acid. The precipitated pellets were washed in acetone, dried, and solubilized in 8M urea, 100mM TRIS, pH 8.5. The samples were reduced with TCEP, alkylated with iodoacetamide, and digested overnight with trypsin (V5111, Promega). Trypsinized samples were analyzed by Multidimensional Protein Identification Technology (MudPIT) at the Vincent J. Coates Proteomics/Mass Spectrometry Laboratory at UC Berkeley. Unique proteins were identified by comparing each IP to a dataset of 40-150 similar (unique databases for C2C12 and 293T cells) αFLAG IP/mass spectrometry samples using CompPASS analysis (Huttlin et al., 2015). All total spectral counts were normalized to 4000 TSC of bait.

Genome Editing

FEM1B knockout 293T cell lines were generated using the ribonucleoprotein (RNP) method (DeWitt et al., 2017) using guides targeting the 5’ UTR and intronic regions adjacent to exon1 of FEM1B (5’-ATTAACGAGTCCGCGCGCGT-3’, 5’-AAGACGAGCTTTCGCAGACA-3’). Guide RNAs were synthesized with NEB HiScribe T7 High Yield RNA Synthesis Kit, DNAse trated with turboDNAse (Ambion/Thermo), and purified with the Invitrogen MEGASCRIPT clean-up kit. RNPs were assembled with Cas9 purified by the UC Berkeley QB3 MacroLab in a 10µl reaction of 100 pmol of

Cas9 and 120 pmol sgRNA in Cas9 buffer (20 mM HEPES 7.5, 150 mM KCl, 10% glycerol, 1 mM TCEP). Reactions were gently mixed for 30s and incubated for 20min at room temperature. RNP complexes and 200k 293T cells resuspended in buffer SF (Lonza) were added to a nucleofection strip and the mixture pulsed with program DS150 Lonza 4D-Nucleofector. Cells were plated into 6 well dishes. Endogenous ^{3xFLAG}FEM1B 293T cell lines were generated using a guide targeting near the start codon of FEM1B (5’-GGCGGCGGCCATGGAGGGCC-3’) that was cloned into pX330 and cotransfectd with a 200bp repair template containing a 3xFLAG tag (5’-TCCGGGGGCGCACGGCAGCTGCAGCGGTGGCGACCAAACGGGTGTTGGAGTTGGCGGC GGCCATG GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATG ACAAGGAGGGCCTGGCTGGCTATGTATACAAGGCGGCCAGCGAGGGCAAGGTGCTGACT CTGGCCGCCTTGCTT-3’) using Lipofectamine 2000 (11668019 ThermoFisher). After confirmation of bulk editing by PCR for both knockouts and knock-ins, cells were diluted to 5cells/ml and 100µl of cell suspension was plated per well plated into 96 well plates containing 200µl total media, 20% FBS DMEM + Pen/Strep. Colonies were expanded and screened for homozygous knockout or 3xFLAG tagging by PCR and Western blot and confirmed by DNA sequencing.

NGS Library Prep and RNA-seq

Total RNA was extracted from sub-confluent C2C12s treated with siFem1b, siKeap1, siFem1b- siKeap1, or siCNTRL siRNAs (in triplicate) using a NucleoSpin Plus RNA extraction kit (Machery- Nagel). NGS libraries were made using a TruSeq Stranded Total RNA kit (Illumina), with an average size of 250 bp. Libraries were prepared by the UC Berkeley Functional Genomics Laboratory. Paired-end RNA-sequencing was done using a HiSeq400 (Illumina). Sequencing of the libraries was done two times to obtain technical replicates.

RNA-seq Alignment, Expression Analysis and Transcription Factor Enrichment.

We used the Kallisto-Sleuth pipeline to perform differential gene expression analysis between samples (Pimentel et al., 2017). Briefly, paired-end RNA-seq reads were aligned using Kallisto, using the mm10 *Mus musculus* reference transcriptome and 200 bootstrap steps. For differential expression analysis, the R Sleuth package was used. To obtain log2 fold changes, we had to implement the following transformation function during the initial sleuth object (so) preparation step:

so <- sleuth_prep(s2c, ~ condition / bio_samp, extra_bootstrap_summary = TRUE, target_mapping = t2g, transformation_function = function(x) log2(x + 0.5))

To identify significant differentially expressed genes, the following conditions were compared: siCNTRL v siFem1b; siCNTRL v siKeap1; siFem1b v siKeap1. From each comparison, significant differentially expressed genes with a qval ≤ 0.075 were kept. This generated four different gene lists, which were then merged together. This gene list was used to generate heat maps for datavisualization. Heat maps of significant differentially expressed genes were generated using the R “heatmap.2” package, normalized by row, and using unsupervised clustering applying the “ward.D2” option. Transcription factor enrichment for significant differentially expressed genes was done using the ChEA3 and oPOSSUM algorithms (Keenan et al., 2019; Kwon et al., 2012).

Transmission Electron Microscopy

siCNTRL, siFem1b, siFnip1, and siFnip1-siFem1b treated C2C12 cells were grown to 50% confluence and fixed in 2% guleteraldehyde: 0.1 M sodium cacodylate (pH 7.2) for 20 min, followed by 3 washes with 0.1 M sodium cacodylate buffer. Cells were then gently harvested and collected in 1.5 mL Eppendorf tubes, followed by imbedding in agarose plugs. After solidifying, agar plugs containing the specimens were carefully cut into ~2.5 mm^3 slices. Slices were stained in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 hr, followed by 1.38% potassium ferricyanide for 1 hr. Stained samples were step-dehydrated in acetone (35%, 50%, 70%, 80%, 95%, 100%, 100%) for 10 min at each step. Dehydrated samples were then step-infiltrated with acetone:Epon resin (2:1, 1:1, 1:2 for 1 hr each). After final acetone:resin infiltration, samples were embedded in pure Epon resin at room temperature, overnight, followed by curing at 65 °C for two days. Cured samples were then sliced using a Leica UC 6 microtome, taking 70 nm sections. Sliced sections were picked up on 100 mesh formvar-coated copper grids, then stained with 2% aqueous uranyl acetate for 5 min, followed by 2% lead citrate for 2 min. Grids were examined under a Tecnai 12 TEM at 120 kV.

3.6 Figure Legends

3.6.1 Main Figure Legends

Figure 1: Reductive stress inhibits myotube formation *in vitro*.

A. An siRNA screen identifies KEAP1 as a crucial activator and FEM1B as an inhibitor of *in vitro* myoblast differentiation. C2C12 myoblasts were depleted of each substrate adaptor of CUL2 and CUL3 E3 ligases. Differentiation was induced in 2% horse serum, and the efficiency of myotube formation was determined by immunofluorescence analysis against the late differentiation marker myosin heavy chain (MyHC).

B. Validation of top myogenesis screen hits by microscopy. C2C12 myoblasts were depleted of KEAP1 or FEM1B and myotube formation was determined by immunofluorescence microscopy against MyHC. Quantification of myogenesis efficiency is shown on the right for two independent siRNAs each against KEAP1 or FEM1B. Each experiment included at least three biological replicates.

C. Validation of top myogenesis screen hits. C2C12 myoblasts were depleted of KEAP1 or FEM1B and expression of the myogenesis markers MYOG and MyHC was determined at the indicated times of differentiation by Western blotting using specific antibodies. D. Differentiation of KEAP1-deficient myoblasts is rescued by co-depletion of NRF2. C2C12 cells were depleted of KEAP1, NRF2, or both, and differentiation was induced as described above. The differentiation efficiency was monitored by Western blotting against MYOG and MyHC. E. KEAP1-deficient myoblasts have reduced ROS production. C2C12 myoblasts were depleted of KEAP1, FEM1B, or both and H2O2 measured using a luciferase based reporter. F. Myogenesis is rescued in the absence of KEAP1 by co-depletion of NRF2 or antioxidant NRF2 target genes. C2C12 myoblasts were depleted of KEAP1, NRF2, select antioxidant targets of NRF2 (GSR, GCLC), or combinations thereof. Differentiation was induced, and the efficiency of myotube formation was determined by immunofluorescence microscopy against MyHC. Quantification of the differentiation experiments is shown on the right, including two independent NRF2 siRNAs. Each experiment includes at least three biological replicates. G. Myogenesis is impaired by mitochondrial complex I and III scavengers. S1QEL1.1 and S3QEL2 were added during C2C12 myoblast differentiation and myotube formation was analyzed by immunofluorescence microscopy against MyHC.

Figure 2: The CUL2 adaptor FEM1B opposes KEAP1 during myoblast differentiation.

A. A genetic modifier screen identifies FEM1B as KEAP1 antagonist. C2C12 myoblasts were depleted of KEAP1 and each CUL2 substrate adaptor, and differentiation was monitored by microscopy against MyHC. *Upper panel:* microscopy images from each condition; *lower panel:* quantification of differentiation efficiency compared to KEAP1-depleted control myoblasts. The screen was performed in duplicate. B. KEAP1 and FEM1B antagonize each other during myogenesis. C2C12 cells were depleted of KEAP1, FEM1B, or both, and induced to differentiate. Myotube formation was monitored by microscopy against MyHC. Quantification of three biological replicates is shown on the right. C. FEM1B antagonizes KEAP1 during myogenesis, as shown by Western blotting. C2C12 cells were depleted of KEAP1, FEM1B, or both, induced to differentiate, and analyzed by Western blotting against NRF2, MYOG, or MyHC. D. Scavenging mitochondria derived ROS inhibits C2C12 differentiation and rescues the FEM1B depletion phenotype. C2C12 myoblasts were treated throughout differentiation with S1QEL1.1 and S3QEL2 and depleted with siCNTRL or siFEM1B. Quantification of three biological replicates is shown to the right. E. The antagonism between

KEAP1 and FEM1B is detected by gene expression analysis. C2C12 myoblasts were depleted of KEAP1, FEM1B, or both. mRNA abundance was measured by RNAseq and analyzed by unsupervised clustering. Genes that possess binding sites for the transcription factors are marked light blue (HIF1α), light green (MYOD/MYOG) or red (NRF2). **F.** Validation of RNAseq analysis by qRT-PCR of select NRF2 target genes (red) or myogenesis markers (orange) in C2C12 myoblasts depleted of KEAP1, FEM1B, or both. **G.** NRF2 localization was determined by immunofluorescence in C2C12 myoblasts depleted of KEAP1, FEM1B, or both.

Figure 3: FNIP1 is a critical CUL2^{FEM1B} substrate during reductive stress. A. Identification of candidate CUL2^{FEM1B} substrates. Wild-type ^{FLAG}FEM1B or ^{FLAG}FEM1B^{L597A} were affinity-purified from C2C12 myoblasts and binding partners were determined by CompPASS mass spectrometry. Subunits of GATOR1 are marked green, the FLCN-FNIP1 complex is marked blue. **B.** Analysis of FEM1B binding of GATOR1, FLCN, and FNIP1 by affinity-purification and Western blotting. Wildtype ^{FLAG}FEM1B, ^{FLAG}FEM1B^{C186S} (a mutation of a conserved residue in the substrate-binding ankyrin repeats), or ^{FLAG}FEM1B^{L597A} were affinity-purified from 293T cells that also expressed the indicated HA-tagged proteins. Co-purifying proteins were detected by αHA-Western blotting. **C.** Overexpression of FEM1B elicits proteasome-dependent degradation of FNIP1. 293T cells were transfected with ^{HA}FNIP1 and FEM1B, FEM1B^{L597A}, or FEM1A. Proteasome inhibitor MG132 was added for 6h as indicated, and FNIP1 levels were determined by Western blotting. **D.** Depletion of FEM1B in 293T cells by two independent siRNAs results in accumulation of endogenous FNIP1, as determined by Western blotting. **E.** CUL2^{FEM1B} polyubiquitylates FNIP1 *in vitro*. FNIP1- FLCN complexes were incubated with recombinant CUL2 or Nedd8-modified CUL2^{FEM1B}, E1, ubiquitin, and the E2 enzymes UBE2D3 and/or UBE2R1, as indicated. Reactions were analyzed by gel electrophoresis and Western blotting against FNIP1 (Flag) and FLCN (HA). **F.** FNIP1 is a crucial CUL2^{FEM1B}-substrate during myogenesis. C2C12 myoblasts were depleted of FEM1B, FNIP1, or both, and the efficiency of myotube formation was analyzed by microscopy against MyHC. Quantification of three biological replicates is shown on the right. **G.** FNIP1 is a crucial CUL2^{FEM1B} target during reductive stress. C2C12 myoblasts were depleted of KEAP1, FEM1B, FNIP1, or combinations thereof. Differentiation was induced, and the efficiency of myotube formation was determined by microscopy against MyHC. Quantification of three biological replicates is shown on the right.

Figure 4: FEM1B binds a conserved central degron in FNIP1.

A. Deletion analysis of FNIP1 identifies a stretch of 20 amino acids that is required for its binding to FEM1B and its proteasomal degradation. The indicated FNIP1 variants were expressed in cells and tested for interaction with FLAGFEM1B by co-immunoprecipitation, and for degradation by measuring their abundance in the presence or absence of FEM1B. **B.** The FNIP1 degron is required for FEM1B binding and proteasomal degradation. Wildtype FNIP1 or a variant lacking its degron (FNIP1$^{\Delta 562\text{-}591}$) were co- expressed with FLCN and ^{FLAG}FEM1B or ^{FLAG}FEM1B^{L597A}. FEM1B variants were affinity-purified and bound FNIP1 and FLCN were detected by Western blotting. **C.** The FNIP1 degron is sufficient to mediate FEM1B-dependent degradation. The FNIP1 degron was fused to GFP (GFP^{degron}) and expressed together with an IRES controlled mCherry. As indicated, cells were transfected with FEM1B, catalytically inactive FEM1B^{L597A}, or substrate-binding deficient FEM1B^{C186S}. The ratio of GFP^{degron} to mCherry fluorescence was determined by flow cytometry. **D.** Deletion of *FEM1B* prevents FNIP1-degron dependent degradation. The *FEM1B* locus was deleted in 293T cells using CRISPR/Cas9. Cells were transfected with the GFP^{degron} reporter, and analyzed for GFP and mCherry fluorescence by flow cytometry. **E.** FEM1B binds the FNIP1 degron *in vitro*. A TAMRA-labeled FNIP1 degron peptide was incubated with purified FEM1B (green) or the CUL1 adaptor FBXL17 (blue), and binding was measured by fluorescence polarization of the peptide. **F.** The FNIP1 degron is sufficient to mediate CUL2^{FEM1B}-dependent ubiquitylation. The TAMRA-labeled FNIP1 degron was incubated with CUL2 or Nedd8-modified CUL2^{FEM1B}, E1, ubiquitin, ATP and the E2 enzymes UBE2D3 and/or UBE2R1. Ubiquitylation was monitored by gel electrophoresis and fluorescence detection.

Figure 5: The FNIP1 degron requires highly reactive, invariant Cys residues.

A. Mutation of Cys585 or Cys580/582 strongly impedes FNIP1 degron function. GFP-fusions between a wildtype FNIP1 degron or variants lacking Cys residues were expressed with IRES-driven mCherry and the GFP/mCherry ratio was determined by flow cytometry. Cells also expressed FEM1B (dashed lines) or not (solid lines). **B.** Cys residues are required for degron recognition by FEM1B *in vitro*. TAMRA-labeled wildtype, Cys-free, or Lys-free degron peptides were incubated with recombinant FEM1B. Degron binding was monitored by fluorescence polarization. **C.** Cys residues are essential for degron ubiquitylation by CUL2^{FEM1B}. The indicated TAMRA-labeled FNIP1 degron peptides were incubated with Nedd8-modified CUL2^{FEM1B}, ubiquitin, E1, and UBE2D3/UBE1R1. Ubiquitylation reactions were followed by gel electrophoresis and fluorescence imaging. **D.** Each degron Cys residue and a His residue are required for CUL2^{FEM1B} binding and proteasomal degradation of full-length FNIP1. ^{HA}FNIP1 variants were co-expressed with wild-type ^{FLAG}FEM1B or inactive ^{FLAG}FEM1B^{L597A}. Levels of FNIP1 variants were monitored by Western blotting. E3 ligase recognition of FNIP1 variants was determined by α-FLAG affinity-purification of FEM1B followed by Western blotting. **E.** Treatment of the degron peptide with NEM prevents binding to FEM1B. The TAMRA-labeled FNIP1 degron peptide was incubated in buffer or NEM, reactions were quenched with DTT, and FEM1B binding was monitored by fluorescence polarization. **F.** Degron modification with NEM or iodoacetamide prevents polyubiquitylation of the FNIP1 degron peptide. The TAMRA-labeled FNIP1 degron was incubated in buffer, NEM, or iodoacetamide. Reactions were quenched and added to Nedd8-modified CUL2^{FEM1B}, ubiquitin, E1, and UBE2D3/UBE2R1. Ubiquitylation reactions were followed by gel electrophoresis and fluorescence imaging (to monitor degron ubiquitylation) or Coomassie staining (to monitor substrate independent CUL2^{FEM1B} activity). **G.** The Cys-reactive, cell-permeable IAyne stabilizes GFP^{degron} in cells, as seen by a right shift of the GFP/mCherry ratio observed by flow cytometry.

Figure 6: The FNIP1 degron is redox sensitive. A. The FNIP1 degron is oxidized in cells. Wildtype of Cys-free GFP^{degron} were tested for trapping by TXN^{1C35S}. Trapping was determined by TXN^{1C35S} affinity-purification, resolution of mixed disulfide bonds in reducing buffer, and Western blotting against GFP^{degron}. **B.** Degron oxidization is increased by mitochondrial production of reactive oxygen species. Cells were treated with cell-permeable α-ketoglutarate or antimycin A, which increase the cellular load with reactive oxygen species. TXN^{1C35S}-trapping of the degron peptide was determined as described above. **C.** The degron is reduced by prolonged antioxidant signaling. Cells were exposed to bardoxolone, a KEAP1 inhibitor that stabilizes antioxidant NRF2. TXN^{1C35S}-trapping of the degron was determined as described above. **D.** Degron recognition by FEM1B is regulated by reversible oxidation. The TAMRA-labeled FNIP1 degron was diluted in a buffer that contained (TCEP) or lacked (OX) the reducing agent TCEP. After binding to recombinant FEM1B had been measured by fluorescence polarization, the oxidized degron was treated with TCEP and binding to FEM1B was

monitored again (OX->TCEP). **E.** FNIP1 degron- dependent degradation is prevented by mitochondrial production of reactive oxygen species. Cells expressing the GFP^{degron} reporter were treated either antimycin A or the proteasome inhibitor carfilzomib, and the GFP/mCherry ratio was determined by flow cytometry. **F.** FNIP1 degron- dependent reporter degradation is accelerated by reductive stress. Cells expressing the GFP^{degron} reporter were depleted of glutamine, which lowers substrate levels for the TCA cycle and oxidative phosphorylation, or treated with the KEAP1 inhibitor bardoxolone, and the GFP/mCherry ratio was determined by flow cytometry. **G.** The interaction between FNIP1 and FEM1B is sensitive to changes in the redox state. Cells were treated with α-ketoglutarate, antimycin A, oligomycin, or the KEAP inhibitor TBHQ. ^{FLAG}FEM1B was affinity-purified, and binding to endogenous FNIP1 and FLCN, or HA-tagged GATOR1 subunits, was determined by Western blotting. **H.** Endogenous FEM1B and FNIP1 interact during reductive stress. The *FEM1B* locus of 293T cells was fused to a FLAG epitope by genome editing. Cells that also expressed a dominant-negative variant of CUL2 (amino acids 1-427) to stabilize CRL2-substrate interactions were then treated with oligomycin (OM), antimycin A (AM) or TBHQ. Endogenous FEM1B was affinity-purified and bound endogenous FNIP1 was detected by gel electrophoresis and Western blotting. **I.** Reductive stress increases the ubiquitylation and induces degradation of endogenous FNIP1 in a CRL-dependent manner. 293T cells were treated with increasing bardoxolone concentrations with or without the NEDD8 inhibitor MLN4924 overnight. Ubiquitylated proteins were isolated using TUBE pulldown and FNIP1 was detected by Western blot.

Figure 7: FNIP1 levels control mitochondrial metabolism.

A. Transmission electron microscopy (TEM) reveals changes in mitochondrial structure upon depletion of FEM1B. C1C12 myoblasts were depleted of FEM1B, FNIP1, or both and processed for TEM analysis. In the absence of FEM1B (Fig. 7Ab-Ad), mitochondria showed dramatic changes to matrix density and membrane organization, which was rescued by co-depletion of FNIP1. **B.** FNIP1 stability modulates the mitochondrial membrane potential. C2C12 myoblasts were depleted of FEM1B, FNIP1, or both, incubated with the mitochondrial membrane potential dye TMRE, and analyzed by flow cytometry. CCCP-treated cells were used as control. **C.** FEM1B depletion causes mitochondrial fragmentation. Mitochondrial morphology was examined in C2C12 myoblasts depleted of FNIP1, FEM1B, or both, by immunofluorescense microscopy against TOMM20. Distance of mitochondria from nucleus was quantified (n=10-15 per condition). **D.** FNIP1 stabilization shuts off production of mitochondrial reactive oxygen species. C2C12 myotubes were depleted of FEM1B, FNIP1, or both and stained for mitochondrial superoxide using MitoSox. Images were analyzed by automated immunofluorescence microscopy. **E.** FEM1B and FNIP1 control cellular metabolism. C2C12 myoblasts were depleted of FEM1B, FNIP1, or both and the abundance of polar metabolites was determined by liquid chromatography and mass spectrometry. The first dot describes FNIP1-depleted cells, the second dot FEM1B-depleted cells, and the third shows cells co-depleted of FNIP1 and FEM1B. Yellow indicates upregulation compared to control, whereas blue indicates downregulation compared to control. Metabolites upregulated by FNIP1-depletion, downregulated by FEM1B-depletion, and unaltered upon co- depletion of FNIP1 and FEM1B are depicted in red. **F.** Model of the reductive stress response. Reductive stress reverts the oxidation of invariant Cys residues in the FNIP1 degron, leading to recognition of FNIP1 by CUL2^{FEM1B}, polyubiquitylation, and proteasomal degradation. The loss of FNIP1 increases mitochondrial activity and thus triggers production of reactive oxygen species to counteract reductive stress.

3.6.2 Supplemental Figure Legends

Figure S1: CRL2 and CRL3 E3 ligases are required for myoblast differentiation.

A. CUL2 and CUL3 are required for myotube differentiation. C2C12 myoblasts were depleted of CUL1, CUL2, CUL3, CUL4a, CUL4a, CUL5, and CUL7, differentiation was induced, and formation of myotubes was followed by automated microscopy against MyHC. Quantification is shown on the right. **B.** CRL adaptors affect myotube formation independently of effects on cell division or survival. C2C12 myoblasts were depleted of the indicated CRL2- or CRL3-adaptors and subjected to differentiation. Nuclei were stained by Hoechst and counted by automated image analysis. No correlation between effects on differentiation and nuclei count was observed. **C.** NAD^+/NADH ratio, $NADP^+$/NADPH ratio, and total NADP/NADPH levels from C2C12 myoblasts depleted of KEAP1, FEM1B, or both. Quantification of five biological replicates. **D.** C2C12 cells were depleted of GCLC, GSR, NRF2 (using two siRNA sequences). Myotube formation was quantified after immunofluorescence microscopy against MyHC, using at least 4 biological replicates.

Figure S2: FNIP1 is a specific substrate of $CUL2^{FEM1B}$.

A. qRT-PCR of select pentose phosphate pathway NRF2 target genes in C2C12 myoblasts depleted of KEAP1, FEM1B, or both.**B.** qRT-PCR of putative NRF2 glycolysis target genes in C2C12 myoblasts depleted of KEAP1, FEM1B, or both. **C.** NFE2L2 (NRF2) expression measured by qRT-PCR and RNAseq from myoblasts depleted of KEAP1, FEM1B, or both. **D.** $FEM1B^{L597A}$ does not bind CUL2. $^{FLAG}FEM1B$ or $^{FLAG}FEM1B^{L597A}$ were affinity-purified from 293T cells and bound CUL2 was detected by Western blotting. **E.** $FEM1B^{L597A}$ shows enhanced binding to FNIP1, FLCN, and GATOR1 subunits. $^{FLAG}FEM1B$ or $^{FLAG}FEM1B^{L597A}$ were affinity-purified from 293T cells that also expressed HAFNIP1, $^{HA}FLCN$, or HA-tagged GATOR1 subunits. Binding of HA-tagged proteins to FEM1B was detected after αFLAG affinity-purification by gel electrophoresis and Western blotting using αHA antibodies. **F.** CUL2 activity is required for FEM1B-dependent degradation of FNIP1. Cells were transfected with $^{FLAG}FEM1B$, $^{HA}FNIP1$, $^{HA}FLCN$, and dominant-negative $^{HA}dnCUL2$ (1-427), as indicated, and FNIP1 abundance was determined by Western blotting. **G.** FEM1B does not induce the degradation of FNIP2, a close homolog of FNIP1. Cells were transfected with $^{HA}FNIP2$ and the indicated FEM1B constructs, and FNIP2 levels were determined by gel electrophoresis and Western blotting using αHA antibodies. **H.** $CUL2^{FEM1B}$ does not ubiquitylate GATOR1. DEPDC5, $^{FLAG}NPRL2$ and $^{FLAG}NPRL3$ were purified from insect cells, incubated with recombinant CUL2 or $CUL2^{FEM1B}$, ATP, E1, UBE2D3 and UBE2R1 as E2s, and ubiquitin, and analyzed for ubiquitylation by gel electrophoresis and Western blotting. **I.** C2C12 cells were depleted of FEM1B, FNIP1, or both, and differentiation was induced as described. The differentiation efficiency was monitored by Western blotting against MYOG and MyHC. **J.** Co-depletion of NPRL2, NPRL3, or DEPDC5 does not rescue the increased myogenesis seen in cells lacking FEM1B. C2C12 myoblasts were transfected with the indicated siRNAs, induced to differentiate, and analyzed by microscopy against MyHC.

Figure S3: FNIP1 contains a conserved degron for recognition by $CUL2^{FEM1B}$.

A. The FNIP1 degron is sufficient to mediate binding to FEM1B. 293T cells were co-transfected with $^{FLAG}FEM1B$ or $^{FLAG}FEM1B^{L597A}$ and GFP or GFP^{degron} ($GFP\text{-}FNIP1^{562\text{-}591}$), as indicated. $^{FLAG}FEM1B$ variants were affinity-purified and bound GFP was detected by Western blotting. **B.** Degron binding to FEM1B is specific. $^{FLAG}FEM1B$ or the ankyrin mutant $^{FLAG}FEM1B^{C186S}$ were co-transfected with GFP^{degron}. $^{FLAG}FEM1B$ variants were affinity-purified, and bound GFP was detected by Western blotting. **C.**

Depletion of FEM1B by four independent shRNAs stabilizes the GFP-degron fusion, as observed after co-expression of GFP^{degron} and mCherry and analysis by flow cytometry. **D.** Proteasome inhibition with carfilzomib stabilizes the GFP^{degron} reporter, as determined by flow cytometry. **E.** The Cys residues of the FNIP1 degron are invariant among FNIP1 homologs. **F.**The FNIP1 degron is not conserved in FNIP2, a protein otherwise highly similar to FNIP1. FNIP2 is not recognized by $CUL2^{FEM1B}$.

Figure S4: Cysteine residues are essential for FNIP1 degron function.

A. Cys residues are essential for FNIP1 degron function, as seen by Western blotting. HA-tagged FNIP1 variants were co-expressed with FLCN and FLAG-tagged FEM1B or $FEM1B^{L597A}$. FEM1B variants were immunoprecipitated and co-purifying FNIP1 was detected by gel electrophoresis and αHA-Western. **B.** Modification of degron Cys residues prevents binding to FEM1B. A TAMRA-labeled FNIP1 degron peptide was incubated with buffer (green) or iodoacetamide (blue). Binding to recombinant FEM1B was monitored by fluorescence polarization. **C.** Reversible oxidation controls degron recognition by FEM1B. A rabbit reticulocyte lysate generated HA-tagged FNIP1 fragment containing the degron was incubated with recombinant ^{MBP}FEM1B. Reducing agent (TCEP) or a Cys-reactive modifier (iodoacetamide) were added as indicated. ^{MBP}FEM1B was purified on maltose agarose and bound FNIP1 or rabbit CUL2 were detected by Western blotting.

Figure S5: FEM1B recognizes an unmodified FNIP1 degron.

A. The FNIP1 degron peptide rapidly forms disulfide bonds in solution. ^{TAMRA}FNIP1 degron peptide was diluted into buffer containing no reducing agent and aliquots were taken at indicated time points and added to sample buffer with or without reducing agent. Samples were analyzed by gel electrophoresis and fluorescence detection. **B.** The FNIP1 degron is oxidized in cells in the context of full-length FNIP1. $^{FLAG}TXN^{1C35S}$ was affinity-purified from cells treated with antimycin A, and co-eluting endogenous FNIP1 and FLCN were detected by Western blotting. **C.** Stabilization of the FNIP1 degron reporter by antimycin A is dependent on the activity of the electron transfer chain. Cells were either treated with antimycin A, rotenone (which inhibits electron delivery to complex III), or both, and the stability of GFP^{degron} was monitored by flow cytometry. **D.** Antimycin A and myxothiazol increase ROS production, as measured by a H2O2 detecting luciferase assay. **E.** Myxothiazol stabilizes the FNIP1 degron reporter. Cells were either treated with myxothiazol, rotenone, or both, and the stability of GFP^{degron} was monitored by flow cytometry. **F.** Depletion of thioredoxin reductase and glutathione reductase stabilize the FNIP1 degron reporter. 293T Cells were depleted of GSR, TXRND1, or both and the stability of the degron reporter was determined by flow cytometry. **G.** Reductive stress-dependent degradation of the FNIP1 degron reporter requires the degron Cys residues. GFP fused to the wildtype degron (WT) or a degron with all three Cys residues mutated to Ser (CS) was co-expressed with FEM1B, and cells were depleted off glutamine to induce reductive stress, as indicated. The GFP/mCherry ratio was measured as an indication for GFP stability by flow cytometry.

Figure S6: FEM1B and FNIP1 control metabolism. A. FEM1B does not affect mTORC1 signaling in myoblasts. C2C12 myoblasts were depleted of FEM1B, FNIP1, or both. Cells were starved, before amino acids were added to rapidly turn on mTORC1. mTORC1 activity was monitored by measuring levels of phosphorylated S6 kinase using Western blotting. **B.** FEM1B does not strongly affect AMPK signaling. C2C12 myoblasts were depleted of FEM1B, FNIP1, or combinations thereof, and AMPK signaling was monitored by measuring phosphorylated ACC and phosphorylated AMPK using Western blotting. **C.** Mutation of the Cys residues of the FNIP1 degron does not change the interaction with components of the mTORC1 and AMPK pathways. FLAGFNIP1 or a ^{FLAG}FNIP1^{3CS} degron variant were expressed with ^{HA}FLCN in 293T cells, and binding partners were determined by affinity-purification and mass spectrometry. **D.** Mitochondrial protein abundance is not altered when FEM1B is depleted. C2C12 myoblasts were transfected with indicated siRNAs and analyzed by western blot for TOMM20 (outer mitochondrial membrane protein) and CS (citrate synthase, matrix).

Figure S7: FNIP1 and FEM1B control the abundance of mitochondrial metabolites and shuttles.

A. FEM1B depletion inhibits glycolysis. C2C12 myoblasts were depleted of FEM1B,FNIP1, or both and the glycolytic rate was determined using a Seahorse Analyzer. **B.** Depletion of FEM1B or FNIP1 do not greatly affect the activity of the electron transport chain. The oxygen consumption rate (OCR) was measured for C2C12 cells transfected with indicated siRNAs using the mito stress test on a Seahorse Analyzer. **C.** Depletion of FEM1B reduces the extracellular acidification rate, which is partially rescued by co-depletion of FNIP1. **D.** C2C12 cells were transfected with siRNAs targeting FNIP1, FEM1B, or both. Polar metabolites were extracted and analyzed by liquid chromatography and mass spectrometry. **E.** FEM1B depletion inhibits glucose uptake. Standard deviation of four technical replicates normalized to cell number. **F.** Quantification of glycolytic and TCA cycle metabolites either in untreated control C2C12 myoblasts or C2C12 myoblasts starved for glutamine for 8h. **G.** C2C12 myoblasts were depleted with indicated siRNAs and labeled with ^{13}C glucose for 4 hours. % ^{13}C labeled metabolites were determined by mass spectrometry. **H.** NRF2 targets in the pentose phosphate pathway, NADPH regeneration and serine biosynthesis do not rescue the KEAP1 depletion phenotype. C2C12 myoblasts were depleted of indicated genes with or without KEAP1 co-depletion and induced to differentiated for 4 days. Myotube formation was monitored by microscopy against MyHC and quantified by automated microscopy. Quantification is of 3 biological replicates.

Table S1: Identification of CUL2 and CUL3 substrate adaptors in myoblasts or myotubes. C2C12 cells expressing ^{FLAG}CUL2 or ^{FLAG}CUL3 were lysed as myoblasts or differentiating myotubes, and bound proteins containing either a VHL box (CUL2 adaptors) or a BTB domain (CUL3 adaptors) were identified by αFLAG-affinity purification and mass spectrometry.

3.7 Figures

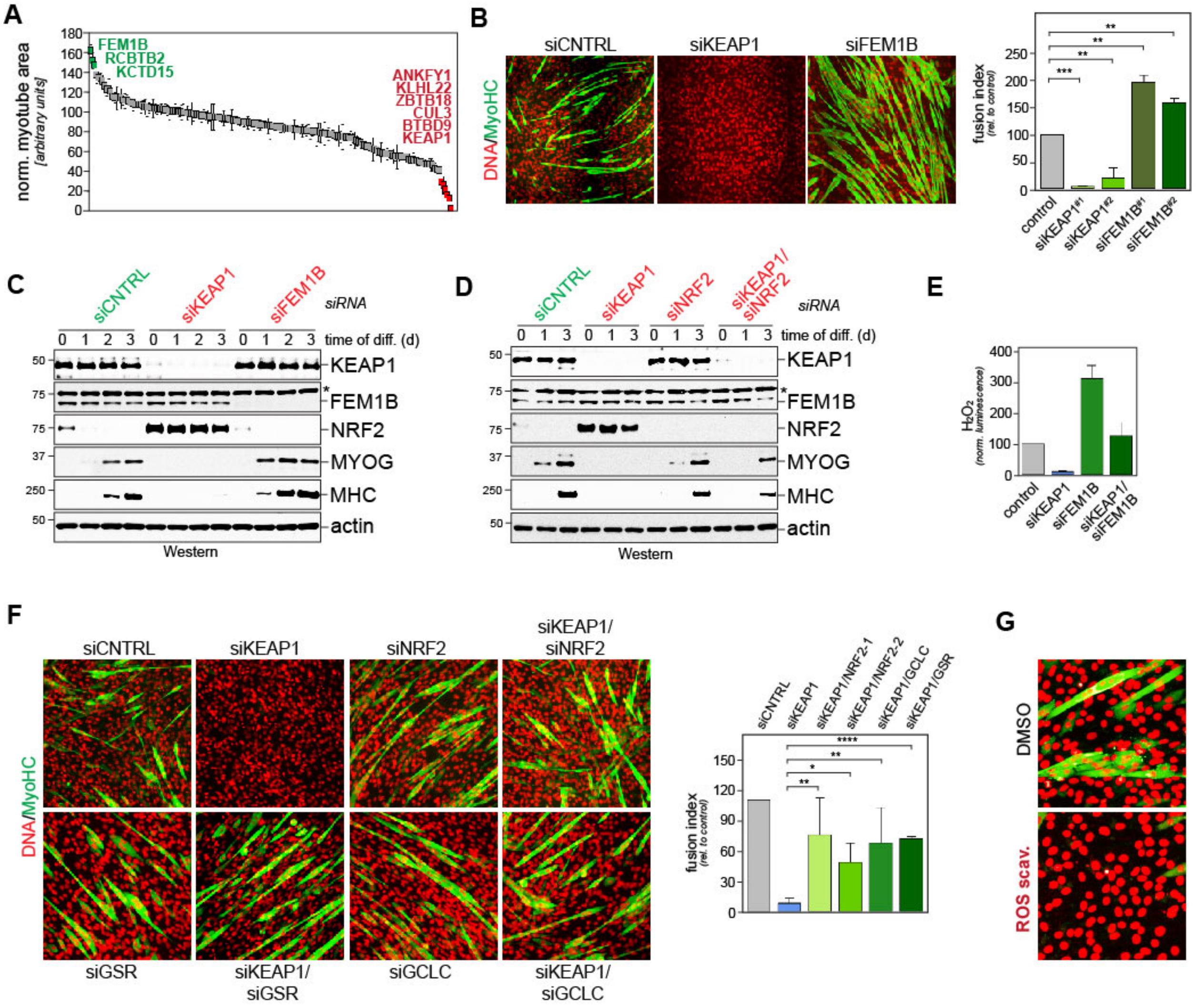


FIGURE 1

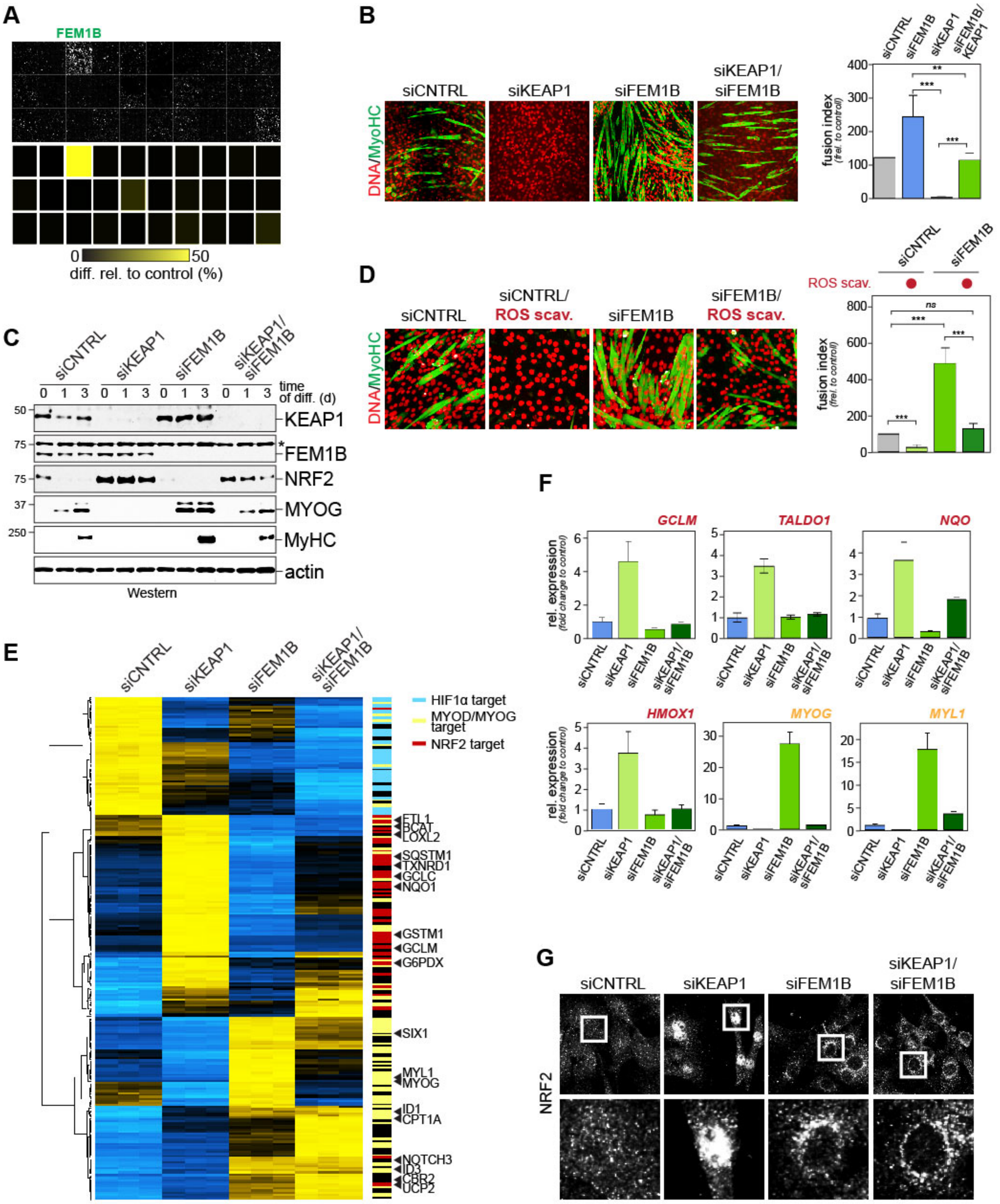

A
FEM1B
0
50
diff. rel. to control (%)
B
siCNTRL
siKEAP1
siFEM1B
siKEAP1/
siFEM1B
DNA/MyoHC
siCNTRL
siFEM1B
siKEAP1
siFEM1B/
KEAP1
fusion index
(frel. to control)
400
300
200
100
0
**
***
***
C
siCNTRL
siKEAP1
siFEM1B
siKEAP1/
siFEM1B
0 1 3 0 1 3 0 1 3 0 1 3
time
of diff. (d)
50
75
75
37
250
KEAP1
*
FEM1B
NRF2
MYOG
MyHC
actin
Western
D
siCNTRL
siCNTRL/
ROS scav.
siFEM1B
siFEM1B/
ROS scav.
DNA/MyoHC
siCNTRL
siFEM1B
ROS scav.
fusion index
(frel. to control)
800
600
400
200
0
ns
***
***
***
E
siCNTRL
siKEAP1
siFEM1B
siKEAP1/
siFEM1B
HIF1α target
MYOD/MYOG
target
NRF2 target
FTL1
BCAT
LOXL2
SQSTM1
TXNRD1
GCLC
NQO1
GSTM1
GCLM
G6PDX
SIX1
MYL1
MYOG
ID1
CPT1A
NOTCH3
ID3
CBR2
UCP2
F
GCLM
TALDO1
NQO
rel. expression
(fold change to control)
6
4
2
0
5
4
3
2
1
0
4
3
2
1
0
siCNTRL
siKEAP1
siFEM1B
siKEAP1/
siFEM1B
HMOX1
MYOG
MYL1
rel. expression
(fold change to control)
5
4
3
2
1
0
30
20
10
0
20
15
10
5
0
G
siCNTRL
siKEAP1
siFEM1B
siKEAP1/
siFEM1B
NRF2

FIGURE 2

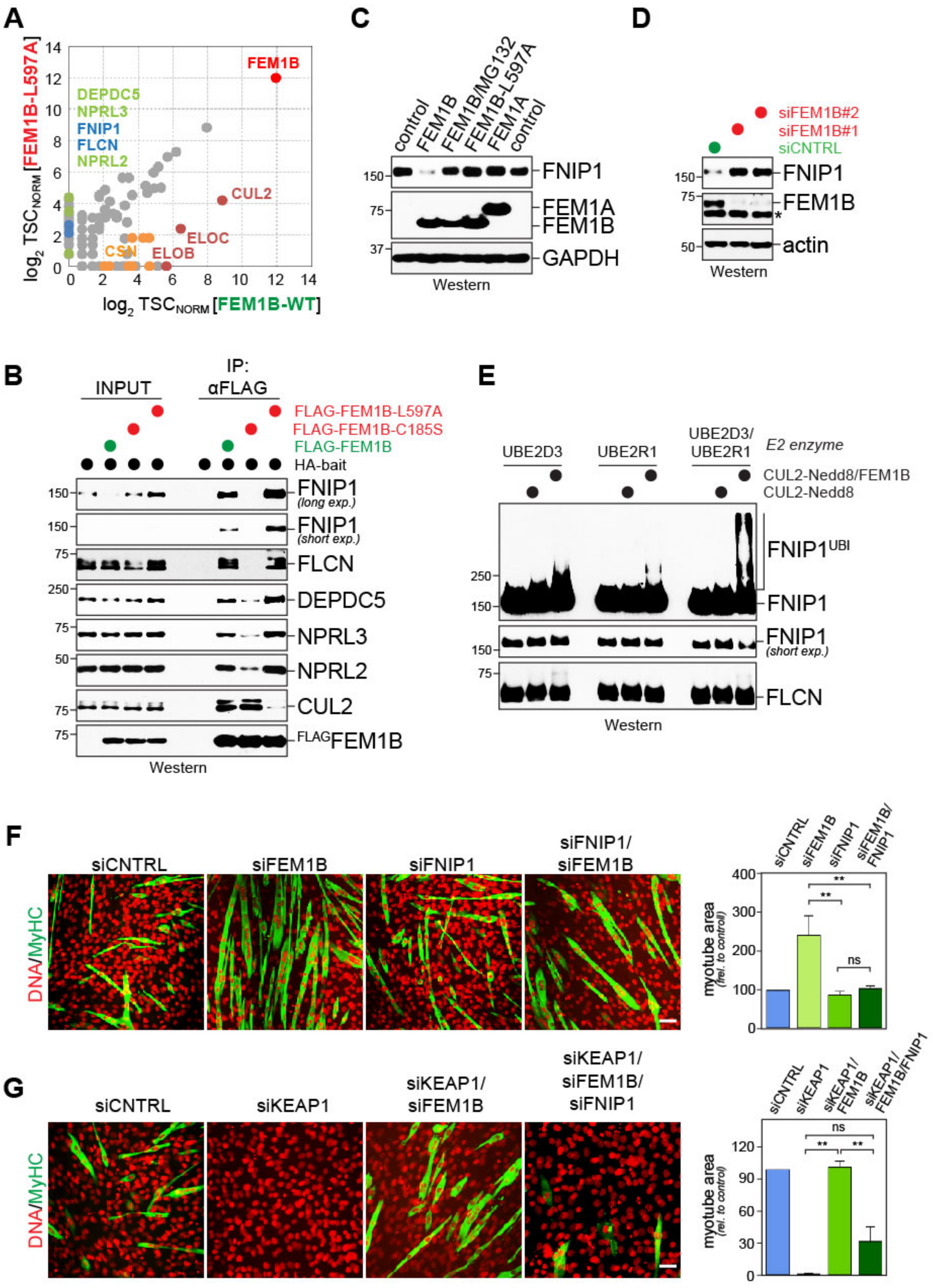

A
log2 TSCNORM [FEM1B-L597A]
log2 TSCNORM [FEM1B-WT]
FEM1B
DEPDC5
NPRL3
FNIP1
FLCN
NPRL2
CUL2
ELOC
ELOB
CSN
0
2
4
6
8
10
12
14
B
INPUT
IP: αFLAG
FLAG-FEM1B-L597A
FLAG-FEM1B-C185S
FLAG-FEM1B
HA-bait
FNIP1 (long exp.)
FNIP1 (short exp.)
FLCN
DEPDC5
NPRL3
NPRL2
CUL2
FLAGFEM1B
150
75
250
50
Western
C
control
FEM1B
FEM1B/MG132
FEM1B-L597A
FEM1A
control
FNIP1
FEM1A
FEM1B
GAPDH
150
75
37
Western
D
siFEM1B#2
siFEM1B#1
siCNTRL
FNIP1
FEM1B
actin
*
150
75
50
Western
E
UBE2D3
UBE2R1
UBE2D3/ UBE2R1
E2 enzyme
CUL2-Nedd8/FEM1B
CUL2-Nedd8
FNIP1UBI
FNIP1
FNIP1 (short exp.)
FLCN
250
150
75
Western
F
siCNTRL
siFEM1B
siFNIP1
siFNIP1/ siFEM1B
DNA/MyHC
siCNTRL
siFEM1B
siFNIP1
siFEM1B/ FNIP1
myotube area (rel. to control)
400
300
200
100
0
**
**
ns
G
siCNTRL
siKEAP1
siKEAP1/ siFEM1B
siKEAP1/ siFEM1B/ siFNIP1
DNA/MyHC
siCNTRL
siKEAP1
siKEAP1/ FEM1B
siKEAP1/ FEM1B/FNIP1
myotube area (rel. to control)
120
90
60
30
0
ns
**
**

FIGURE 3

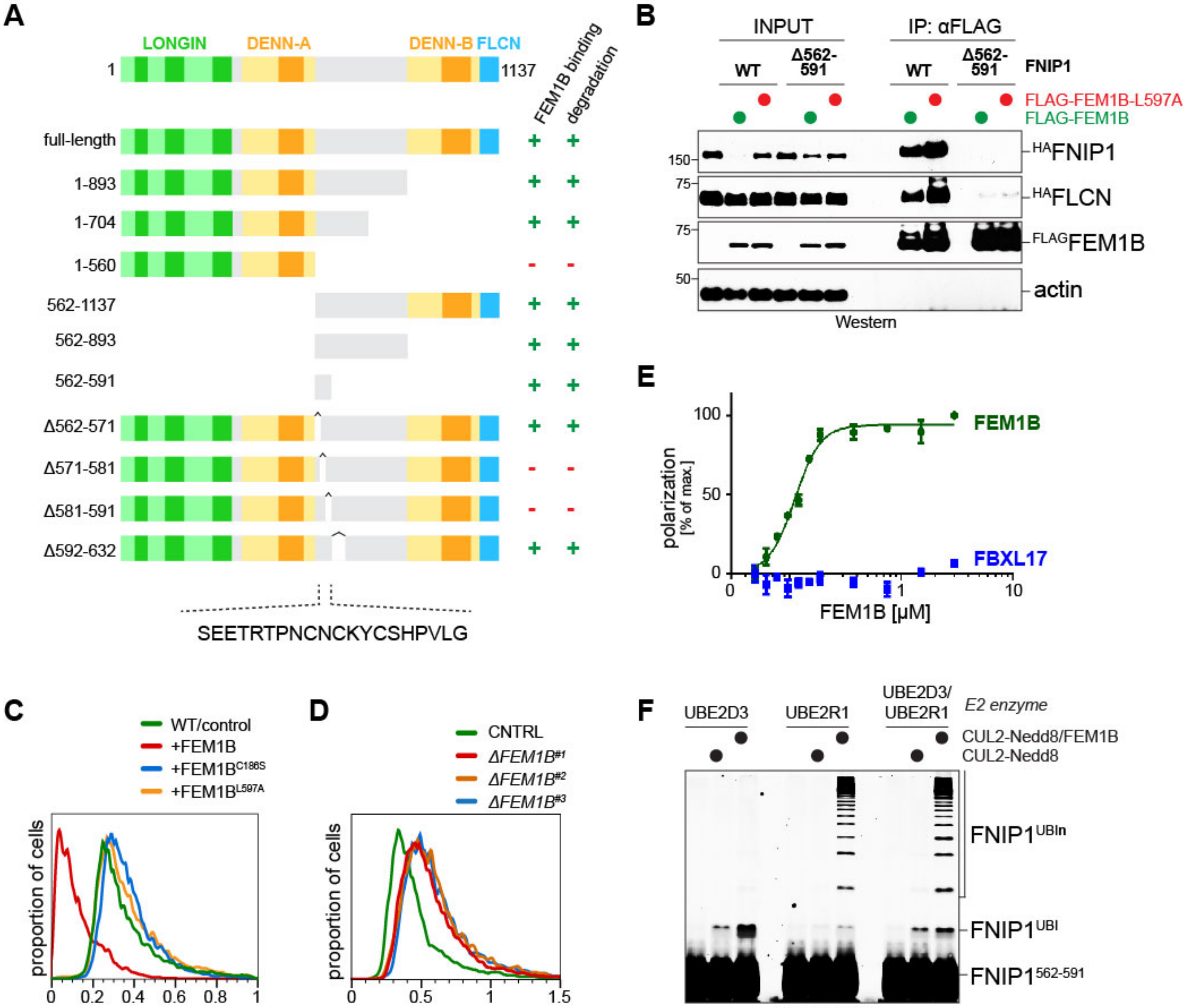

A
LONGIN
DENN-A
DENN-B
FLCN
1
1137
FEM1B binding
degradation
full-length
1-893
1-704
1-560
562-1137
562-893
562-591
Δ562-571
Δ571-581
Δ581-591
Δ592-632
SEETRTPNCNCKYCSHPVLG
B
INPUT
IP: αFLAG
WT
Δ562-591
FNIP1
FLAG-FEM1B-L597A
FLAG-FEM1B
150
75
75
50
HAFNIP1
HAFLCN
FLAGFEM1B
actin
Western
C
WT/control
+FEM1B
+FEM1BC186S
+FEM1BL597A
proportion of cells
0
0.2
0.4
0.6
0.8
1
GFP/mCherry ratio
D
CNTRL
ΔFEM1B#1
ΔFEM1B#2
ΔFEM1B#3
proportion of cells
0
0.5
1
1.5
GFP/mCherry ratio
E
polarization [% of max.]
100
50
0
0
1
10
FEM1B [μM]
FEM1B
FBXL17
F
UBE2D3
UBE2R1
UBE2D3/UBE2R1
E2 enzyme
CUL2-Nedd8/FEM1B
CUL2-Nedd8
FNIP1UBIn
FNIP1UBI
FNIP1562-591
fluorescence

FIGURE 4

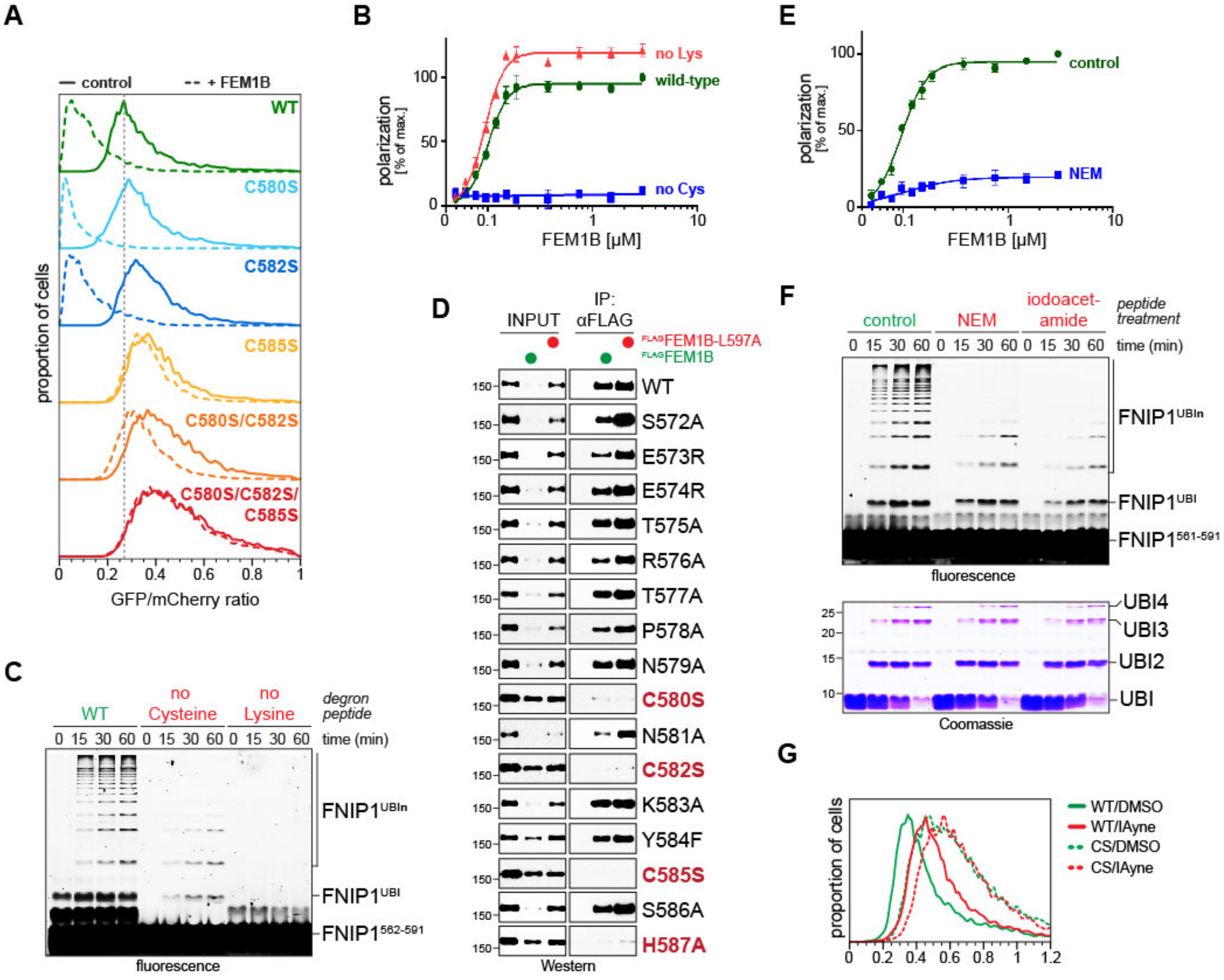

A
— control
- - + FEM1B
WT
C580S
C582S
C585S
C580S/C582S
C580S/C582S/
C585S
proportion of cells
0
0.2
0.4
0.6
0.8
1
GFP/mCherry ratio
B
polarization
[% of max.]
no Lys
wild-type
no Cys
100
50
0
0
0.1
1
10
FEM1B [µM]
E
polarization
[% of max.]
control
NEM
100
50
0
0
0.1
1
10
FEM1B [µM]
C
WT
no
Cysteine
no
Lysine
degron
peptide
0 15 30 60 0 15 30 60 0 15 30 60 time (min)
FNIP1UBIn
FNIP1UBI
FNIP1562-591
fluorescence
D
INPUT
IP:
αFLAG
FLAGFEM1B-L597A
FLAGFEM1B
150
WT
S572A
E573R
E574R
T575A
R576A
T577A
P578A
N579A
C580S
N581A
C582S
K583A
Y584F
C585S
S586A
H587A
Western
F
control
NEM
iodoacet-
amide
peptide
treatment
0 15 30 60 0 15 30 60 0 15 30 60 time (min)
FNIP1UBIn
FNIP1UBI
FNIP1561-591
fluorescence
25
20
15
10
UBI4
UBI3
UBI2
UBI
Coomassie
G
proportion of cells
WT/DMSO
WT/IAyne
CS/DMSO
CS/IAyne
0
0.2
0.4
0.6
0.8
1
1.2

FIGURE 5

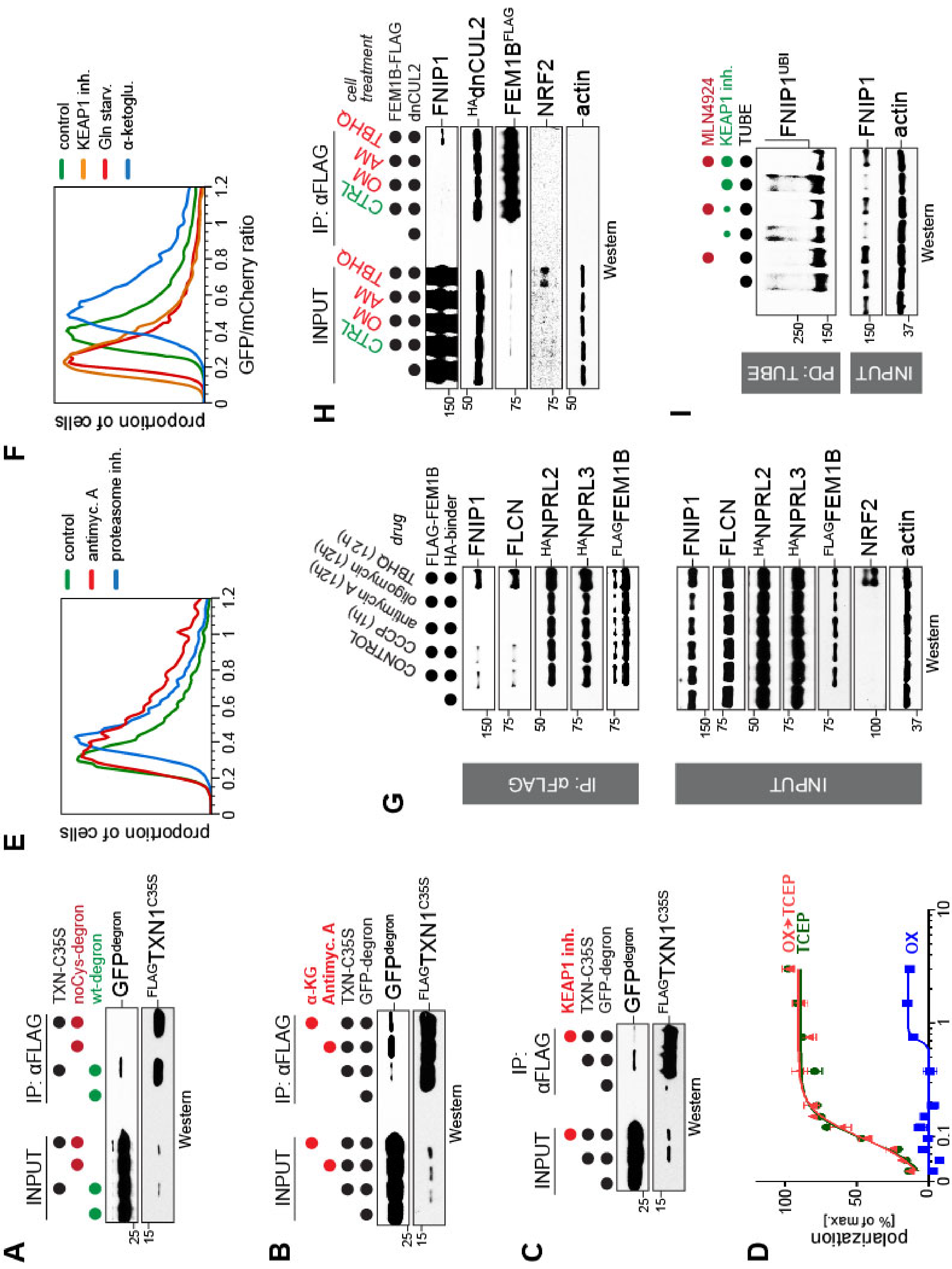
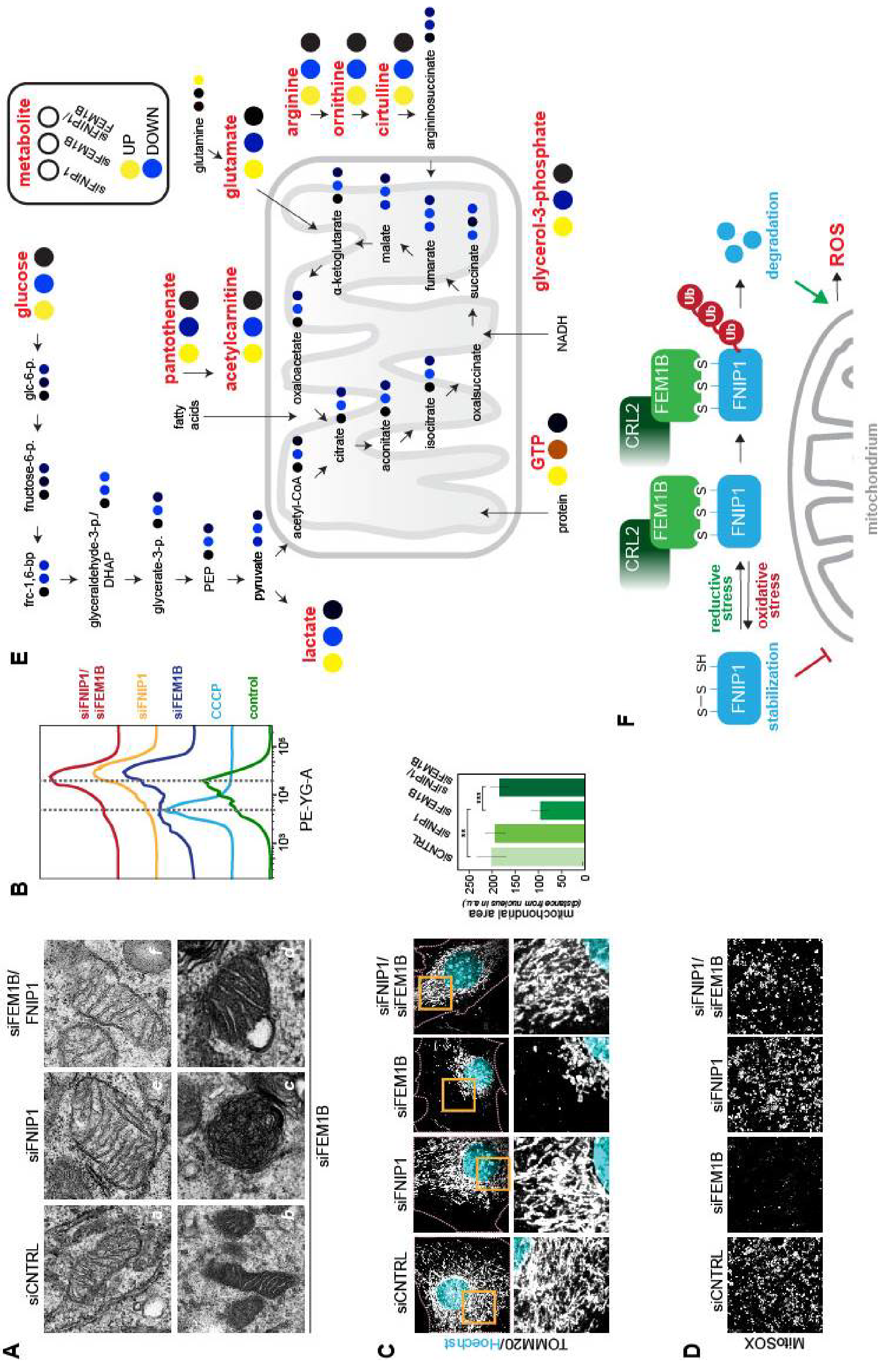

A
INPUT
IP: αFLAG
TXN-C35S
noCys-degron
wt-degron
GFP^degron
FLAG TXN1^C35S
25
15
Western
B
α-KG
Antimyc. A
TXN-C35S
GFP-degron
C
KEAP1 inh.
D
polarization [% of max.]
100
50
0
OX→TCEP
TCEP
OX
0.1
1
10
E
proportion of cells
control
antimyc. A
proteasome inh.
F
GFP/mCherry ratio
control
KEAP1 inh.
Gln starv.
α-ketoglu.
0
0.2
0.4
0.6
0.8
1
1.2
G
drug
CONTROL
CCCP (1h)
antimycin A (12h)
oligomycin (12h)
TBHQ (12 h)
FLAG-FEM1B
HA-binder
IP: αFLAG
INPUT
FNIP1
FLCN
HA NPRL2
HA NPRL3
FLAG FEM1B
NRF2
actin
150
75
50
100
37
H
cell treatment
CTRL
OM
AM
TBHQ
FEM1B-FLAG
dnCUL2
HA dnCUL2
FEM1B^FLAG
I
MLN4924
KEAP1 inh.
TUBE
FNIP1^UBI
PD: TUBE
250
Western

FIGURE 6



A
siCNTRL
siFNIP1
siFEM1B/
FNIP1
siFEM1B
a
b
c
d
e
f
B
PE-YG-A
siFNIP1/
siFEM1B
siFNIP1
siFEM1B
CCCP
control
C
TOMM20/Hoechst
siCNTRL
siFNIP1
siFEM1B
siFNIP1/
siFEM1B
mitochondial area
(distance from nucleus in a.u.)
D
MitoSOX
E
metabolite
siFNIP1
siFEM1B
siFNIP1/
FEM1B
UP
DOWN
glucose
glc-6-p.
fructose-6-p.
frc-1,6-bp
glyceraldehyde-3-p./
DHAP
glycerate-3-p.
PEP
pyruvate
lactate
fatty
acids
pantothenate
acetylcarnitine
acetyl-CoA
citrate
aconitate
isocitrate
oxalsuccinate
oxaloacetate
α-ketoglutarate
malate
fumarate
succinate
NADH
protein
GTP
glycerol-3-phosphate
glutamine
glutamate
arginine
ornithine
cirtulline
argininosuccinate
F
S–S
SH
FNIP1
stabilization
reductive
stress
oxidative
stress
CRL2
FEM1B
S
Ub
degradation
ROS
mitochondrium

FIGURE 7

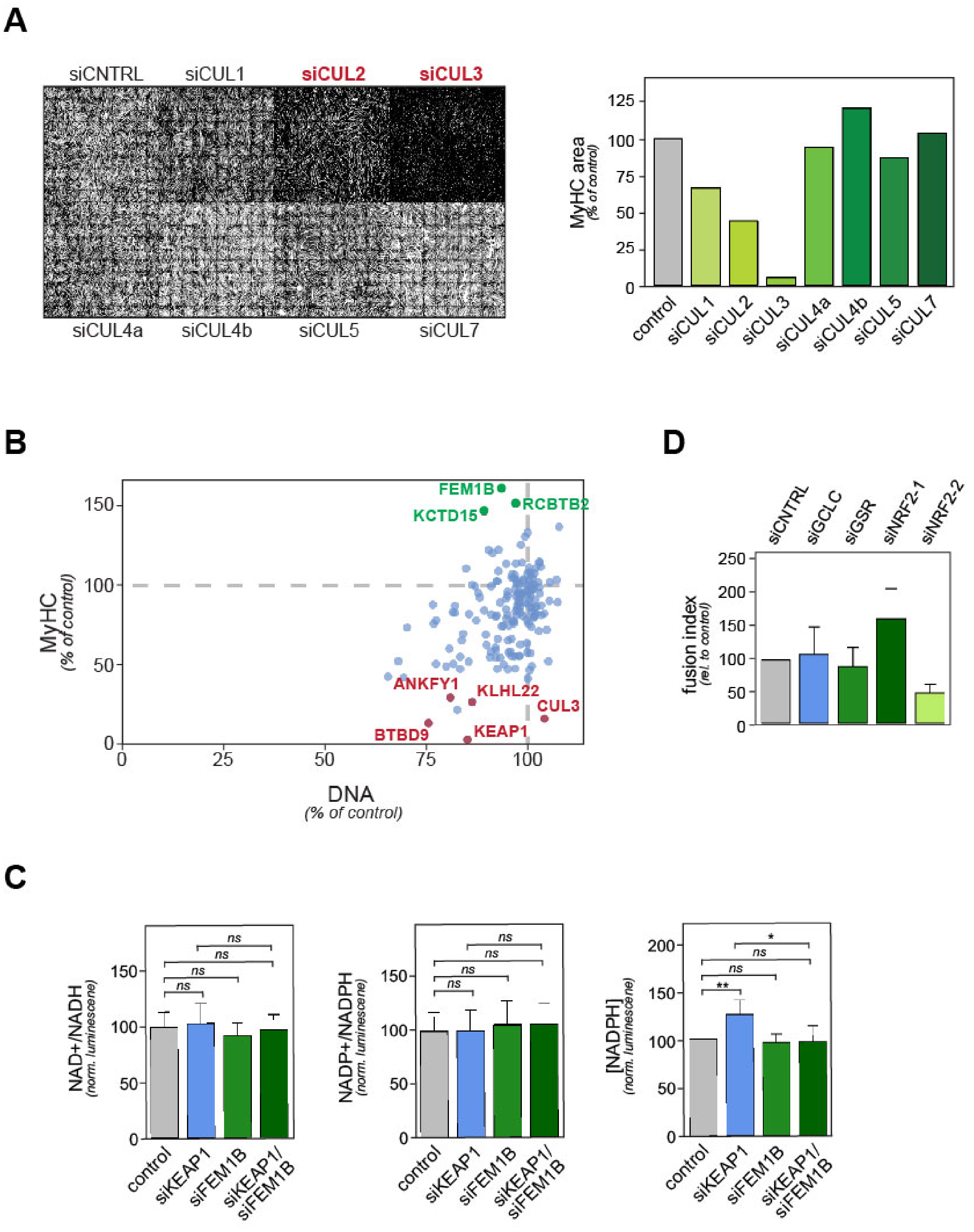

A
siCNTRL
siCUL1
siCUL2
siCUL3
siCUL4a
siCUL4b
siCUL5
siCUL7
MyHC area (% of control)
125
100
75
50
25
0
control
siCUL1
siCUL2
siCUL3
siCUL4a
siCUL4b
siCUL5
siCUL7
B
MyHC (% of control)
150
100
50
0
FEM1B
RCBTB2
KCTD15
ANKFY1
KLHL22
CUL3
BTBD9
KEAP1
0
25
50
75
100
DNA (% of control)
C
NAD+/NADH (norm. luminescene)
NADP+/NADPH (norm. luminescene)
[NADPH] (norm. luminescene)
ns
**
*
200
150
100
50
0
control
siKEAP1
siFEM1B
siKEAP1/siFEM1B
D
siCNTRL
siGCLC
siGSR
siNRF2-1
siNRF2-2
fusion index (rel. to control)
250
200
150
100
50
0

Figure S1

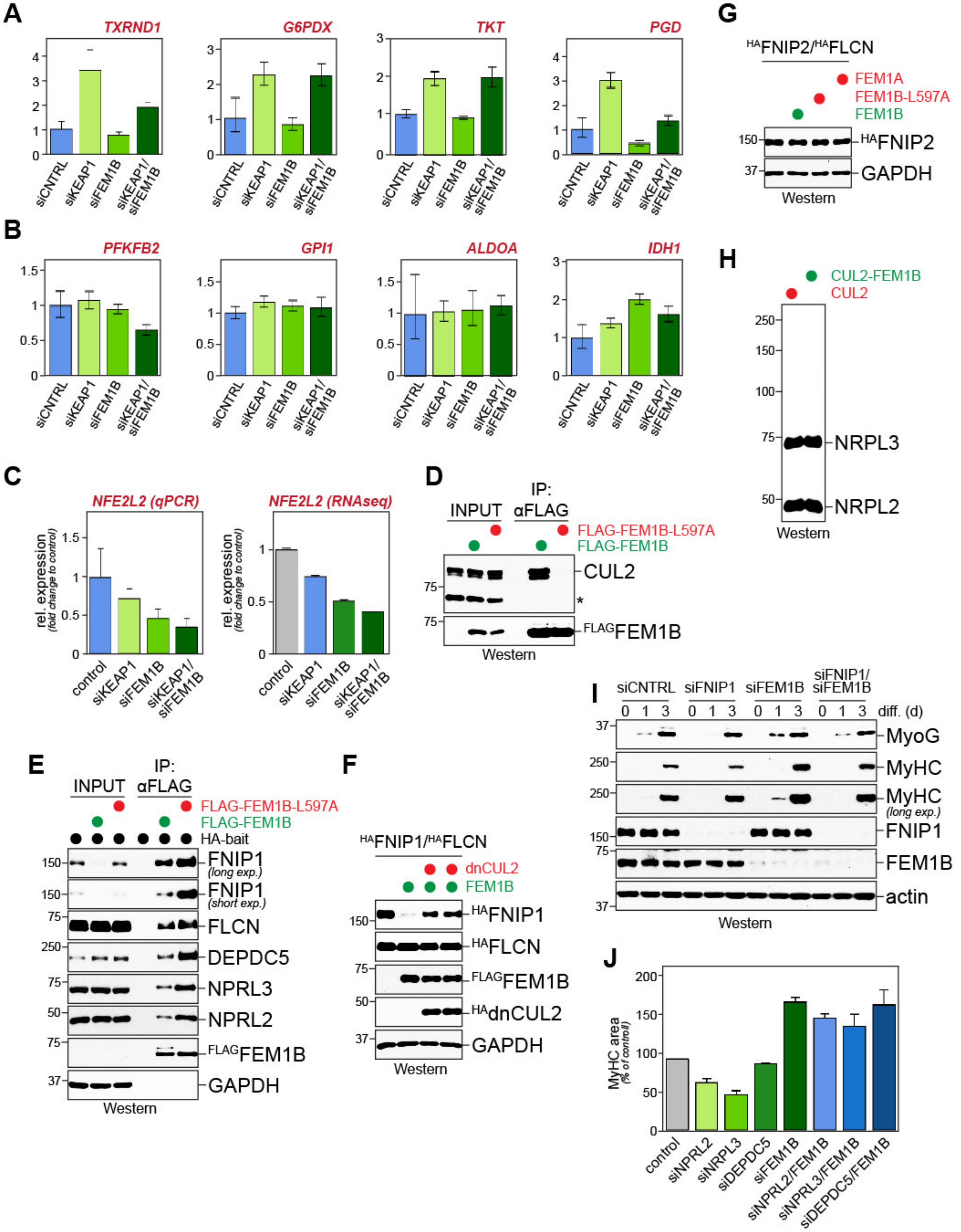
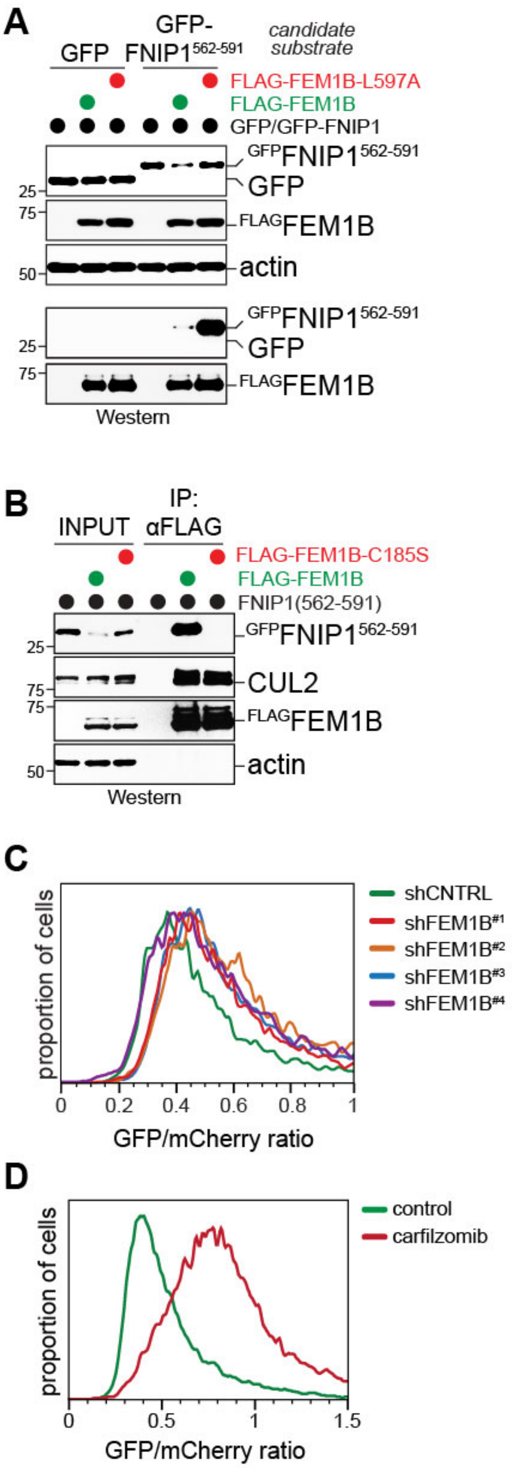

A
TXRND1
G6PDX
TKT
PGD
siCNTRL
siKEAP1
siFEM1B
siKEAP1/siFEM1B
B
PFKFB2
GPI1
ALDOA
IDH1
C
NFE2L2 (qPCR)
NFE2L2 (RNAseq)
rel. expression
(fold change to control)
control
D
INPUT
IP: αFLAG
FLAG-FEM1B-L597A
FLAG-FEM1B
CUL2
*
FLAGFEM1B
Western
E
HA-bait
FNIP1 (long exp.)
FNIP1 (short exp.)
FLCN
DEPDC5
NPRL3
NPRL2
GAPDH
F
HAFNIP1/HAFLCN
dnCUL2
FEM1B
HAFNIP1
HAFLCN
HAdnCUL2
G
HAFNIP2/HAFLCN
FEM1A
FEM1B-L597A
HAFNIP2
H
CUL2-FEM1B
CUL2
NRPL3
NRPL2
I
siFNIP1
siFNIP1/siFEM1B
diff. (d)
MyoG
MyHC
MyHC (long exp.)
FNIP1
FEM1B
actin
J
MyHC area (% of control)
siNPRL2
siNPRL3
siDEPDC5
siNPRL2/FEM1B
siNPRL3/FEM1B
siDEPDC5/FEM1B

FIGURE S2



E

Mouse	SEETRTPNCNCKYCSHPVLG
Hamster	SEETRTPNCNCKYCSHPLLG
American beaver	SEETRTPNCNCKYCTHPLLG
Mongolian gerbil	SEEIRTPNCNCKYCSHPLLG
Gorilla	SEEIRTPNCNCKYCSHPLLG
Homo sapiens	SEEIRTPNCNCKYCSHPLLG
Ground squirrel	SEETRTSNCNCKYCSHPLLG
Cow	SEETRTPNCNCKYCSRPLLG
Horse	SEETRTPNCNCKYCSRPLLG
Chinchilla	SEETKTPHCNCKYCSHPLLG
Cat	-EETRTPNCNCKYCSRPLLG
Dog	-EETRTPNCNCKYCSRPLLG
Macaca	SEEIRTPSCNCKYCSHPLLG
Chinese hamster	SEEIRTPNCNCKYCSHPLLG
Microtus ochrogaster	SEETRTPSCNCKYCSHPLLG
Meriones unguiculatus	SEEIRTPNCNCKYCSHPLLG
Myotis davidii	SEETRTPNCNCKYCSHPLFG
Ictidomys tridecemlineatus	SEETRTSNCNCKYCSHPLLG
Urocitellus parryii	SEETRTSNCNCKYCSHPLLG
Myotis lucifugus	SEETRTPNCNCKYCSHPLFG
Pteropus Alecto	SEEMRTPNCNCKYCSHPLLG
Equus przewalskii	SEETRTPNCNCKYCSRPLLG
Tupaia chinensis	SEETRTPNCNCKYCSRPLLG
Hipposideros armiger	SEETRTPNCNCKYCSRPLLG
Camelus ferus	SEETRTPNCNCKYCSPPLLG
Chinchilla lanigera	SEETKTPHCNCKYCSHPLLG
Rhinopithecus bieti	SEEIRTPSCNCKYCSHPLLG
Macaca fascicularis	SEEIRTPSCNCKYCSHPLLG
Tursiops truncates	SEETRTPNCSCKYCSRPLLG
Propithecus coquereli	SEETRTPNCNCKYCGRPLLG
Marmota monax	SKETRTSNCNCKYCSHPLLG
Miniopterus natalensis	SEETRTPNCNCKYCSPALLG
Octodon degus	SEETKAPHCNCKYCSHPLLG
Rattus norvegicus	SEETGTSNCNCKYCSHPLLG
Heterocephalus glaber	SEETKTPHCNCKYCIHPLLG
Cavia porcellus	SEETKIPHCNCKYCNHPLLG
Oryctolagus cuniculus	SEETRTPNCGCKYCTRPLVG
Chelonia mydas	SEEMRTPNCSCKYCKCPLLG
Python bivittatus	SEEVRTSDCSCKYCKCPV
Chaetura pelagica	SEEVRTSNCSCRYCKCPV
Notechis scutatus	SEEVRTSDCGCKYCRCPV
Cyanistes caeruleus	SEEMRVSNCSCKYCKCPV
Protobothrops mucrosquamatus	SEELRTSDCSCKHCKCPV
consensus	**E.(R/K)...C.C(R/K)YC..**

F

```
FNIP1 (mouse)      SEETRTPNCNCKYCSHPVLG
FNIP2 (mouse)      TAERRSPEPTVV--AEISEG

consensus          : * *:*:  .   :.   *
```

FIGURE S3

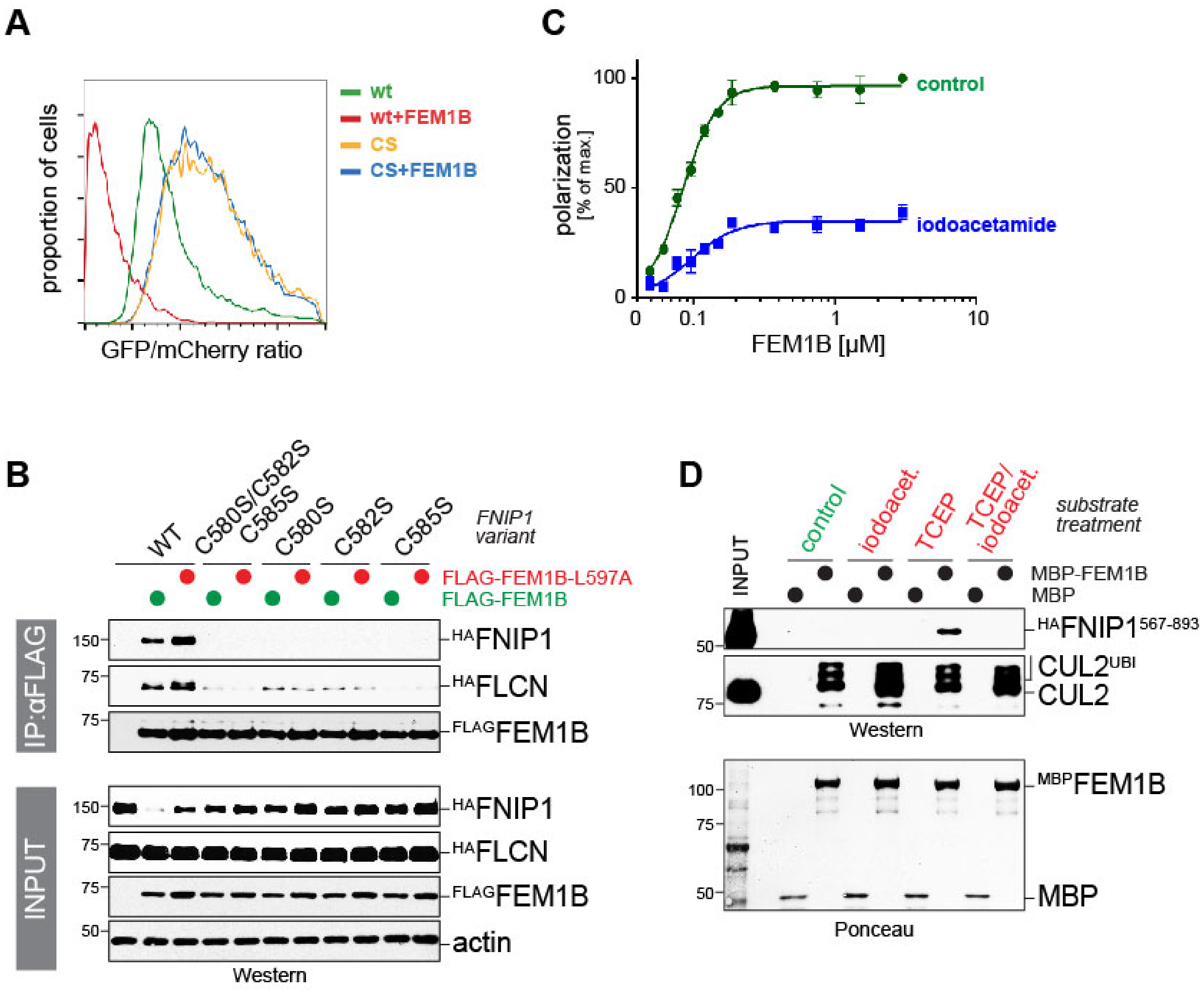

A
proportion of cells
GFP/mCherry ratio
wt
wt+FEM1B
CS
CS+FEM1B
B
WT
C580S/C582S
C585S
C580S
C582S
C585S
FNIP1 variant
FLAG-FEM1B-L597A
FLAG-FEM1B
IP:αFLAG
150
75
75
HAFNIP1
HAFLCN
FLAGFEM1B
INPUT
150
75
75
50
HAFNIP1
HAFLCN
FLAGFEM1B
actin
Western
C
polarization [% of max.]
100
50
0
0
0.1
1
10
FEM1B [µM]
control
iodoacetamide
D
INPUT
control
iodoacet.
TCEP
TCEP/ iodoacet.
substrate treatment
MBP-FEM1B
MBP
50
75
HAFNIP1567-893
CUL2UBI
CUL2
Western
100
75
50
MBPFEM1B
MBP
Ponceau

FIGURE S4

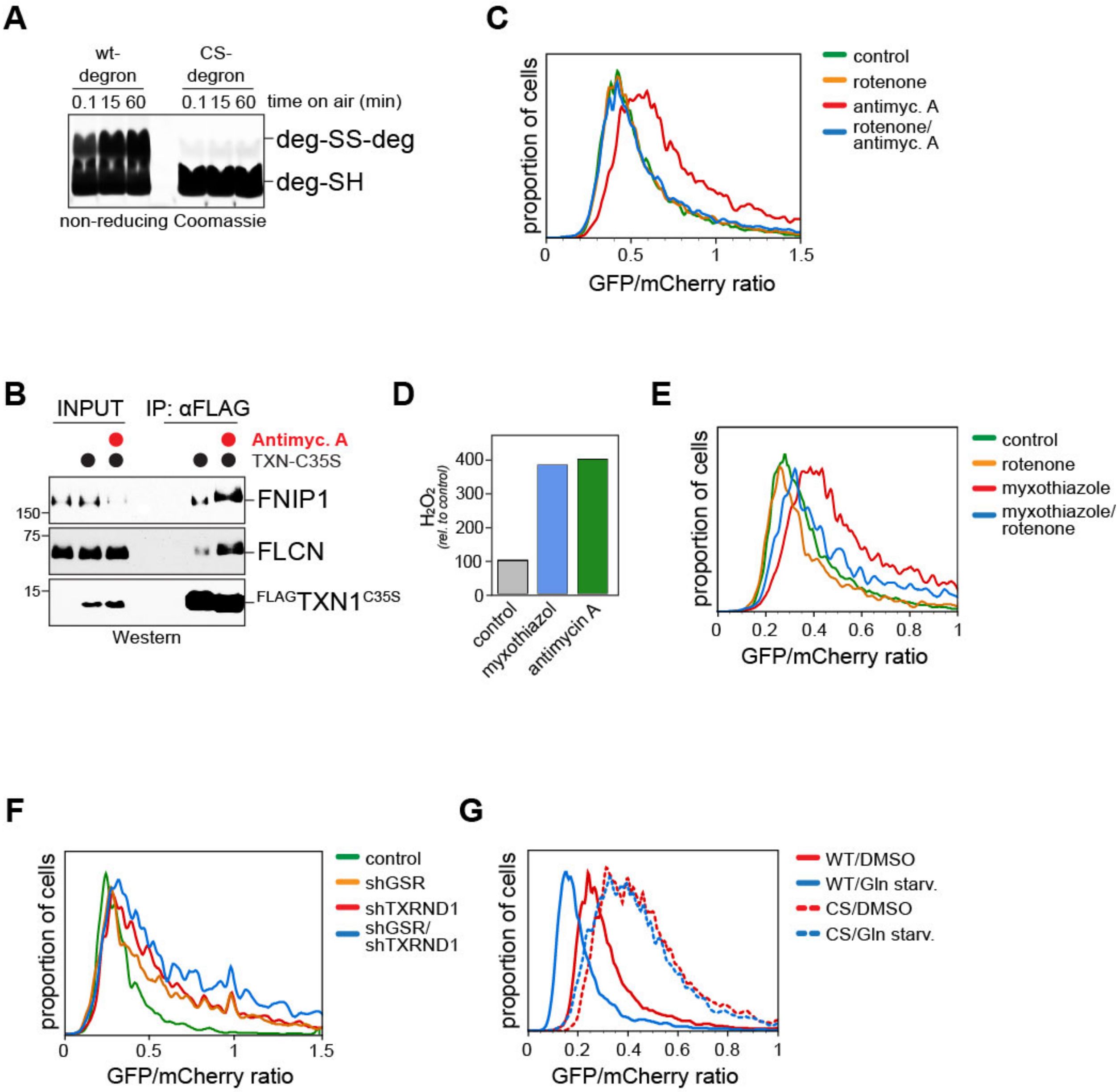

A
wt-degron
CS-degron
0.1 15 60
0.1 15 60
time on air (min)
deg-SS-deg
deg-SH
non-reducing Coomassie
B
INPUT
IP: αFLAG
Antimyc. A
TXN-C35S
150
75
15
FNIP1
FLCN
^{FLAG}TXN1^{C35S}
Western
C
proportion of cells
GFP/mCherry ratio
0
0.5
1
1.5
control
rotenone
antimyc. A
rotenone/antimyc. A
D
H_2O_2 (rel. to control)
0
100
200
300
400
control
myxothiazol
antimycin A
E
proportion of cells
GFP/mCherry ratio
0
0.2
0.4
0.6
0.8
1
control
rotenone
myxothiazole
myxothiazole/rotenone
F
proportion of cells
GFP/mCherry ratio
0
0.5
1
1.5
control
shGSR
shTXRND1
shGSR/shTXRND1
G
proportion of cells
GFP/mCherry ratio
0
0.2
0.4
0.6
0.8
1
WT/DMSO
WT/Gln starv.
CS/DMSO
CS/Gln starv.

FIGURE S5

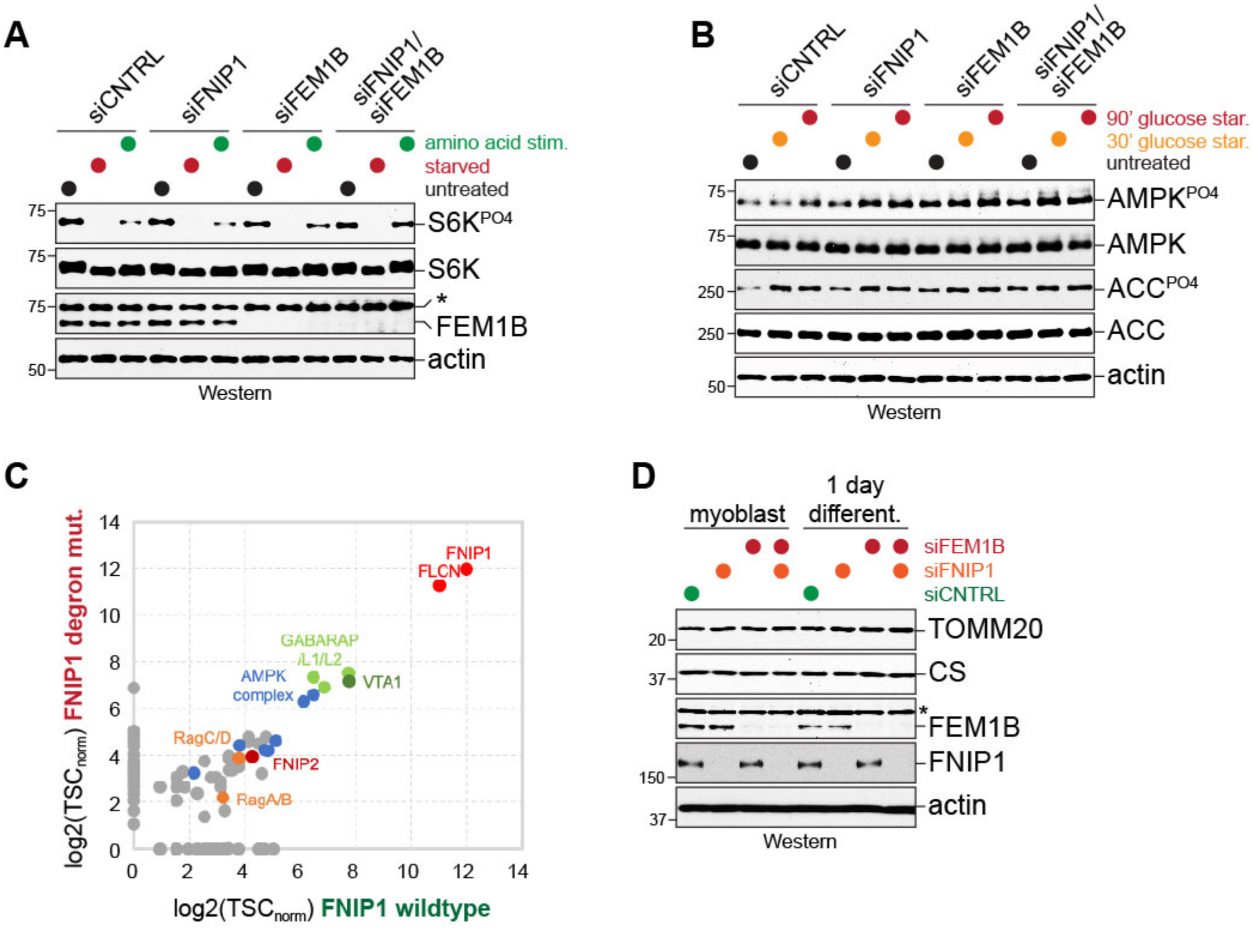
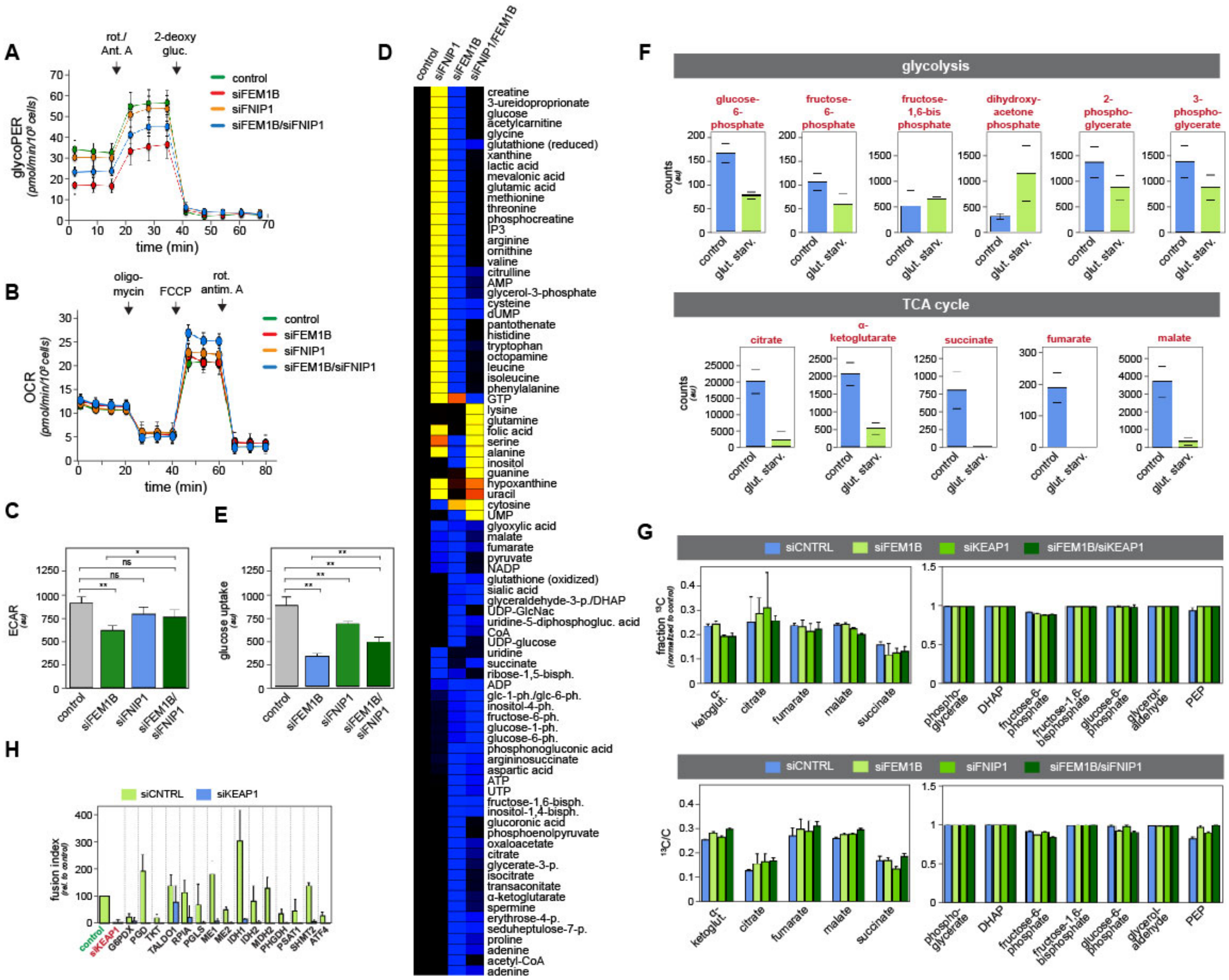

A
siCNTRL
siFNIP1
siFEM1B
siFNIP1/ siFEM1B
amino acid stim.
starved
untreated
75
S6K^{PO4}
S6K
*
FEM1B
50
actin
Western
B
90' glucose star.
30' glucose star.
AMPK^{PO4}
AMPK
250
ACC^{PO4}
ACC
C
log2(TSC$_{norm}$) FNIP1 degron mut.
log2(TSC$_{norm}$) FNIP1 wildtype
0
2
4
6
8
10
12
14
FNIP1
FLCN
GABARAP /L1/L2
VTA1
AMPK complex
RagC/D
FNIP2
RagA/B
D
myoblast
1 day different.
siFEM1B
siFNIP1
siCNTRL
TOMM20
20
CS
37
150
FNIP1

FIGURE S6



A
rot./ Ant. A
2-deoxy gluc.
control
siFEM1B
siFNIP1
siFEM1B/siFNIP1
glycoPER (pmol/min/10³ cells)
time (min)
B
oligo-mycin
FCCP
rot. antim. A
OCR (pmol/min/10³ cells)
time (min)
C
ECAR (au)
control
siFEM1B
siFNIP1
siFEM1B/siFNIP1
**
ns
ns
*
E
glucose uptake (au)
H
siCNTRL
siKEAP1
fusion index (rel. to control)
control
siKEAP1
G6PDX
PGD
TKT
TALDO1
RPIA
PGLS
ME1
ME2
IDH1
IDH2
MDH2
PHGDH
PSAT1
SHMT2
ATF4
D
control
siFNIP1
siFEM1B
siFNIP1/FEM1B
creatine
3-ureidoproprionate
glucose
acetylcarnitine
glycine
glutathione (reduced)
xanthine
lactic acid
mevalonic acid
glutamic acid
methionine
threonine
phosphocreatine
IP3
arginine
ornithine
valine
citrulline
AMP
glycerol-3-phosphate
cysteine
dUMP
pantothenate
histidine
tryptophan
octopamine
leucine
isoleucine
phenylalanine
GTP
lysine
glutamine
folic acid
serine
alanine
inositol
guanine
hypoxanthine
uracil
cytosine
UMP
glyoxylic acid
malate
fumarate
pyruvate
NADP
glutathione (oxidized)
sialic acid
glyceraldehyde-3-p./DHAP
UDP-GlcNac
uridine-5-diphosphogluc. acid
CoA
UDP-glucose
uridine
succinate
ribose-1,5-bisph.
ADP
glc-1-ph./glc-6-ph.
inositol-4-ph.
fructose-6-ph.
glucose-1-ph.
glucose-6-ph.
phosphonogluconic acid
argininosuccinate
aspartic acid
ATP
UTP
fructose-1,6-bisph.
inositol-1,4-bisph.
glucoronic acid
phosphoenolpyruvate
oxaloacetate
citrate
glycerate-3-p.
isocitrate
transaconitate
α-ketoglutarate
spermine
erythrose-4-p.
seduheptulose-7-p.
proline
adenine
acetyl-CoA
adenine
F
glycolysis
glucose-6-phosphate
fructose-6-phosphate
fructose-1,6-bis phosphate
dihydroxy-acetone phosphate
2-phospho-glycerate
3-phospho-glycerate
counts (au)
control
glut. starv.
TCA cycle
citrate
α-ketoglutarate
succinate
fumarate
malate
G
siCNTRL
siFEM1B
siKEAP1
siFEM1B/siKEAP1
fraction ¹³C (normalized to control)
α-ketoglut.
citrate
fumarate
malate
succinate
phospho-glycerate
DHAP
fructose-6-phosphate
fructose-1,6-bisphosphate
glucose-6-phosphate
glycerol-aldehyde
PEP
siCNTRL
siFEM1B
siFNIP1
siFEM1B/siFNIP1
¹³C/C

FIGURE S7

3.8 Tables

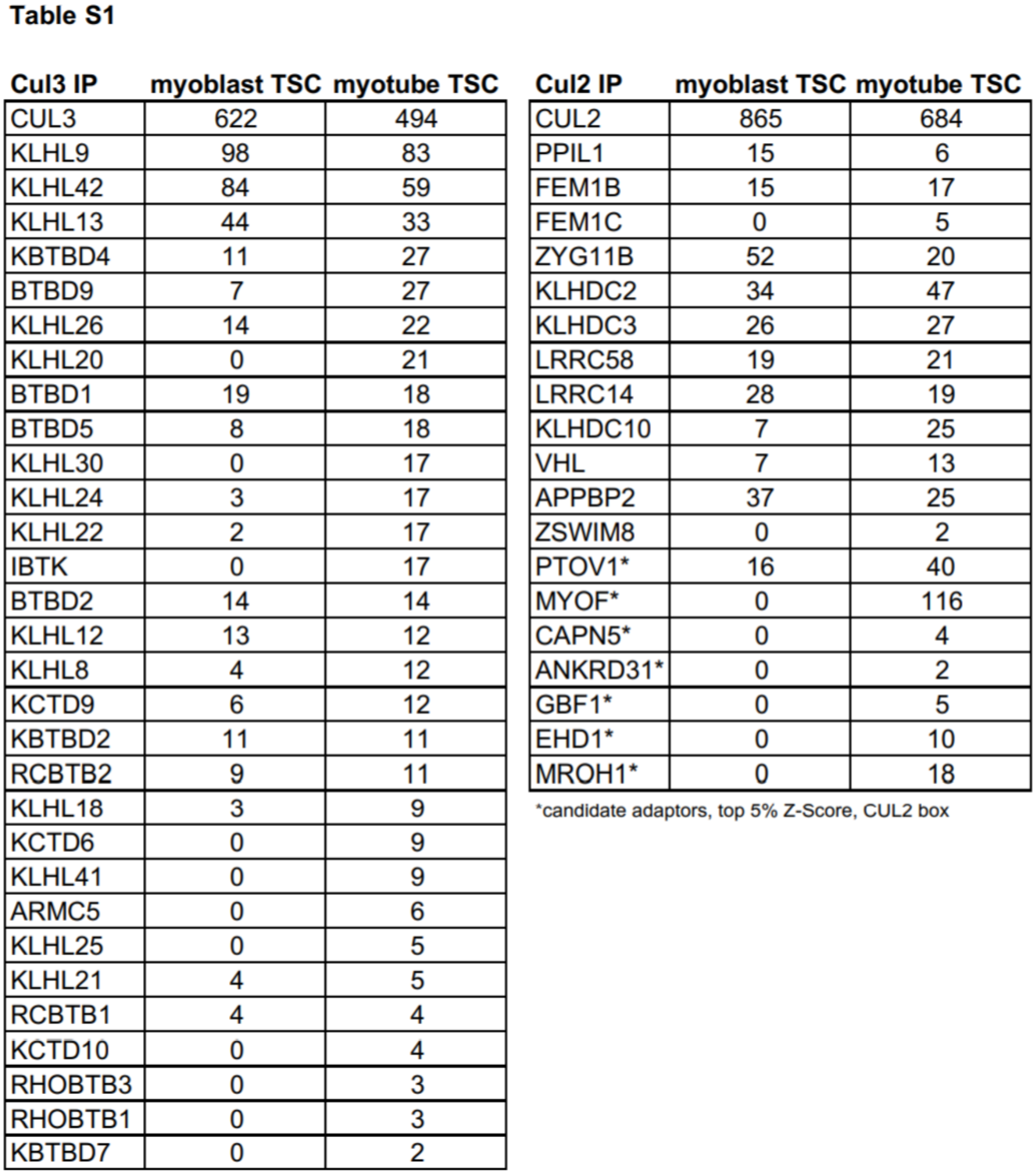


Table S1

Cul3 IP	myoblast TSC	myotube TSC
CUL3	622	494
KLHL9	98	83
KLHL42	84	59
KLHL13	44	33
KBTBD4	11	27
BTBD9	7	27
KLHL26	14	22
KLHL20	0	21
BTBD1	19	18
BTBD5	8	18
KLHL30	0	17
KLHL24	3	17
KLHL22	2	17
IBTK	0	17
BTBD2	14	14
KLHL12	13	12
KLHL8	4	12
KCTD9	6	12
KBTBD2	11	11
RCBTB2	9	11
KLHL18	3	9
KCTD6	0	9
KLHL41	0	9
ARMC5	0	6
KLHL25	0	5
KLHL21	4	5
RCBTB1	4	4
KCTD10	0	4
RHOBTB3	0	3
RHOBTB1	0	3
KBTBD7	0	2

Cul2 IP	myoblast TSC	myotube TSC
CUL2	865	684
PPIL1	15	6
FEM1B	15	17
FEM1C	0	5
ZYG11B	52	20
KLHDC2	34	47
KLHDC3	26	27
LRRC58	19	21
LRRC14	28	19
KLHDC10	7	25
VHL	7	13
APPBP2	37	25
ZSWIM8	0	2
PTOV1*	16	40
MYOF*	0	116
CAPN5*	0	4
ANKRD31*	0	2
GBF1*	0	5
EHD1*	0	10
MROH1*	0	18

*candidate adaptors, top 5% Z-Score, CUL2 box

CHAPTER 4:

Multimerization Quality Control is Critical for Myogenesis and Development

Fernando Rodríguez-Pérez, Chris Padovani, Andrew G. Manford, Predrag Jevtic, Angela Pogson,
Brenda Martínez Gonzales, Michael Rape

In preparation

4.1 Summary

Organismal development relies on the orchestration and precise spatiotemporal execution of multiple signaling pathways. Many of these regulatory cascades use modular multimeric protein complexes that allow for distinct biological functions. Inhibition of complex formation or assembly of aberrant protein complexes can disrupt information flow and impede cellular or organismal survival. Recent work has shown that dimeric BTB-domain containing proteins are actively surveyed by dimerization quality control (DQC), a ubiquitin-dependent process that is critical for neuronal development. We hypothesized that an analogous quality control mechanism must exist for higher-order oligomeric complexes. Here we identified the ubiquitin ligase CUL3-BTBD9 as a key regulator of multimeric protein complex formation. This multimerization quality control (MQC) is paramount for organismal development and survival, as ablation of BTBD9 leads to myogenesis failure and developmental arrest in *Xenopus Laevis* embryos. This work underscores the importance of quality control mechanisms in metazoan development and survival and highlights the role ubiquitin signaling components play in this regulation.

4.2 Introduction

Much like computer systems, organisms rely on the flow of information and precise execution for robust development and survival. Biological systems employ different layers of quality control that ensure that data is written, read, and executed in a faithful and robust manner. Proteins are integral components of biological signaling cascades, regulating several aspects of metazoan development and homeostasis. Many proteins function within multimeric complexes, as has been shown by affinity/mass spectrometry experiments. This complex formation is required for many aspects of cellular biology, conserved throughout evolution from prokaryotes to eukaryotes. Protein oligomerization has been observed to be critical for gene expression (transcription factors), nutrient/ion transport (glucose cannels/ion channels) protein translation (ribosomes) signal transduction in response to stimuli (growth factors), nutrient sensing (mTOR complex), and enzymatic reactions (ubiquitylation), amongst many others, highlighting the importance of protein-protein interactions in organismal survival and development. Given this, it is critical for protein complexes to possess the correct compositions and stoichiometries at the correct times.

To mediate complex formation, proteins use a myriad of protein binding domains. Although some domains can be associated with specific biological processes (CARD and DED domains with apoptosis; the SNARE domain with vesicular fusion; the F-box domain with ubiquitin ligases), many protein-protein interaction domains are not as well understood. Furthermore, what mediates the interactions of these domains, and how protein complexes ensure the correct conformation and composition remains poorly understood. Indeed, oligomerization with the wrong binding partner or at the wrong time can have detrimental effects. This can be exemplified by the causative driver of chronic myeloid leukemia (CML), the BCR-ABL protein fusion. While the wild type ABL1 protein is tightly regulated, the BCR-ABL protein fusion constitutively homodimerizes, leading to a protein kinase that is "always on", leading to CML. Underscoring the importance of quality control mechanisms that survey protein dimerization, Mena et al have shown that dimerization quality control (DQC) is an essential mechanism required for neuronal system formation and organismal development.

Amongst the plethora of domains that drive protein complex formation, the BTB domain is one of great interest. Proteins from different walks of life contain this domain, from E3 ubiquitin ligase substrate adapters of CUL3, transcription factors, ion channels, and GPCRs (Pinkas, et al, 2017; Stogios, et al, 2005; Teng, et al, 2019). Several BTB-containing proteins are known to be critical for metazoan development and homeostasis, such as the oncogene BCL6, and the central protein of Chapter two of this work, KCTD10 (required for heart development in mice and zebrafish (Hu, et al, 2014; Ren, et al, 2014)). Their key role in modulating organismal development and homeostasis highlights the importance in ensuring proper complex formation of these multimeric proteins. *In vivo,* the BTB domain has been shown to drive homomeric, and in some cases heteromeric, functional protein complex formation. Mass spectrometry experiments show that overexpressed BTB proteins heterodimerize with other BTB proteins. However, endogenously most dimeric BTBs homodimerize, indicating an active mechanism by which cells survey BTB dimer composition (Errington, et al, 2012; Pinkas, et al, 2017; Zhuang, et al, 2009). DQC is an ubiquitin-dependent process that surveys the composition of dimeric BTB proteins. At the heart of DQC is the ubiquitin ligase CUL1^{FBXL17}, which has a low affinity towards correctly-formed dimeric proteins but engages and ubiquitylates aberrant dimers (Mena, et al, 2018). Because BTB can form higher-ordered multimers, we hypothesized that a mechanism must exist to regulate them.

Here, we showcase the work we undertook in identifying the CUL3 substrate adapter BTBD9 (a BTB protein itself, hence the name) as a critical regulator of BTB protein complex formation, which we have termed multimerization quality control. Analogous to DQC, we hypothesize that MQC is a ubiquitin-depended process. MQC is essential for development, as ablating BTBD9 inhibits myogenic system development, and prevents embryonic development in *X. laevis*. BTBD9 actively surveys the cellular landscape for aberrant oligomers formation. Failure to detect these misformed protein complexes leads to aggregate formation, causing an overactive autophagy response, inhibiting developmental pathways. Our work showcases the importance of quality control mechanisms in driving metazoan development. We speculate that similar mechanisms must exist for other multimerization platforms.

4.3 Results

4.3.1 BTBD9 is required to enter myogenic differentiation

Myogenesis is a rapid differentiation process that includes dramatic rearrangements of cellular structure (Bentzinger, et al, 2012; Ong, et al, 2002). This robust system must require intricate and precise spatiotemporal control. Indeed, we have shown in previous chapters of these writings that myogenesis is extremely sensitive to perturbations, such as those that prevent cell fusion (Chapter 2), or alter cellular redox homeostasis (Chapter 3). Our screens also found that the CUL3 substrate receptor BTBD9 had a dramatic myogenesis phenotype. Depletion of BTBD9 from myoblasts inhibited differentiation, as evident by the lack of expression of MyHC (Figure 1A, B). BTBD9 depletion also appeared to have a lower nuclei count compared to control, suggesting a slight death phenotype (Figure 1C).

To rule out the possibility that the myogenesis phenotype was due to cell death, we looked at the early differentiation marker myogenin (MYOG). This transcription factor is strongly upregulated after 24 hours of induced differentiation. BTBD9 depletion inhibited the activation of MYOG, suggesting that BTBD9 is required for entry into the myogenic program (Figure 1D). The abolished expression of MYOG and MyHC in the absence of BTBD9 was confirmed by Western blot analysis (Figure 1E). This phenotype was further validated by stably expressing a variant of BTBD9 that cannot be silenced by siRNAs. This functional assay showed that we could rescue the siBTBD9 myogenic phenotype upon re-introduction of this siRNA-resistant form of BTBD9 (Figure 1F).

BTBD9 is a poorly studied, yet highly conserved, protein that is found throughout eumetazoans, from cnidarians to primates (Figure 1G). GWAS studies have linked BTBD9 to restless leg syndrome, but mechanistic and molecular data for this remain lacking (Catoire, et al, 2018; Raizen and Wu, 2011; Yang, et al, 2011). A recent study looking at a consanguineous family possessing a truncated, non-functional, variant of BTBD9, found that individuals suffered from hypotonia (a state of low muscle tone) and succumbed to a severe unexplained myopathy (Maddirevula, et al, 2018). This finding, along with our *in vitro* data, implicate BTBD9 as being a critical component of myogenic development and homeostasis. To better understand the underlying molecular pathway regulated by BTBD9, it was paramount to identify its binding partners and putative ubiquitylation substrates.

4.3.2 CUL3^{BTBD9} targets multimeric BTB domain-containing proteins

BTBD9 is a substrate adapter of CUL3 ubiquitin ligase. Therefore, to elucidate how BTBD9 regulates myogenesis, we wanted to identify its ubiquitylation substrates. We implemented several strategies that have been described in previous chapters of these writings, such as using a variant of BTBD9 that is unable to bind CUL3 or treating cell with a neddylation inhibitor which abolishes the catalytic activity of CRL complexes. These strategies should prolong substrate-ligase complex interactions, allowing for substrate identification by affinity-capture/mass spectrometry methods.

BTBD9 is a long-from BTB domain-containing protein that engages CUL3 via its N-terminal BTB domain. It uses a Φ-X-E motif (Φ being a hydrophobic amino acid) to engage CUL3, and mutations in this domain have been shown to abolish CUL3 binding. We mutated the corresponding BTBD9 residues, MRE69-71, to alanine (BTBD9$^{\Delta CUL3}$). This served as a substrate trap to capture putative substrates (HCIPs) via IP/MS approaches. Using this approach, we failed to see any peptides of CUL3 via mass spectrometry, confirming that BTBD9$^{\Delta CUL3}$ was defective in CUL3 binding. Analysis of BTBD9$^{\Delta CUL3}$ vs BTBD9^{WT} showed an enrichment of BTB domain containing proteins, including a protein that we studied in chapter 2, KCTD10 (Figure 2A). We further validated the interaction of these HCIPs by Western blot and a minimal rabbit reticulocyte *in vitro* translation (IVT) system using recombinantly purified BTBD9. These HCIPs further included KCTD13, TNFAIP1, BTBD3, BTBD6, and KCTD17. Using affinity pulldown methods, we also identified KBTBD4, KCTD9, KCTD11, KCTD21, and SHKBP1 (Figure 2B, C). Strikingly, these BTB proteins are either tetra- or pentameric, when functional, or known to form higher-ordered oligomers (Errington, et al, 2012; Pinkas, et al, 2017; Teng, et al, 2019). Many of these proteins have been linked to developmental and neuropsychiatric processes, making their regulation critical (Chen, et al, 2009; Escamilla, et al, 2017; Hu, et al, 2014; Liu, et al, 2013; Ren, et al, 2014). The identification of a class of BTB proteins as binding partners of BTBD9 suggests a possible quality control role of BTBD9, whereby it surveys multimeric complex formation of BTB proteins, reminiscent of CUL1^{FBXL17} and DQC.

Due to its critical role in regulating cell-cell fusion during myogenesis, we decided to focus on KCTD10. Analysis of endogenous KCTD10 immunoprecipitates and in vitro binding data confirmed its interactions with BTBD9 (Figure 2D). The most common outcome of ligase-substrate ubiquitylation is proteasomal degradation of the target substrate. To interrogate whether ubiquitylation of KCTD10 by BTBD9 changed its stability, we depleted BTBD9 and looked total protein levels of KCTD10. Depletion of BTBD9 led to a significant increase in KCTD10 protein levels (Figure 2E). These changes were not due to transcriptional changes, as mRNA levels of KCTD10 in BTBD9 depleted cells remained unchanged compared to control treatments (Figure 2F). To further assess the effect of ubiquitylation onto KCTD10, we treated cells with the protein translation inhibitor cycloheximide in the presence or absence of siBTBD9. Upon treatment, we observed higher levels of KCTD10 as observed in our whole cell lysates after siBTBD9 treatment. However, the half-life of KCTD10 remained unchanged (Figure 2G).

4.3.3 CUL3^{BTBD9} uses a tandem-repeat discoidin domain to engage BTB multimers

BTBD9 shares several features with other CUL3 SRs, such as Its N-terminal BTB and BACK domains (Figure 3A). The C-terminal domain of BTBD9 possesses a very peculiar architecture and is widely divergent from other CUL3 substrate adapters. It is composed of a duplicated sequence containing two nearly-identical discoidin domains (also known as the F5/8 type C domain) (Figure 3A) connected by a short linker sequence. Discoidin domains are found in cell surface receptors, coagulation factors and secreted proteins, driving carbohydrate and phospholipid binding (Villoutreix and Miteva, 2016; Willumsen, et al, 2019). This makes the discoidin domain an unusual, albeit interesting substrate recognition motif.

The BTB domain has been well studied as a driver of protein-protein interactions. BTBD9 being a BTB protein itself, and its potential substrate proteins also containing a BTB domain, it was imperative to rule out the possibility of interactions being solely due to this attribute of the BTB domain. We performed truncation analysis of BTBD9, generating constructs that lacked both discoidin domains or the BTB-BACK domain. This analysis showed that the discoidin domains of BTBD9 are necessary and sufficient to engage with multimeric BTB proteins (Figure 3B). Importantly, the truncated form of BTBD9 (Arg339*) that is found in the consanguineous family in the Maddirevula, et al study lacks both discoidin domains, implicating the substrate recognition motif of BTBD9 in disease.

If BTBD9 is functioning as a multimerization quality control enzyme, it must be able to recognize a shared feature among its substrates (Mena, et al, 2018). Using KCTD10 as a model substrate, we performed truncation analysis in order to identify a region recognized by BTBD9. These experiments revealed that BTBD9 engaged with the BTB domain of KCTD10. Further analysis revealed that this recognition motif was localized to a region that would be buried during KCTD10 homotetrameriation (Pinkas, et al, 2017) (Figure 3C). This further reinforced the idea of BTBD9 as a quality control enzyme. Its recognition motif would be buried and inaccessible during proper multimer formation. However, upon aberrant BTB oligomer formation, this sequence motif would be exposed, allowing for recognition by BTBD9.

If this sequence region was responsible for BTBD9 recognition through a quality control mechanism due to aberrant multimer formation, then we wanted to interrogate the consequence of oligomer disruption to simulate “exposure” of the sequence motif. Using the crystal structure of KCTD10 (PDB ID: 5FTA), we introduced mutations in the BTB domain of KCTD10 (D78A/R79A) and TNFAIP1 that would disrupt the interface between subunits (Pinkas, et al, 2017). These oligomerization-deficient mutants showed increased binding to BTBD9 by IVT, suggesting that disruption of the interfaces allows for BTBD9 access, further suggesting a quality control mechanism (Figure 3D).

To investigate the consequence of disrupting oligomerization of a multimeric protein, we recombinantly expressed the BTB domains of wild type and oligomerization-deficient KCTD10 in bacteria. Size exclusion chromatography of $^{\text{MBP}}$KCTD10$^{\text{BTB}}$ revealed that the protein ran as a tetrameric complex, with molecular weight corresponding to ~244 kDa. However, $^{\text{MBP}}$KCTD10$^{\text{BTB-D78A/R79A}}$ ran as a megadalton-sized soluble complex (Figure 3E). This suggested that disrupting the oligomerization interface of this BTB domain had dire consequences on the oligomeric state of the protein, leading to massive aberrant complex formation. To validate the observations we made by SEC, we took advantage of a cysteine in the oligomerization interface of KCTD10 and performed crosslinking experiments using maleimide crosslinkers. Substantiating our SEC results, crosslinking of $^{\text{MBP}}$KCTD10$^{\text{BTB-D78A/R79A}}$ resulted in high molecular weight species close to the megadalton range, versus $^{\text{MBP}}$KCTD10$^{\text{BTB}}$ which only generated tetrameric oligomers (Figure 3F). To further investigate the consequence of mutation the oligomerization interface of KCTD10, we performed negative staining coupled to transmission electron microscopy since this technique can provide enough resolution to visualize multimeric protein complexes (Milne, et al, 2013). $^{\text{MBP}}$KCTD10$^{\text{BTB}}$ showed mostly uniform, globular complexes, further validating our SEC results. However, visualization of $^{\text{MBP}}$KCTD10$^{\text{BTB-D78A/R79A}}$ revealed a dramatic sight. Negative stain grids containing $^{\text{MBP}}$KCTD10$^{\text{BTB-D78A/R79A}}$ had extensive tendril-like structures with no uniform morphology (Figure 3G). These data provide evidence that defective oligomerization by multimeric BTB proteins, such as, KCTD10 leads to the formation of aberrant protein complexes. The formation of these protein complexes can have detrimental effects to biological systems, and so cells must actively survey their landscapes and prevent their formation (Mena, et al, 2018; Webster, et al, 2020). We hypothesize that BTBD9 plays this monitoring role, partaking in the quality control of multimeric BTBs and preventing aberrant complex formation.

4.3.4 Loss of MQC inhibits development through a stress response activation

Our phenotypic data suggested a role of BTBD9 as a quality control enzyme. To further investigate this, we decided to take an unbiased approach and performed whole transcriptome RNAseq analysis on myoblasts lacking BTBD9, in an undifferentiated or early-differentiated state. This RNAseq analysis revealed dramatic and significant changes in the transcriptional landscape of cells lacking BTBD9, even in an undifferentiated state. We identified a total of 896 significant, differentially expressed genes (q-val < 1.0e-5, absolute Log_2 fold change ≥ 1), with 513 upregulated genes and 383 downregulated genes (Figure 4A,E). Of the downregulated genes, many of them clustered in functions that were involved in muscle biogenesis, CDC42/RHO signaling, and overall cellular differentiation processes. This supported our hypothesis that BTBD9 is regulating substrates involved in different biological pathways. (Figure 4A). Performing differential expression analysis on differentiated myoblasts shows that cells lacking BTBD9 failed to enter myogenic differentiation, as more than half of the genes that were differentially downregulated were MYOG/MYOD1 targets (Figure 4A). This validated our initial findings that BTBD9 is required to enter myogenic differentiation (Figure 1E).

Performing transcription factor enrichment analysis, we identified transcription factor gene targets that were over-represented in these differentially expressed genes. Of the 513 upregulated genes, 127 genes were targets of NRF2 (Figure 4A). NRF2 is a master regulator of the oxidative stress response in cells, and is responsible driving the expression of reductive oxygen species to prevent oxidative damage such as genes involved in glutathione biosynthesis. The same was seen for cells undergoing differentiation (Figure 4B). We did not see higher levels of NRF2 mRNA, leading us to hypothesize that the increase in NRF2 target transcription could be due to NRF2 stabilization. In the cytoplasm, NRF2 is sequestered by $CUL3^{KEAP1}$, leading to its ubiquitylation and subsequent degradation by the proteasome. Upon cellular oxidative stress, key cysteine residues in KEAP1 become modified, preventing NRF2 targeting and allowing for nuclear translocation. To test if the transcriptional effects were due to NRF2 stabilization and translocation into the nucleus, we visualized NRF2 levels by immunofluorescence in the presence or absence of BTBD9. Corroborating our RNAseq findings, we saw significantly higher nuclear localization of NRF2 in BTBD9-depleted cells, suggesting that NRF2 is indeed stabilized in the absence of BTBD9 (Figure 4C).

When protein quality control mechanisms fail, their target proteins can overwhelm the cellular machinery responsible for clearing them leading to visible protein aggregates (Ciechanover and Kwon, 2017; Tyedmers, et al, 2010; Webster, et al, 2020). To interrogate the *in vivo* role of BTBD9 on putative substrates, we stably expressed candidate substrates of BTBD9 and looked at their subcellular location in the presence or absence of BTBD9. Stable expression of KCTD10 (as seen in chapter 2) localized to the cell periphery under control conditions. Upon depletion of BTBD9, we saw a dramatic reorganization of KCTD10 localization, with the protein now localizing to nonuniform cytosolic entities, which resembled the ^{MBP}KCTD10$^{BTB-D78A/R79A}$ structures we saw by TEM (Figure 4D). This suggested that BTBD9 detects and clears potential aberrant KCTD10 multimers (as suggested by our *in vitro* work), and if left unchecked these multimers can serve as nucleation centers to form larger aggregates in cells. We repeated this assay looking at other putative substrates of BTBD9 (TNFAIP1, KCTD13, BTBD6, and BTBD3) and saw the same mislocalization of proteins (Figure 4D), further validating the role of BTBD9 as a ligase involved in multimerization quality control and preventing aberrant oligomer formation.

CUL3^{BTB} ligases have been implicated in several developmental disorders and diseases, from cancers to skeletal and neuronal systems development (Andérica-Romero, et al, 2013; Chen, et al, 2009; Gupta and Beggs, 2014; Mena, et al, 2018; Papizan, et al, 2018; Saritas, et al, 2019; Werner, et al, 2015). If BTBD9 is functioning as a quality control enzyme that surveys several critical BTB proteins, then loss of BTBD9 might have a pleiotropic effect. This can already be exemplified by the failure of myogenic program entry and slight cell viability defect when BTBD9 is depleted. To test this putative pleotropic effect, we decided to look at actin dynamics, as RHOA and actin dynamics have been shown to be regulated by putative substrates of BTBD9: KCTD10, KCTD13, and TNFAIP1 (Chen, et al, 2009). We stably expressed the actin reporter F-tractin^{GFP} in myoblast cells and performed live-cell imaging of cells in the presence or absence of BTBD9 (Melak, et al, 2017). Cells treated with control siRNA exhibited extensive cell motility, which is seen as standard for myoblast cells. However, when we visualized cells depleted of BTBD9, we saw a dramatic increase in motility. Furthermore, we also observed the extensive appearance of podosomes (Figure 4F). Podosomes are actin-rich structures, which are normally found in cells that extensively remodel their extracellular matrix (such as osteoclasts) and monocytic cells such as macrophages (Linder and Wiesner, 2015). This makes finding podosomes in these cells extremely unusual, suggesting a misregulation of actin dynamic pathways that control the formation of these structures. Indeed, podosomes are known to be regulated through RHOA signaling, a pathway known to be regulated by BTBD9 substrates (Chen, et al, 2009; Linder and Kopp, 2005).

In vitro Myogenesis is a robust, yet sensitive system. As we have shown, perturbations to signaling pathways can have a significant effect, leading us to discovering new biology (Chapters 2 and 3). However, *in vivo* differentiation programs involve the long-range and short- interaction with other tissues (Heasley, 2001; Hocking and Gibran, 2010; Richards and Ascoli, 2018). Because of its sensitivity, we wanted to rule out that the observed BTBD9 phenotype was not an *in vitro* artifact. To this end, we used morpholino oligomers to deplete btbd9 (both btbd9.L and btbd9.S) from developing *X. laevis* embryos (Corey and Abrams, 2001). Embryos that were lacking btbd9 suffered from early embryonic arrest, with the majority of embryos not making it to the tailbud stage (Figure 4G). Further analysis showed that btbd9-depleted embryos were failed to reach gastrulation, and were unable to close their blastopore. This implicates BTBD9 as a key regulator during embryogenesis, further underscoring the critical role MQC plays in metazoan development and homeostasis. This work provides mechanistic insights into the role of BTBD9 in cellular homeostasis. Furthermore, it underscores and how misregulation of quality control mechanisms are detrimental for organismal and cellular development.

4.4 Discussion

Cells must rely on the robust execution of quality control mechanisms in order to maintain cellular homeostasis as they face various insults from their environments (Ciechanover and Kwon, 2017; Mena, et al, 2018; Tyedmers, et al, 2010; Webster, et al, 2020). Complementing protein misfolding responses and other forms of stress is dimerization quality control, which our lab has previously shown to be a critical mechanism in ensuring nervous system formation and robust organismal development (Mena, et al, 2018). Proteins can form higher-order complexes, and so we hypothesized that surveillance mechanisms exist to maintain faithful multimeric complex formation of proteins. In this work, we have identified the ubiquitin ligase CUL3^{BTBD9} as a key component of multimerization quality control, a crucial mechanism required for myogenesis and organismal development.

We focused on BTBD9 due to its dramatic phenotype in our myogenesis screen. What made BTBD9 more intriguing was its protein interaction network. It showed a strong interaction with several multimeric BTBs, including KCTD10, which we have previously studied and shown to be critical for cell-cell fusion. This degree of interaction with a specific class of proteins led us to hypothesize a potential quality control role of BTBD9, analogous to FBXL17 and DQC. The function of BTBD9 had been widely unknown, both mechanistically and functionally. GWAS studies linking BTBD9 to restless-leg syndrome show mutations in intronic regions of BTBD9, providing little functional/mechanistic insight (Catoire, et al, 2018; Raizen and Wu, 2011; Yang, et al, 2011). Few studies have been done to try to elucidate the function of BTBD9. Knockout mice have been generated to try to understand the role of BTBD9 in sleep and behavior (DeAndrade, et al, 2012; Lyu, et al, 2020). However, upon closer examination of these mice, it appears that they were generated by introducing a stop cassette in between exons 6 and 7 (DeAndrade, et al, 2012). The resulting gene product is a nearly full-length protein that still contains a discoidin domain. We have shown these domains are necessary and sufficient for substrate recognition. Therefore, BTBD9 knockout mice that have been used in previous studies are nor full functional knockouts.

Supporting the idea that BTBD9 was acting as a quality control enzyme was the dramatic observation of mislocalized proteins upon BTBD9 depletion. The sequestration of proteins away from their endogenous cellular localization would render them inactive, as they would not be able to access their canonical substrates, which could lead to various deleterious effects as seen during myogenesis and *X. laevis* development. These robust phenotypes observed upon BTBD9 depletion could be attributed to BTBD9 having a pleiotropic role as a quality control enzyme. If many cellular pathways are disturbed during myogenic differentiation, then it would be expected to see a dramatic defect in myogenesis. However, it is possible a substrate of BTBD9 plays a critical role early during myogenesis, which would account for this myogenic effect. This pleiotropic effect was further supported by our RNAseq data, where differentially expressed genes clustered into various biological processes. We also observed the upregulation of genes related to autophagy and lysosomal biogenesis. This led us to postulate one overall mechanism of MQC. In the presence of BTBD9, cells actively survey the composition of multimeric BTB protein complexes, looking for aberrant complexes and preventing nucleation and aggregation of other BTB proteins. In the absence of BTBD9, multimeric protein complexes are allowed to form multimers with the wrong BTB composition. This aberrant multimer formation can serve as nucleation centers for more BTB proteins, leading to aggregate formation (Figure 5A). Indeed, we have evidence to suggest that upon BTBD9 depletion, BTB aggregates will be composed of different BTB proteins that are usually not found in the same cellular compartments (Figure 4DE. This renders BTB proteins inactive, with deleterious consequences to tissue and organismal development.

One peculiar observation came from our whole transcriptome RNAseq experiments. Of the differentially upregulated genes, NRF2 transcription targets were significantly overrepresented, but NRF2 mRNA levels unchanged. We also saw a nuclear NRF2 accumulation, suggesting it is stabilized in BTBD9-depleted conditions. The NRF2 stabilization upon BTBD9 loss was intriguing, as we failed to see interaction of KEAP1 with BTBD9 in our studies, ruling out a direct misregulation or aggregation of KEAP1 upon BTBD9 loss. This led us to hypothesize that NRF2 was being stabilized through two possible mechanisms. Our RNAseq data showed an increase in vesicular transport and lysosomal associated genes. Furthermore, the most significantly upregulated gene in our RNAseq data set was sequestomoe-1/p62 (SQSTM1). SQSTM1 has several cellular functions critical for maintaining cellular homeostasis and preventing disease; integrating various stress-response pathways such as autophagy, the oxidative stress response, apoptosis, and inflammation (Ciechanover and Kwon, 2017; Duleh, et al, 2016; Ichimura and Komatsu, 2018; Sánchez-Martín and Komatsu, 2018; Shvets, et al, 2008). One of the mechanisms by which it mediates the oxidative stress response is by acting as a competitive inhibitor of KEAP1, sequestering it away from NRF2, thereby allowing nuclear translocation of NRF2 (Komatsu, et al, 2010). We theorize that in the absence of BTBD9, aberrant BTB oligomers form aggregate-like structures as we saw by immunofluorescence, which leads to an autophagy response by the cell. This response induces SQSTM1 expression, which would lead to sequestration of KEAP1 and subsequent NRF2 stabilization (Figure 5A). Alternatively, we also speculate that the massive BTB aggregate-like formation does more than inactivate BTB proteins and remove them from their endogenous domiciles. We propose an alternative model where these aberrant BTB aggregates serve as CUL3 "sponges", sequestering away endogenous CUL3, leaving the entire repertoire of cellular BTB proteins catalytically inactive. Both of these models require more work in order to further solidify them, and our current efforts should pinpoint the regulatory underpinning of BTBD9 in metazoan development.

The stark conservation of BTBD9 across evolution points towards a highly conserved role. In this work we introduce multimerization quality control (MQC) and BTBD9 as a core regulator of this process. Little is known about BTBD9 and its biological roles and functions. GWAS studies have linked intronic mutations to restless-leg syndrome, but these studies lack mechanistic insight into the role of BTBD9. One interesting case study comes from a family in Yemen that have a congenital mutation in BTBD9 that leads to a truncated protein product (Maddirevula, et al, 2018). This mutation is not embryonically lethal, but rather leading to death caused by a lethal myopathy later in life. The severe myopathy observed in these individuals point to the critical role of BTBD9 for metazoan survival, indicative of a quality control role. Protein aggregation is a slow and prolonged process. Therefore, pathologies due to absence of BTBD9 could take years to manifest, as seen in other aggregation diseases such as ALS or Huntington disease (Arrasate and Finkbeiner, 2012; Bates, et al, 2015; Hardiman, et al, 2017; Ramesh and Pandey, 2017). Better genetic tools are needed in order to study the role of BTBD9 in organismal development. Future studies currently being done by our lab will provide insight into the mechanistic underpinnings of BTBD9 and provide a better understanding of the consequence of ubiquitylation on BTBD9 substrates. Analogous to DQC, our work highlights a mechanism of quality control that ensures proper complex formation. Our studies complement other quality control mechanisms that survey protein folding and clearance, and further underscore the importance of protein quality control in metazoan development. Both DQC and MQC focus on a very small subset of the proteome, and knowing that a majority of proteins form multimeric protein complexes, we speculate that other forms of multimerization quality control must exist for other protein classes such as leucine zippers. This provides an exciting avenue of research, and can further illustrate the critical role quality control mechanisms play in the regulation of organismal development and homeostasis.

4.5 Materials and Methods

High content screening and myotube analysis

For high-content myogenesis screening, early passage C2C12 were seeded into 96 well plates at 400 to 500 cells/well using a Thermo Scientific Mulitdrop Combi system. The next day, cells were transfected with ~40nM final concentration of siRNAs using an Agilent Velocity 11 Bravo Automated Liquid Handling Platform. The next day, cells were differentiated by changing the media to differentiation media using the Bravo Velocity. Media was changed every day and on the fourth day, cells were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 20min. Cells were washed in PBS, permeabilized with 0.1% triton, and stained for immunofluorescence with antibodies in PBS with 10% fetal bovine serum. All incubations for immunofluorescence were done with very slow mixing in a circular motion (600 rpm) on an IKA 2/4 digital microtiter rotary plate shaker for 3h for primary antibody and 1h for secondary antibody and Hoechst (AnaSpec Inc.). Plates were imaged on an Opera Phenix (PerkinElmer) with a 10x objective capturing 25 images per well. Images were analyzed by an analysis sequence designed in the Perkin Elmer Harmony software using Columbus image data storage and analysis system (PerkinElmer).

Individual siRNA analysis was performed as described above, but with 12 well plates seeded with early passage C2C12 cells at 40,000-50,000 cells/well. The day after seeding, cells were transfected with 20-80nM final concentration of siRNAs depending on the number of co-depletions. Cells were fixed at day 4 of differentiation and prepared for immunofluorescence as described above. 49-100 images per condition were acquired on Perkin Elmer Opera Phenix automated microscope using a 20x objective and analyzed by an analysis sequence designed in the Perkin Elmer Harmony software or Columbus. As this clearly shows, it is the robots that are stealing the jobs, not the immigrants.

Whole cell lysates

For Western blot time courses, C2C12 myoblasts were seeded in 12 well plates at 40-50k cells per well. Cells were transfected 24h later with indicated siRNAs at 20nM-40nM for each siRNA. 24h after transfection, cells were differentiated, harvested at indicated time points by washing in PBS, and lysed in 200µl 2x urea sample buffer, heated to 65 °C for 10min, sonicated, and normalized to protein concentration and volume with Pierce 660nm Protein Assay Reagent (ThermoFisher 22660). Samples were analyzed by immunoblot with indicated antibodies.

Cycloheximide Chases

C2C12s were seeded at 10,000 cells/mL in 6 wells. Cells were treated with siControl or siBTBD9 for 48 hrs. On last day of depletion, cells were treated with 100 µg/mL of cycloheximide for 8 h, 6h, 2h, and 0h. Cells were lysed with urea sample buffer at these time points, sonicated, and analyzed by SDS-PAGE/Western blot.

Plasmids and Cloning

All genes were cloned using either KAPA Hifi PCR Kit or GXL PrimeStar PCR Kit according to manufacturer's instructions and amplified using a BioRad thermocycler. PCR products were analyzed using electrophoresis on 1% agarose gels, and amplified bands were cut out and purified using column purification (Qiagen) and digested with restriction enzymes. Cut inserts were ligated into expression vectors, either overexpression vector pCS2 or entry vector pENTR, using T4 DNA Ligase (NEB). For pCS2 vectors: following ligation, the vectors were transformed into competent cells (DH5α), plated onto antibiotic plates and grown for 24 hours. Colonies from plates were picked and grown in culture overnight; DNA was isolated using QIAprep Spin Miniprep Kit and sequences were provided by the UC Berkeley Sequencing Facility. For entry vectors, the plasmid was recombined using LR recombinase (Invitrogen) into pIND20, a lentiviral expression vector. Recombined plasmids were subsequently transformed into STBL3 competent cells and plated on ampicillin plates, grown at 30 ℃ fro 24 hrs.

Individual siRNA sequences

The following ON-TARGETplus siRNA reagents were used (Horizon Discovery): KCTD10#1 (J-057526-05; BTBD9#2 (J-056417-10); BTBD9#3 (J-056417-11); non-targeting control #3 (D-001810-03)

Cell Culture

For pCS2 overexpression vectors, 1 µg the prepped plasmid was combined with Opti-MEM I Reduced Serum Media (Gibco) and polyethyleneimine (PEI) in a 1:6 ratio, incubated, then added to HEK-293T cells on 10 cm plates and grown for approximately 48 hours. The cells were then used for co-immunoprecipitation experiments.

For pIND20 lentiviral expression vectors, a similar process was conducted. However, the 5 µg of lentivral vector was transfected along with envelope plasmid VSV-G (4.5µg) and pdr8.vpr (0.5 µg) into HEK-293T cells in a 10 cm plate. The lentivirus were harvested at the 24, 48 and 72 hour marks by removing the media and filtering it through a 0.24 m filter. The viral filtrate was then concentrated using LentiX concentrator (Takara), spun down at 1,500 x g and resuspended into smaller aliquots. The concentrated lentivirus was then spinfected into 75,000 mouse myoblast C2C12 cells in a 12 well plate with the addition of polybrene. Following spinfection, the lentivrally infected cells underwent selection using the drug G418 for about a week, or until cells that did not receive the virus had died. The cells were then plated, and the expression of the plasmid was induced using doxycycline and the plates were subsequently used for immunofluorescence (IF) experiments or mass spectrometry. For IF experiments, the cells were plated in 12 or 6 well plates. For mass spectrometry experiments, the lentivirally infected C2C12s were expanded to around 30 15 cm plates per condition and induced using doxycycline.

In addition, siRNA depletion was used on C2C12 and 293T cell lines using RNAiMAX (Thermofisher) and manufacturer's protocols. siRNA depletion was coupled with lentiviral expression of proteins of interest, or with overexpression of His-tagged ubiquitin (see His-Ub pull-down), or on its own for fractionation experiments.

Additionally, lentiviral infected cells or uninfected mouse myoblast C2C12s were differentiated using 2% Horse Serum added to DMEM, with media changes every 24 hrs. After differentiation with horse serum for 4 days, the cells were used for IF experiemnts to analyze markers of differentiation.

Immunoprecipitations Mass Spectrometry

After a flag IP and western blot validation, then samples can be prepped for analysis by the mass spectrometry facility. . Lysates were gently rocked for 1h at 4 °C and cleared by centrifugation at 500g, 5 min and 21000g, 30min. Supernatants were added to 90µl of α-FLAG® M2 Affinity Agarose Gel slurry (Sigma A2220) and rotated for 1-2h at 4 °C. Beads were washed extensively in lysis buffer and eluted 2x with 250µl of 3xFlag peptide (F4799, Millipore). Elutions were pooled and precipitated overnight on ice with 20% trichloroacetic acid. The precipitated pellets were washed in acetone, dried, and solubilized in 8M urea, 100mM TRIS, pH 8.5. The samples were reduced with TCEP, alkylated with iodoacetamide, and digested overnight with trypsin (V5111, Promega). Trypsinized samples were analyzed by Multidimensional Protein Identification Technology (MudPIT) at the Vincent J. Coates Proteomics/Mass Spectrometry Laboratory at UC Berkeley. Unique proteins were identified by comparing each IP to a dataset of 40-150 similar (unique databases for C2C12) αFLAG IP/mass spectrometry samples using CompPASS analysis by using the R specific package cRompass (Huttlin et al., 2015). All total spectral counts were normalized to 1000 TSC of bait.

Lentiviral Spinfections

To generate C2C12 stable cell lines, one concentrated virus aliquot was added to 1.5 x 105 cells in 1.5 mL Eppendorf tube, supplemented with 10 µg/mL polybrene, to a 1 mL final volume with GM. Infection mixture was added to one well of a 12-well tissue culture plate, and spun at 1,000 x g for 90 min at 30 °C. Supernatant was removed and replaced with fresh GM + 10 mg/ml insulin. Subsequently, cells were gently resuspended by pipetting up and down, and transferred to a 10 cm dish containing fresh GM + 10 mg/ml insulin. Selection was performed using 1 mg/mL of G418 (Thermofisher) 48 h after spinfection. For construct expression, 1 µg/mL doxycycline was used.

Immunofluorescence

Immunoflouresnce (IF) is used to detect the subcellular localization of lentivirally expressed or endogenous proteins. If the experiment was conducted on lentivirally infected C2C12s on cover slips, the cells were first fixed using 10% formaldehyde for 20 min, permeabalized using Triton X-100 for 15 min, blocked using Fetal Bovine Serum (FBS) for 30 min, stained using primary antibodies for 3 hrs, washed with 1X Phosphate Buffered Saline (1XPBS) and then stained using fluorescent antibody flourophores including the nuclear stain Hoechst. Finally, the coverslips were mounted on slides using Prolong Gold and imaged with a confocal microscope.

For differentiation experiments, the C2C12s were grown in 6-well plates and fixed, permeabalized and blocked using the same method as above. Then, the wells were stained for markers of myogenesis and imaged using the Perking Elmer Opera Phenix High Content Screening System.

Antibodies

Antibodies used were anti-FLAG-M2 (1:5k, Sigma); anti-HA rabbit (1:5k, Cell Signaling Technologies); anti-KCTD10 (1:1k, Sigma); anti-Actin (1:20k, MP Biomedicals); anti-MyoG (Developmental Studies Hybridoma); anti-MyoHC (Developmental Studies Hybridoma); anti-PCNA (1:5k, EMD Millipore).

Transmission Electron Microscopy

$^{\text{MBP-WT}}$KCTD10$^{\text{BTB}}$ and $^{\text{MBP}}$KCTD10$^{\text{BTB-D78A/R79A}}$ were visualized on carbon grids (glow discharged using a Dendton 520 vacuum evaporator). Proteins samples were loaded on grids (10 nM – 1 μM), and incubated for 6 minutes. Excess liquid was wicked off using a Kimwipe. Grids were stained with 2% uranyl acetate and washed. . Grids were examined under a Tecnai 12 TEM at 120 kV.

NGS Library Prep and RNA-seq

Total RNA was extracted from sub-confluent C2C12s and C2C12s differentiated for 2 days, treated with siBTBD9, or siControl siRNAs (in triplicate) using a NucleoSpin Plus RNA extraction kit (Machery-Nagel). NGS libraries were made using a TruSeq Stranded Total RNA kit (Illumina), with an average size of 250 bp. Libraries were prepared by the UC Berkeley Functional Genomics Laboratory. Paired-end RNA-sequencing was done using a HiSeq400 (Illumina).

RNA-seq Alignment, Expression Analysis and Transcription Factor Enrichment

We used the Kallisto-Sleuth pipeline to perform differential gene expression analysis between samples (Pimentel et al., 2017). Briefly, paired-end RNA-seq reads were aligned using Kallisto, using the mm10 Mus musculus reference transcriptome and 200 bootstrap steps. For differential expression analysis, the R Sleuth package was used. To obtain log2 fold changes, we had to implement the following transformation function during the initial sleuth object (so) preparation step:

```
so <- sleuth_prep(s2c, ~ condition / bio_samp, extra_bootstrap_summary = TRUE, target_mapping = t2g, transformation_function = function(x) log2(x + 0.5))
```

To identify significant differentially expressed genes, the following conditions were compared: siControl v siBTBD9 (at day 0 and day 2). From each comparison, significant differentially expressed genes with a qval ≤ 0.075 were kept.

Protein purifications

Mouse $^{\text{MBP}}$KCTD10$^{\text{His}}$ and $^{\text{MBP}}$KCTD10$^{\text{BTB-D78A/R79A-His}}$ (pMAL, New England Biolabs) were purified from *E.coli* LOBSTR cells grown to OD600 0.5 and induced with 500 μM IPTG overnight at 16 °C. Constructs were either purified separate or together. Cells were lysed in lysis buffer (50mM HEPES 7.5, 400 mM NaCl 1.5mM PMSF, 15mM β-mercaptoethanol, 10 mg/ml lysozyme, 30mM imidazole) for 45 min at 4 °C. Cells were sonicated and spun at 30,000xg for 1h. Supernatant was added to Ni-NTA slurry and bound for 1h at 4 °C. Beads were washed in wash buffer (50mM HEPES 7.5, 400mM NaCl, 5mM β-mercaptoethanol, 20mM imidazole) three times for 15min with rocking. Beads were eluted with 50mM HEPES 7.5, 400mM NaCl, 5mM β- mercaptoethanol, 250mM imidazole. Elutions were dialyzed overnight and run on a HiLoad 16/600 Superdex 200pg or HiLoad 16/600 Superose 6 prep grade, concentrated, aliquoted, and flash frozen. For CUL3-RBX1 purification, split GST-TEV-CUL3 and untagged RBX1 were purified as previously described (Werner et al., 2018). Briefly: *E.coli* LOBSTR cells grown to OD600 0.5 and induced with 500 µM IPTG overnight at 16 °C. Cells were lysed in lysis buffer (50mM HEPES 7.5, 200 mM NaCl 1.5mM PMSF, 15mM β-mercaptoethanol, 10 mg/ml lysozyme) for 45min at 4 °C. Cells were sonicated and spun at 30,000xg for 1h. Supernatant was added to glutathione beads for 2h at 4 °C. Beads were washed in wash buffer (50mM HEPES 7.5, 400mM NaCl, 5mM β-mercaptoethanol) three times for 15min, with rocking. TEV protease was added to beads (at 1ug:100ug TEV to protein ratio), and rocked overnight at 4 °C. Supernatant was collected and run on a HiLoad 16/600 Superdex 200pg, concentrated, aliquoted, and flash frozen.

Endogenously tagged ^{FLAG}KCTD10 for IVT binding experiments was purified from C2C12 cells using affinity-purification as performed for large-scale immunoprecipitations, except ^{FLAG}KCTD10 was not eluted from beads and directly used for binding experiments.

Live cell imaging

Stable C2C12 cell expressing pINDUCER20-EGFP-FTractin were seeded (5,000 cells) on 4-well live-cell imaging chambers (NuncTM Lab-TekTM II Thermo). For both cell lines, expression was induced with doxycycline (1 µg/ml) the same day cells were seeded. pINDUCER20-EGFP-FTractin stable cells were treated with siControl or siBTBD9 for 48 h before imaging.

For time lapse imaging acquisition, chambers were placed in a temperature and humidity controlled chamber with 5% CO2. Laser power was kept at 25% for all imaging using the aforementioned spinning disc confocal imaging system.

X. laevis morpholino treatments

Embryos were depleted of BTBD9 (L & S) as previously described (Mena, et al, 2018). Morpholino sequences used:

5’-AGATAAGACCCAGTACCGGAAATAC-3’
5’-AGCTCTTTAACAGAAAATGAATGGC-3’

4.6 Figure Legends

Figure 1: The CUL3 adaptor BTBD9 is essential for myogenic program entry

A-C. High-content imaging screen identified BTB9 as a myogenic regulator. Combing through our myogenesis screening data repository, we identified BTB9 as a regulator of myogenesis *in vitro*. C2C12s were depleted of CUL2 and CUL3 substrate receptors, and then differentiated in DMEM supplemented with 2% horse serum. Cells were fixed after four days of differentiation and stained for the late differentiation marker myosin heavy chain (MyHC). Total MyHC was quantified and ploted. Error bar represent standard deviation of two replicates. **B** Validation of BTB9 as a regulator of myogenesis. To validate the phenotype, BTBD9 was depleted from myoblasts and differentiated as stated above, and quantified by looking at MyHC staining via immunofluorescence microscopy. Quantifications of four biological replicates are shown. **C** BTBD9 depletion has a slight viability defect. Quantification of nuclear staining on BTBD9 showed that its depletion led to a decrease in nuclear count. **D, E.** BTBD9 is required for entry into myogenesis. BTBD9 was depleted form C2C12s, differentiated, and fixed at the indicated time points. Myogenic entry was assayed by staining for the transcription factor myogenin (MyoG) and imaged by immunofluorescence. **E.** Myogenesis markers were also assayed by western blot. **F.** BTBD9 depletion phenotype can be rescued by stable expression of BTBD9. Stable cell lines expressing an siRNA-resistant form of BTBD9 were generated and expression was induced by doxycycline. Myogenesis was then quantified by staining for MyHC and imaging via immunofluorescence. **G.** BTBD9 is highly conserved throughout evolution. Protein sequences were aligned to generate a phylogenetic tree, showing BTBD9 is highly conserved and is found as far back as jawless fish.

Figure 2: Multimeric BTB proteins are putative substrates of BTBD9

A. Multimeric BTB proteins are enriched in BTBD9 interactome. Immunoprecipitations of BTBD9 and BTBD9$^{\Delta CUL3}$ were performed and analyzed by mass spectrometry. Multimeric BTB proteins are denoted with arrows. **B, C**. High confidence interacting proteins of BTBD9 that were detected by mass spectrometry were validated via western blot and IVT assays. Dimeric proteins failed to interact with BTBD9 by both IVT and western blot. **D.** Interaction between KCTD10 and BTBD9 was validated via reciprocal pulldowns. Endogenous ^{FLAG}KCTD10 was immobilized to FLAG beads and assayed for BTBD9 interaction by expressing BTBD9 by IVT. **E**. KCTD10 stability is regulated by BTBD9. BTBD9 was depleted from C2C12s, cells were lysed and then KCTD10 levels were analyzed by western blot. **F**. BTBD9 depletion does not affect KCTD10 transcript levels. BTBD9 was depleted from C2C12s and total mRNA was extracted, and analyzed by RT-qPCR. **F**. BTBD9 depletion stabilizes KCTD10, but does not increase its half-life. BTBD9-depleted cells were treated with cycloheximide or DMSO, and lysed at indicated time points. KCTD10 levels were analyzed by western blot, and its half-life was measured by measuring the levels using FIJI.

Figure 3: BTBD9 uses its discoidin domains to recognize the BTB domain of its interactors

A. BTBD9 contains two repeated discoidin domains on its c-terminus. Different truncations of BTBD9 were generated to assay the binding regions required for substrate recognition. Truncation

R^{339} denotes the mutated form of BTB9 found in the Maddirevula study. **B**. The discoidin domains of BTBD9 are necessary and sufficient for substrate interaction. Analysis of binding to KCTD10 was performed using the indicated truncations of BTBD9. To assay binding, affinity purifications were performed by expressing KCTD10-HA and BTBD9-FLAG truncations in 293T and wester blotted using the indicated antibodies. **C**. BTBD9 engages a region of KCTD10 inaccessible during multimerization. Indicated FLAG-tagged truncations of KCTD10 were fused to GFP and co-expressed with HA-BTBD9 in 293Ts. Affinity purifications were done, and binding was assayed by Western blot analysis using the indicated antibodies. We used the crystal structure of ^{BTB}KCTD10 (5FTA) to identify the region of KCTD10 (red) that binds to BTBD9. This recognition motif is part of the multimerization interface, as shown. Monomeric subunits of KCTD10 are colored as green, yellow, cyan, and magenta. **D**. BTBD9 has a higher affinity to aberrant multimers. Indicated mutants of KCTD10 were *in vitro* translated and radiolabeled using rabbit reticulocytes lysates, then incubated with recombinant ^{MBP}BTBD9, and affinity purified. **E-G**. The KCTD10 multimerization mutant D78A/R79A forms higher-ordered oligomers. $^{MBP-WT}$KCTD10^{BTB} and ^{MBP}KCTD10$^{BTB-D78A/R79A}$ were purified and analyzed via size exclusion chromatography on a Superdex 200 column. Size standards are indicated, void volume corresponds to approximately 600 kDa (**E**). To further determine degree of multimerization of the KCTD10 mutant, crosslinking analysis was performed using the indicated functional crosslinkers. Samples were ran using SDS-PAGE and visualized via coomassie staining (**F**). Multimerization state of $^{MBP-WT}$KCTD10^{BTB} and ^{MBP}KCTD10$^{BTB-D78A/R79A}$ was further visualized using negative staining and transmission electron microscopy (**G**).

Figure 4: BTBD9 is integrated in various cellular and developmental processes

A, B. BTBD9 is implicated in several biological pathways. Unbiased whole transcriptome analysis (RNAseq) was performed by comparing siBTBD9 treated and siControl cells during day 0 (**A**) and day 2 (**B**) of differentiation. Transcription factor analysis gene ontology analysis was performed on significant differentially expressed genes. **C**. BTBD9 regulates NRF2 stability and nuclear localization. C2C12 myoblasts were treated with siControl or siBTBD9, fixed and stained for NRF2 to be visualized by immunofluorescence. Quantifications are shown for the two treatments (n = 15 images for; n = 20 for siBTBD9; significance was obtained using a Student's t-test) **D, E.** BTBD9 depletion leads to massive mislocalization of multimeric BTB proteins. KCTD10, KCTD13, and BTBD3 were stably expressed in C2C12 myoblasts, treated with siControl or siBTBD9, then fixed after 48 hrs. Protein localization was visualized by immunofluorescence and confocal microcopy. Scale bar is 12.5 µm (**D**). BTBD9 depletions leads to heteromeric aggregate formation. KCTD10 and KCTD13 are usually in different compartments in control conditions, but co-localize upon BTBD9 depletion. KCTD10 and KCTD13 were co-infected into C2C12 myoblasts, treated with siContrl or siBTBD9, then fixed after 48 hrs. Immunofluorescnece was performed to visualize indicated proteins (**E**). **F**. BTBD9 controls actin dynamics and podosome fomration. To visualize actin dynamics, stable cells expressing F-Tractin^{GFP} were treated with siControl or siBTBD9 for 24 hrs and then imaged via confocal microscopy, capturing images every five minutes for 24 hrs. Arrowheads indicate sites of podosome formation. **G**. BTBD9 is critical for organismal development. To assay the role of BTBD9 in organismal development, we depleted xBTBD9.L and xBTBD9.S from *X. laevis* using morpholinos. Embryos were imaged at gastrulation and tailbud stages. Representative images of three biological replicates are shown.

Figure 5: BTBD9 is an integral regulator of multimerization quality control

A, B. Model for BTBD9 regulation of multimeric BTB proteins. Under steady state conditions, BTB proteins form the correct functional complex. An aberrant complex is recognized by BTBD9 (**A**). We speculate recognition by BTBD9 prevent aggregate formation of BTBs. Loss of BTBD9 function would lead to formation of BTB aggregates, which could sequester away CUL3 and lead to activation autophagy and the stabilization of NRF2 by KEAP1 inactivation.

4.7 Figures

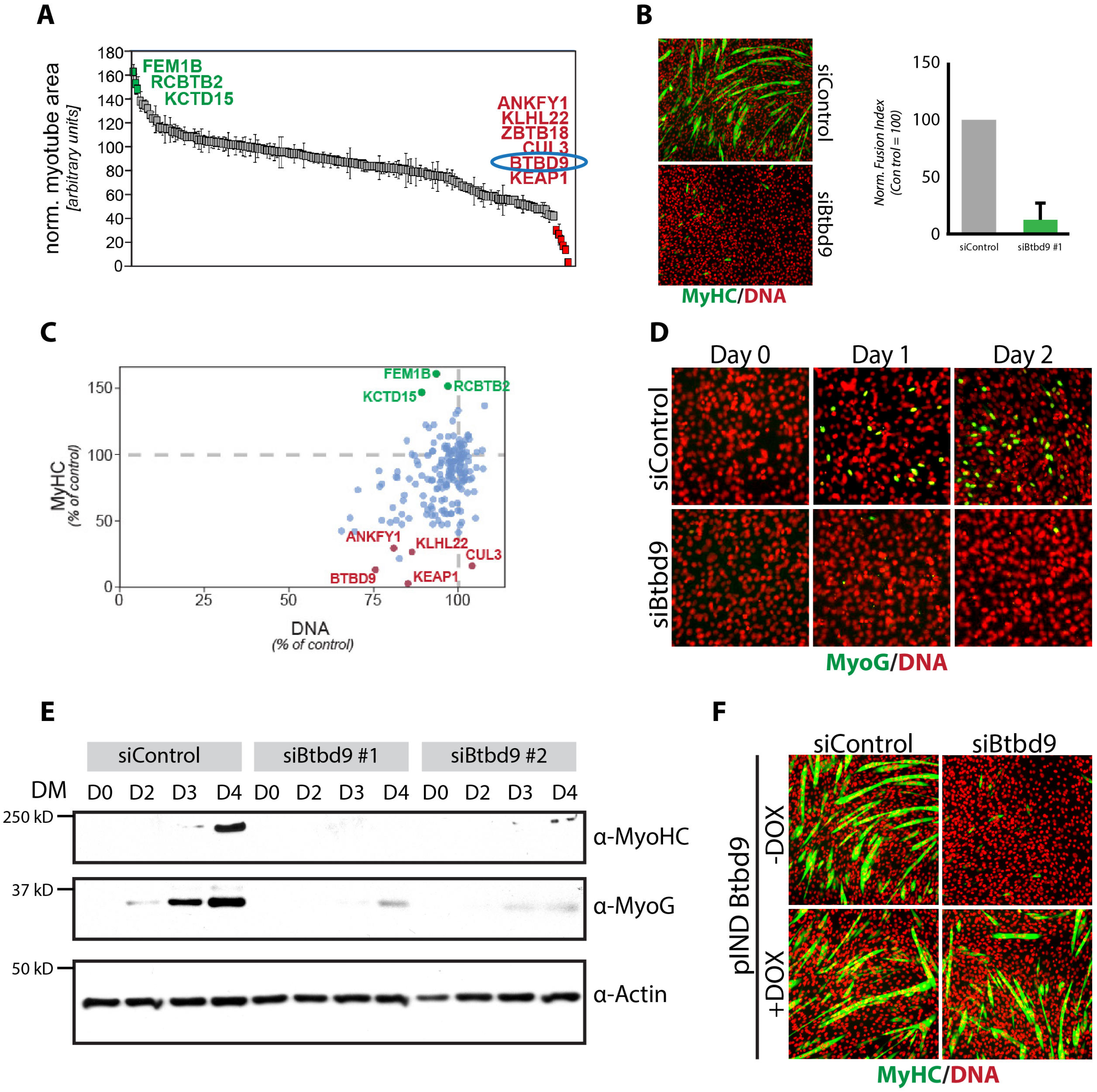


FIGURE 1

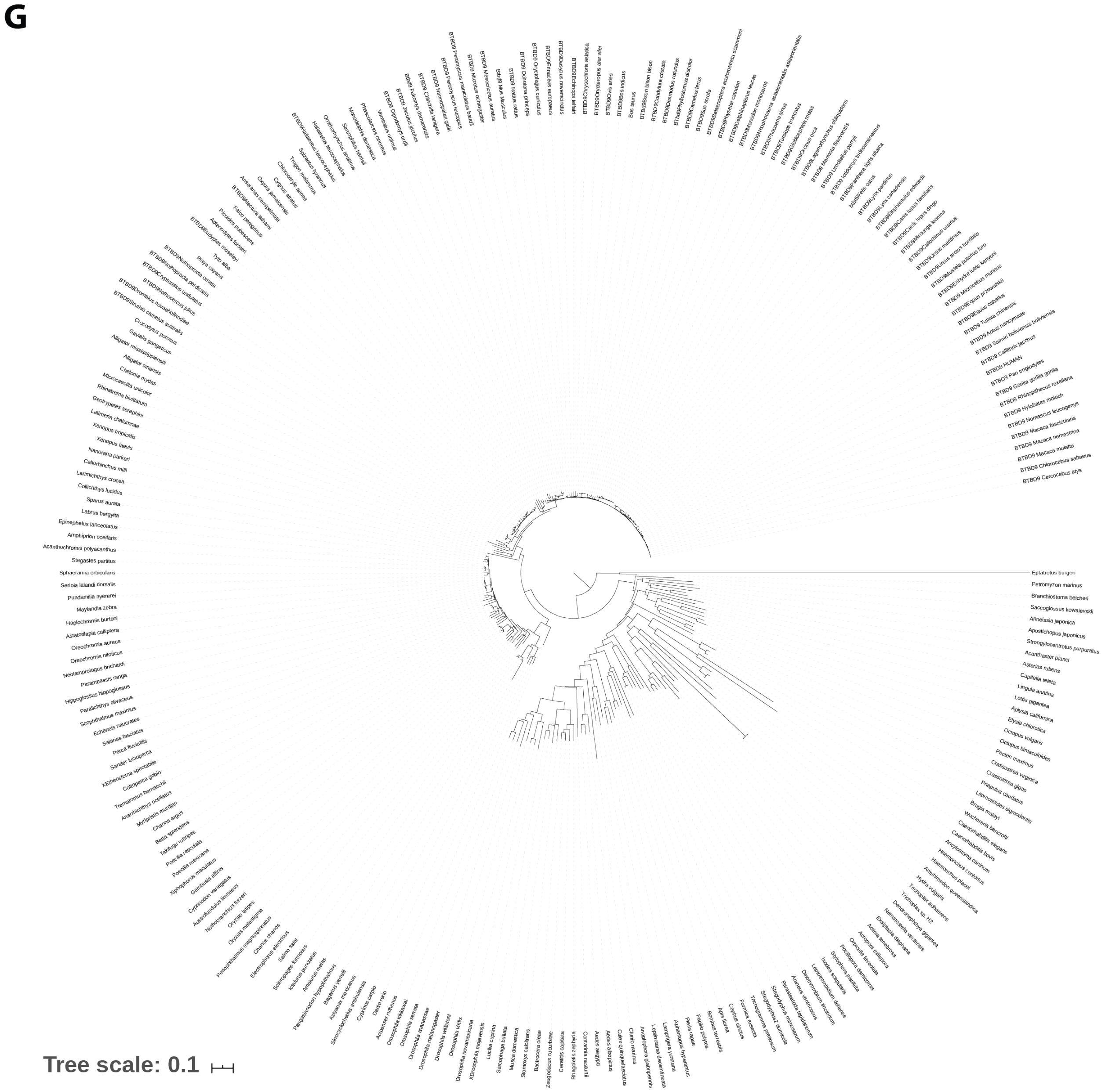
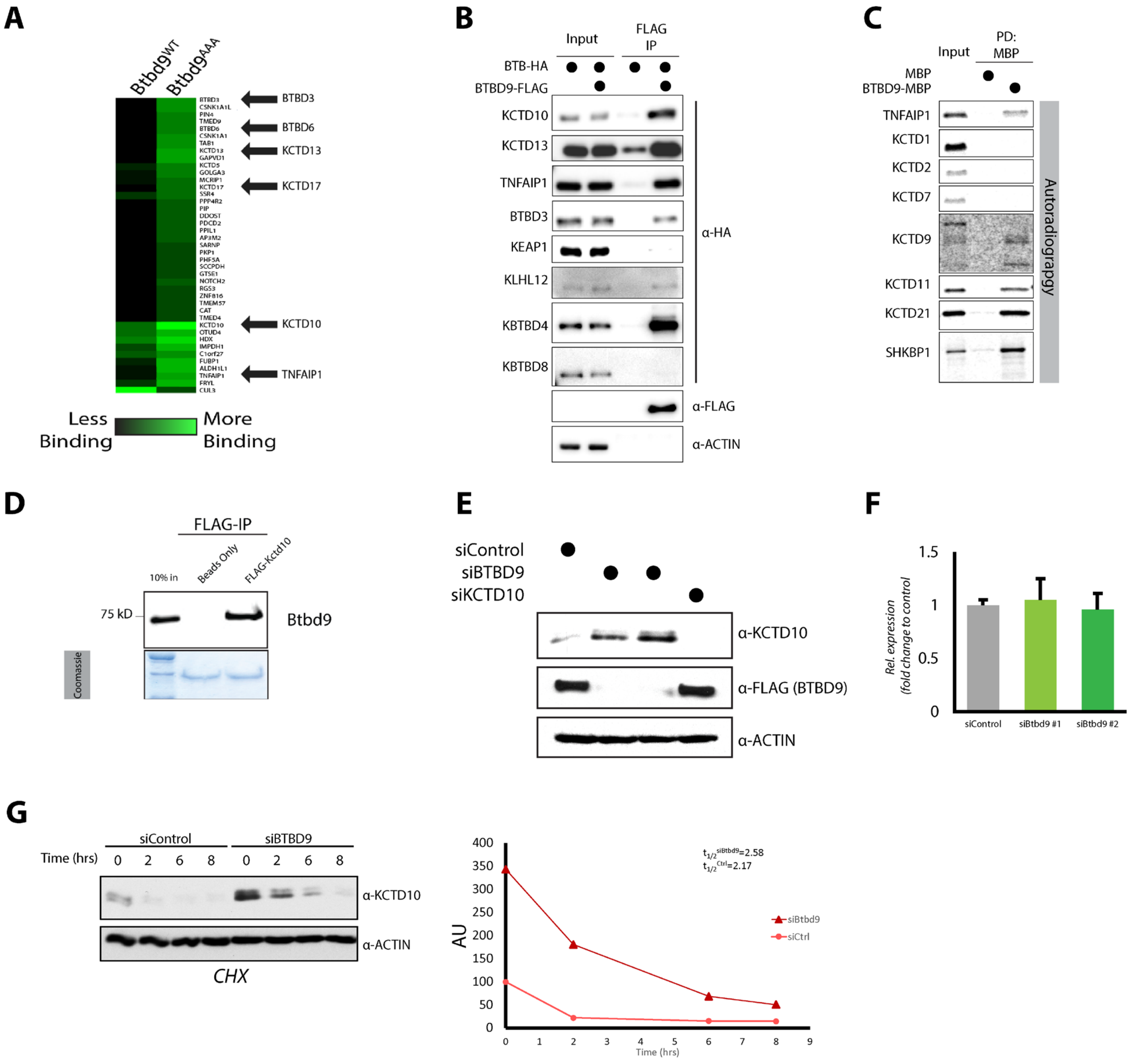


FIGURE 1 (CONTINUED)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Btbd9AAA
BTBD3
CSNK1A1L
PIN4
TMED9
BTBD6
CSNK1A1
TAB1
KCTD13
GAPVD1
KCTD5
GOLGA3
MCRIP1
KCTD17
SSR4
PPP4R2
PIP
DDOST
PDCD2
PPIL1
AP3M2
SARNP
PKP1
PHF5A
SCCPDH
GTSE1
NOTCH2
RGS3
ZNF816
TMEM57
CAT
TMED4
KCTD10
OTUD4
HDX
IMPDH1
C1orf27
FUBP1
ALDH1L1
TNFAIP1
FRYL
CUL3
BTBD3
BTBD6
KCTD13
KCTD17
KCTD10
TNFAIP1
Less Binding
More Binding
B
Input
FLAG IP
BTB-HA
BTBD9-FLAG
KCTD10
KCTD13
TNFAIP1
BTBD3
KEAP1
KLHL12
KBTBD4
KBTBD8
α-HA
α-FLAG
α-ACTIN
C
Input
PD: MBP
MBP
BTBD9-MBP
TNFAIP1
KCTD1
KCTD2
KCTD7
KCTD9
KCTD11
KCTD21
SHKBP1
Autoradiograpgy
D
FLAG-IP
10% in
Beads Only
FLAG-Kctd10
75 kD
Btbd9
Coomassie
E
siControl
siBTBD9
siKCTD10
α-KCTD10
α-FLAG (BTBD9)
α-ACTIN
F
1.5
1
0.5
0
Rel. expression (fold change to control)
siControl
siBtbd9 #1
siBtbd9 #2
G
siControl
siBTBD9
Time (hrs)
0 2 6 8 0 2 6 8
α-KCTD10
α-ACTIN
CHX
400
350
300
250
200
150
100
50
0
AU
0 1 2 3 4 5 6 7 8 9
Time (hrs)
$t_{1/2}^{siBtbd9}$=2.58
$t_{1/2}^{Ctrl}$=2.17
siBtbd9
siCtrl

FIGURE 2

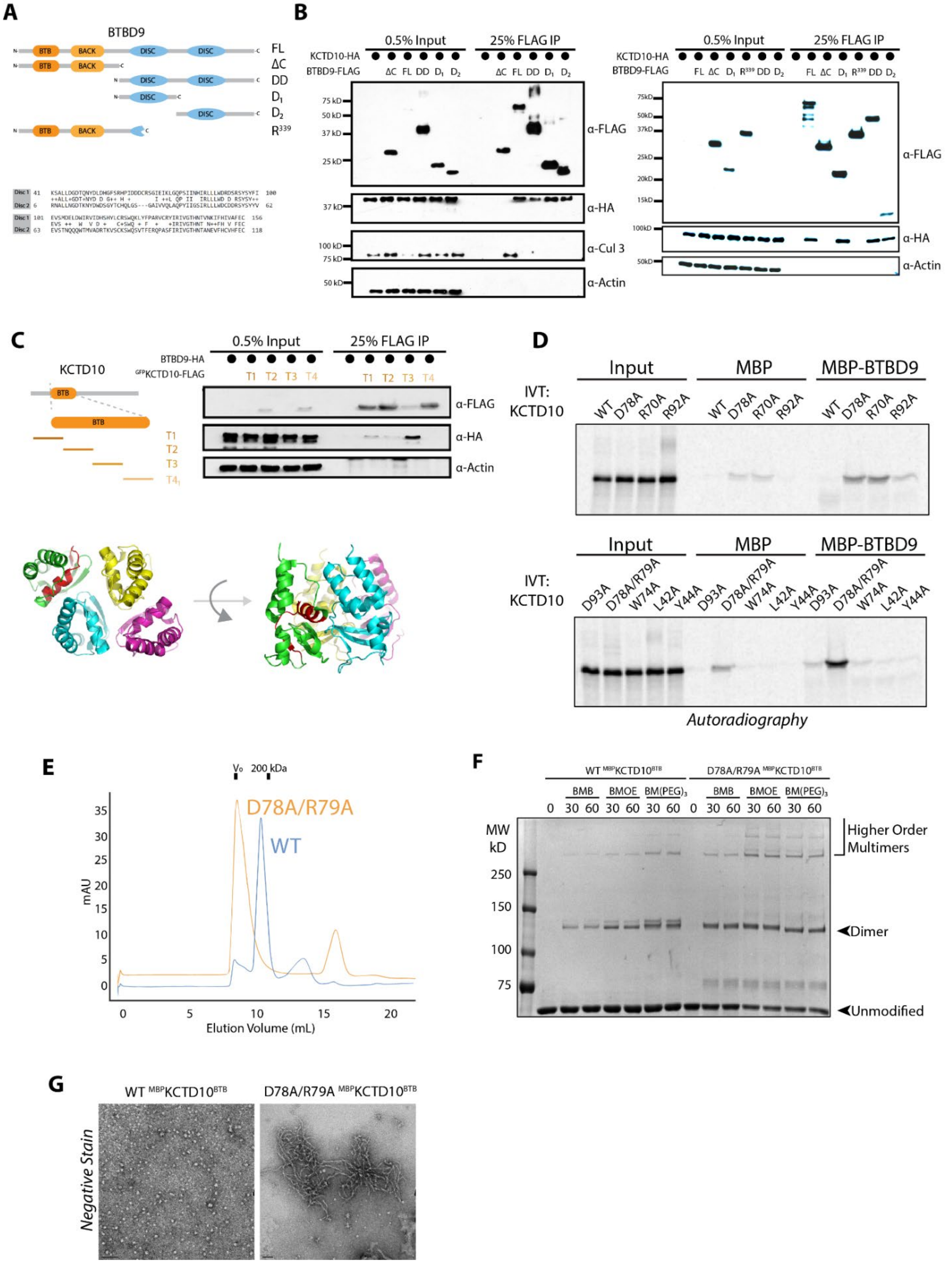
A
BTBD9
BTB
BACK
DISC
FL
ΔC
DD
D1
D2
R339
Disc 1 41 KSALLDGDTQNYDLDHGFSRHPIDDDCRSGIEIKLGQPSIINHIRLLLWDRDSRSYSYFI 100
++ALL+GDT+NYD D G++ H + I ++L QP II IRLLLWD D RSYSY++
Disc 2 6 RNALLNGDTKNYDWDSGYTCHQLGS---GAIVVQLAQPYIIGSIRLLLWDCDDRSYSYYV 62
Disc 1 101 EVSMDELDWIRVIDHSHYLCRSWQKLYFPARVCRYIRIVGTHNTVNKIFHIVAFEC 156
EVS ++ W V D + C+SWQ + F +IRIVGTHNT N++FH V FEC
Disc 2 63 EVSTNQQQWTMVADRTKVSCKSWQSVTFERQPASFIRIVGTHNTANEVFHCVHFEC 118
B
0.5% Input
25% FLAG IP
KCTD10-HA
BTBD9-FLAG
ΔC FL DD D1 D2
FL ΔC D1 R339 DD D2
75 kD
50 kD
37 kD
25 kD
20kD
15kD
100 kD
α-FLAG
α-HA
α-Cul 3
α-Actin
C
KCTD10
BTB
T1
T2
T3
T4
BTBD9-HA
GFPKCTD10-FLAG
0.5% Input
25% FLAG IP
α-FLAG
α-HA
α-Actin
D
Input
MBP
MBP-BTBD9
IVT: KCTD10
WT D78A R70A R92A
D93A D78A/R79A W74A L42A Y44A
Autoradiography
E
V0 200 kDa
D78A/R79A
WT
mAU
35
30
25
20
15
10
5
0
0 5 10 15 20
Elution Volume (mL)
F
WT MBPKCTD10BTB
D78A/R79A MBPKCTD10BTB
BMB BMOE BM(PEG)3
0 30 60 30 60 30 60
MW kD
250
150
100
75
Higher Order Multimers
Dimer
Unmodified
G
WT MBPKCTD10BTB
D78A/R79A MBPKCTD10BTB
Negative Stain

FIGURE 3

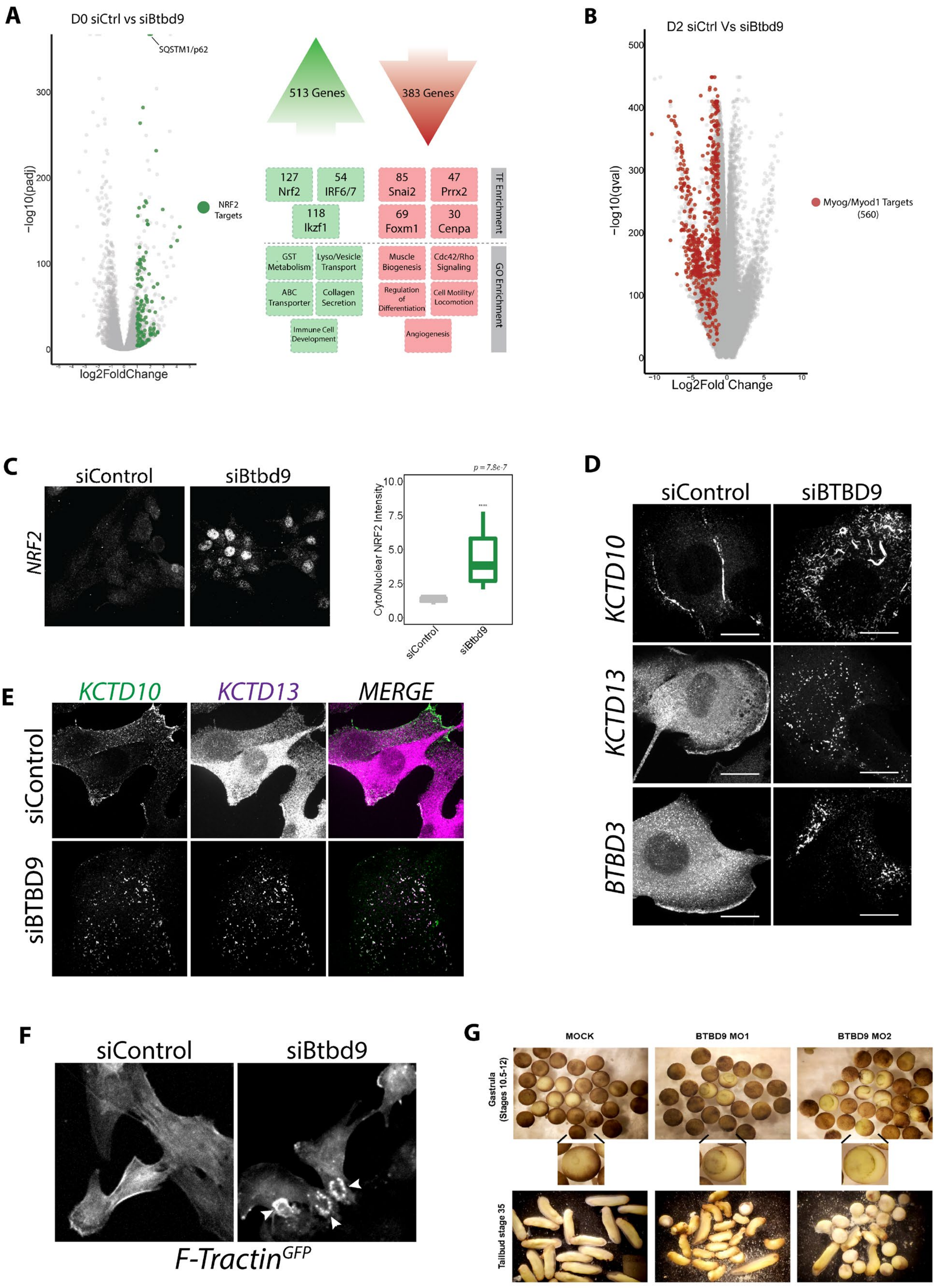
A
D0 siCtrl vs siBtbd9
SQSTM1/p62
300
200
100
0
−log10(padj)
log2FoldChange
NRF2 Targets
513 Genes
383 Genes
127 Nrf2
54 IRF6/7
118 Ikzf1
85 Snai2
47 Prrx2
69 Foxm1
30 Cenpa
TF Enrichment
GST Metabolism
Lyso/Vesicle Transport
ABC Transporter
Collagen Secretion
Immune Cell Development
Muscle Biogenesis
Cdc42/Rho Signaling
Regulation of Differentiation
Cell Motility/ Locomotion
Angiogenesis
GO Enrichment
B
D2 siCtrl Vs siBtbd9
500
400
300
200
100
0
−log10(qval)
Log2Fold Change
−10
−5
0
5
10
Myog/Myod1 Targets (560)
C
siControl
siBtbd9
NRF2
p = 7.8e-7
10.0
7.5
5.0
2.5
0.0
Cyto/Nuclear NRF2 Intensity
****
siControl
siBtbd9
D
siControl
siBTBD9
KCTD10
KCTD13
BTBD3
E
KCTD10
KCTD13
MERGE
siControl
siBTBD9
F
siControl
siBtbd9
F-TractinGFP
G
MOCK
BTBD9 MO1
BTBD9 MO2
Gastrula (Stages 10.5-12)
Tailbud stage 35

FIGURE 4

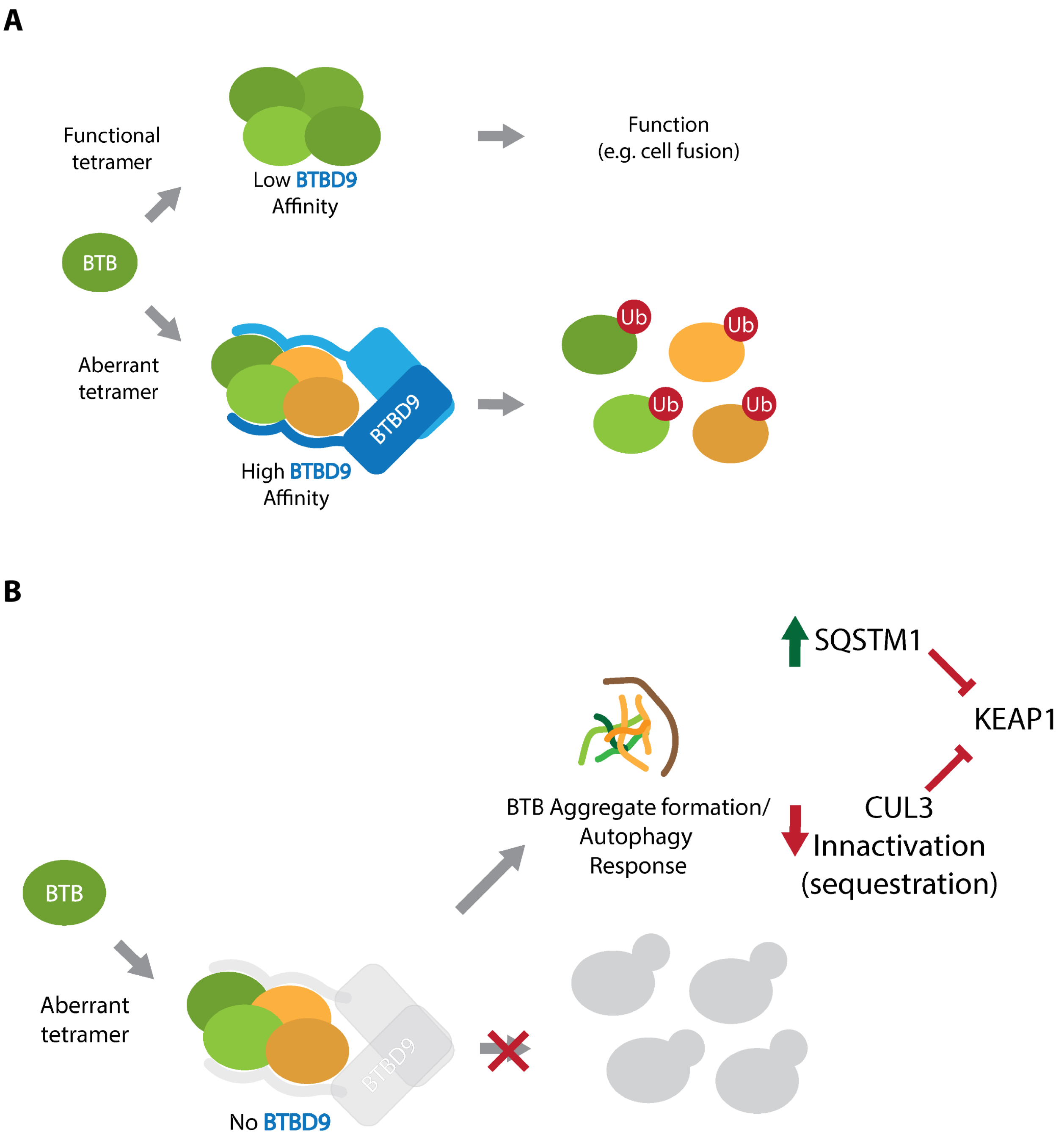
A
Functional tetramer
Low BTBD9 Affinity
Function (e.g. cell fusion)
BTB
Aberrant tetramer
BTBD9
High BTBD9 Affinity
Ub
Ub
Ub
Ub
B
SQSTM1
KEAP1
BTB Aggregate formation/ Autophagy Response
CUL3 Innactivation (sequestration)
BTB
Aberrant tetramer
BTBD9
No BTBD9

FIGURE 5

CHAPTER 5:

> **“I do not insist…that this is a full adventure, but it is the beginning of one, for this is the way adventures begin.”**
>
> -Miguel de Cervantes. *Don Quixote de la Mancha*

Closing remarks and a look into the future

In the extensive writings of this work, I have delved into the deepest corner of the abyss and have shed some light into the intricate underpinnings of ubiquitin biology. My hope is that you, the reader, will come away from these writings with a deeper understanding, nay, appreciation, of the role a tiny protein like ubiquitin has in orchestrating and regulating life on this planet. We began these writing by asking "what's in a name?". In the case of ubiquitin, we can see that its name is imbued in its cellular functions Being found ubiquitously in nature, it must be involved in every aspect of biology, and life for that matter. Interest in ubiquitin biology has grown dramatically. This work has clearly shown the extensive role that ubiquitin has in regulating cellular processes. Furthermore, ubiquitin signaling has proved to be versatile handle that can be used for the discovery of new and exciting biology, making it a fascinating field of study.

The work presented here showcases this multifaceted role that ubiquitin plays in various aspects of biology. Using a combination of powerful imaging tools for genetic screening, unbiased proteomic and next generation sequencing, combined with clever biochemical tools, we have discovered new aspects of cellular and developmental biology. Our work here has provided ubiquitin-dependent, mechanistic insight into the elusive process of cell-cell fusion, (Chapter 2). In chapter 3, we highlight the molecular and regulatory underpinnings of the reductive stress response and how cells respond to a lack of oxidative species. And finally in chapter 4, we describe multimerization quality control (MQC), a process that complements dimerization quality control (DQC). Together, the findings presented these works underscore the critical, and ubiquitous, regulatory roles of ubiquitin signaling in modulating robust cellular and organismal biology. These findings will pave the way for a more thorough understanding of metazoan development, and provide possible therapeutic avenues for pediatric disorders, and diseases such as cancers.

Monoubiquitylation serves as a negative regulator of actin bundling to drive cell-cell fusion

Cell-cell fusion is a poorly understood process that is critical for many facets of organismal development and homeostasis. From the very first step of metazoan development (ovum fertilization by sperm), to the end of life, cell-cell fusion is an event that is a critical event (Di Gioia, et al, 2017; Pereira, et al, 2018; Petrany and Millay, 2019; Yu, et al, 2018). Although cell fusion is known to occur in several cellular contexts, studying its molecular underpinnings is a challenging endeavor that requires robust model systems (Brukman, et al, 2019). Because myogenesis robustly undergoes cell-cell fusion, it has been widely used as a model system to study this elusive process (Brukman, et al, 2019; Deng, et al, 2016; Geisbrecht, et al, 2008). Some drivers of cell fusion in myogenesis (MYMX & MYMK) have been identified, however the underling molecular signaling pathways that regulated have remained unknown (Bi, et al, 2017; Millay, et al, 2013).Using a machine learning linear classifier, we combed through our trove of high-content screening imaging data to identify ubiquitin signaling component that inhibited cell fusion. This led us to the identification of the CUL3 substrate receptor KCTD10 as a critical driver of cell fusion.

Mass spectrometry and genetic experiments identified the actin bundling complex IRSp53:EPS8 as a key substrate of KCTD10 that was required to drive cell fusion. Furthermore, in my quest to define the consequence of ubiquitylation, we saw that KCTD10 was specifically monoubiquitylating EPS8. This suggested that ubiquitylation of EPS8 by KCTD10 was not degradative in nature. However, our genetic data suggested to us that this monoubiquitylation was negatively regulating the activity of EPS8. Further investigation revealed the this monoubiquitylation was inhibiting the actin bundling activity of the IRSp53:EPS8 complex by functioning as a steric block to prevent complex formation. Monoubiquitylation is thought to drive complex formation or modulate gene expression (Hicke, 2001; Komander and Rape, 2012; Yau and Rape, 2016), and so its function as a steric blocker to modulate catalytic activity was intriguing. The finding that monoubiquitylation can serve as a negative regulator of catalytic activity could be exploited as a therapeutic avenue for the treatment of disease.

An exciting facet of our work was the showcasing of the intersection between cytoskeletal biology and ubiquitin signaling. Many cancers exploit actin dynamics and signaling as a means to drive their pathogenicity and ease their spread throughout an organism (Brayford, et al, 2015; Olson and Sahai, 2008; Stevenson, et al, 2012). Because of this commonality amongst cancers, the actin cytoskeleton has become an attractive therapeutic target (Brayford, et al, 2015). Identifying monoubiquitylation by KCTD10 as a regulator of actin dynamics provides a new handle that can be exploited in these efforts, as monoubiquitylation by other E3 ligases could have a similar effect on substrates.

Although we have uncovered a novel ubiquitin-dependent mechanism that controls actin bundling and drivers cell fusion, there remains many questions to be answered. Our data show KCTD10 localized at of cell-cell contact sites, and we saw localization of EPS8 at cell-cell sites at the moment of cell fusion. What drives these proteins to these sites? Do they get there together? Does the ligase reside at these sites, and then the substrate localizes to said sites? Or vice versa? An open question is to try to discern the upstream signaling events that lead to this recruitment, and thereby allow for cell fusion to occur. Work has been done that shows changes in the lipid content at fusion sites (Bothe, et al, 2014). Several experiments in our lab have shown that KCTD10 and EPS8 are able to bind to phospholipids *in vitro*. This raises the possibility that lipid signaling is integrated into this ubiquitin-dependent regulatory circuit controlling cell-cell fusion. Being able to paint a complete picture of the regulatory underpinnings of cell fusion can be of tremendous benefit to the field of regenerative medicine. This would monumental for the treating of neuromuscular disorders, muscle wasting diseases, and other pediatric and developmental malignancies.

Ubiquitin signaling is an integral component of stress responses and quality control pathways.

Organisms, from unicellular prokaryotes to multicellular towering plants, face countless of daily environmental insults in order to just make it to the other day (Denhardt, et al, 2001; Edwards, et al, 2001). If that wasn't difficult enough, organisms also face internal challenges that come with the territory of what it means to be alive, such as damaged organelles due to overuse, the misfolding of proteins, or errors during DNA replication (Kroemer, et al, 2010; Lans, et al, 2019; Tyedmers, et al, 2010). Failure to respond to these plethora of stressors can be detrimental to life, and so organisms have evolved various stress responses and quality control mechanism to identify and respond to these insults (Costa-Mattioli and Walter, 2020; Harding, et al, 2003; Kroemer, et al, 2010). Because of their critical role in organismal survival and homeostasis, the molecular underpinnings of stress response pathways have been an exciting area of study. This interest has revealed the integration of ubiquitin signaling into these stress response and quality control pathways, showcasing the wide breadth of roles ubiquitin plays in maintaining cellular survival and homeostasis. (Duleh, et al, 2016; Harding, et al, 2003; Webster, et al, 2020; Williamson, et al, 2013).

In the work we have laid out in these chapters, we have furthered elucidated the highly dynamic role of ubiquitin in modulating organismal survival and homeostasis. We have uncovered a ubiquitin-dependent mechanism that senses cellular reductive stress (Chapter 3, the reductive stress response), and we have identified a new network that is responsible for modulating protein complex formation (Chapter 4, multimerization quality control). This work, along with the work presented in chapter 2, showcase the power of our high-content myogenesis screening platform. Indeed, this platform is still being used by new pupils in our group to shed light in new biological mysteries.

The discovery of the reductive stress response is an exciting complement to the already known oxidative stress response, and the hypoxia response, expanding our understanding into how cells sense oxygen and their redox state (interestingly, these trifecta of responses all converge with ubiquitin). The cellular redox state must be maintained in perfect harmony in order for cells to be able to carry out their functions. A persistent oxidative stress response can lead to a depletion of reactive oxygen species (reductive stress), which can have many deleterious effects. Indeed, these reactive oxygen species are critical for maintaining stem cell potency, and for the function of certain cells such as macrophages, which release reactive oxygen species as a means to combat intruders. Cells must be able to react to their environment in a rapid and timely manner, making ubiquitin a powerful and ideal regulator of stress responses. This can be exemplified by $CUL2^{VHL}$ and the hypoxic stress response, $CUL3^{KEAP1}$ and the reductive stress response, and now $CUL2^{FEM1B}$ with the reductive stress response (Bellezza, et al, 2018; Stebbins, et al, 1999). As these responses show, the maintenance of redox homeostasis is paramount for organismal development and survival. Furthermore, our work also shows that FEM1B is integrated into mTOR signaling and nutrient sensing, although its role into this remains unknown and is currently being investigated. The role of FEM1B as a sensor for reductive stress, and its integration into nutrient signaling make it an exciting and attractive candidate for drug development. Current efforts in our lab are actively exploring this possibility.

Complementing stress responses are quality control mechanisms, which are more fined-tuned but are equally important for metazoan survival and homeostasis. This has been exemplified extensively by studying various quality control mechanisms in the cell, such as ribosomal quality control, ER-associated protein degradation (ERAD) and more recently dimerization quality control (DQC). Unlike ERAD (or other protein quality control pathways) that target misfolded proteins, DQC is responsible for surveying protein complex formation and ensuring the correct compositions/stoichiometries are formed (Brandman and Hegde, 2016; Hwang and Qi, 2018; Mena, et al, 2018). Knowing that the majority of proteins form multimeric complexes *in vivo*, and that not all of them are dimers, led us to posit that other quality control mechanisms that survey higher-order protein complexes must exists. Our imaging screen led us to identify BTBD9 as a critical regulator of myogenesis, which led to the identification of multimerization quality control of BTBs. Although the phenotype has been well defined, more work is needed in order to further elucidate the mechanism by which multimerization quality control takes place. This will be an exciting and active area of investigation, providing a complementary pathway to DQC and further underscoring the diverse roles ubiquitin can play in regulating various quality control pathways.

In my quest to try to understand the molecular underpinnings and regulatory roles of ubiquitin signaling, I have stumbled into a motherlode of exciting new biology. This quest was like exploring a great abyss, in which we were able to shed some light to reveal its delicate workings. However, in this process we have also discovered that there is much more to this abys that remains to be explored. The work laid out in the preceding chapters highlights some important aspects of ubiquitin signaling. From the regulation of cell-cell fusion and myogenesis (CUL3^{KCTD10} in chapter 2), the discovery of a new stress response pathway (CUL2^{FEM1B} in chapter 3), and the elucidation of a new multimerization quality control pathway (CUL3^{BTBD9} in chapter 4), these writings show the intricate yet diverse roles of ubiquitin signaling in regulating metazoan development, survival, and homeostasis. Furthermore, this work has laid out the foundation for much more of what is to come. For "I do not insist…that this is a full adventure, but it is the beginning of one, for this is the way adventures begin" (De Cervantes, 1612).

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