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Rubicon Homology Proteins and their Role in Neurodegeneration

By

Dan Alexandru Tudorica

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Biophysics

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor James Hurley, Chair

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Abstract

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Professor James Hurley, Chair

Autophagy is a fundamental process of cellular quality control and maintenance that is used to protect cells during natural, healthy aging. Defects in autophagy are implicated in a host of pathologies of aging, notably neurodegenerative diseases. The Rubicon Homology (RH) proteins are a class of autophagy-regulating proteins defined by their RAB7A binding RH domains, consisting of the proteins Rubicon, Pacer, and PLEKHM1. These proteins bidirectionally regulate autophagy, as determined by the phosphorylation state of RAB7A.

Here, I demonstrate a mitochondrial autophagy (mitophagy) specific form of regulation for RH family proteins. Activation of PINK1 and Parkin in response to mitochondrial damage initiates a response that includes phosphorylation of RAB7A at Ser72. Rubicon is a RAB7A binding negative regulator of autophagy. The structure of the Rubicon:RAB7A complex suggests that phosphorylation of RAB7A at Ser72 would block Rubicon binding. Indeed, *in vitro* phosphorylation of RAB7A by TBK1 abrogates Rubicon:RAB7A binding. Pacer, a positive regulator of autophagy, has an RH domain with a basic triad predicted to bind an introduced phosphate. Consistent with this, Pacer-RH binds to phospho-RAB7A but not to unphosphorylated RAB7A. In cells, mitochondrial depolarization reduces Rubicon:RAB7A colocalization whilst recruiting Pacer to phospho-RAB7A-positive puncta. Pacer knockout reduces Parkin mitophagy with little effect on bulk autophagy or Parkin-independent mitophagy. Rescue of Parkin-dependent mitophagy requires the intact pRAB7A phosphate-binding basic triad of Pacer. Together these structural and functional data support a model in which the TBK1-dependent phosphorylation of RAB7A serves as a switch, promoting mitophagy by relieving Rubicon inhibition and favoring Pacer activation.

The stability of the Rubicon protein has recently been established as a natural regulator of autophagy flux in a variety of tissues (adipose, hepatic, cardiac, and neuronal), where cells will stabilize or degrade Rubicon as a means of inhibiting or promoting autophagy flux respectively. To develop a novel pharmacological inducer of autophagy, I sought to mimic this form of regulation by creating a small molecule heterobifunctional degrader of Rubicon that colocalizes Rubicon with the E3 ligase Cereblon, inducing ubiquitinylation and subsequent degradation.

To my family.

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Chapter 1

Mitochondrial autophagy and its role in neurodegeneration

Introduction – mechanisms of autophagy

Non-dividing, terminally differentiated cells are faced with a key problem: how to remain healthy and robust over the course of many decades. Human health depends on the ability of these cells—including fat cells, muscle cells, bone cells, and, indeed, neurons—to maintain strict watch over the biochemical processes that churn along within them for the entirety of a human's life. This is no small feat. The basic biology of these cells entails metabolic processes that generate reactive and dangerous intermediates, the strict partitioning of macromolecules, and a sometimes-fraught interplay between cells, tissues, and organ systems. Cells can face internal challenges in the form of variable genetic profiles and mutations that alter the effectiveness of the cell's machinery. Cells can also face external challenges: exposure to toxins, invasion by pathogens, and physical injury.

My work focuses on a mechanism by which cells surveil and protect themselves from these insults: the process known as “autophagy”. Autophagy, or cellular “self-eating” is a fundamental process by which the cell can isolate a portion of its cytoplasm in a double-membraned vesicle called the “autophagosome”. This autophagosome is then trafficked to the vicinity of the lysosome. The autophagosome and the lysosome fuse into a single vesicle, and the enzymes of the lysosomes reduce the contents of the autophagosome to their fundamental molecular building blocks. The amino acids, lipids, and carbohydrates generated by this process are released into the broader cellular pool and reused. This process originated as a starvation response: when nutrients are sparse, degrading existing macromolecules can help make up the deficit. Autophagy is thus regulated by many of the classic catabolic signaling pathways, including phosphorylation of autophagy proteins by the energy-sensing kinase AMPK, and inhibition of the master autophagy transcription factor TFEB by the amino acid-sensing complex mTORC1^{1,2}. Similarly, autophagy has a specialized role in the liberation of fat from adipocytes and the conversion of inert triglycerides to metabolically active free fatty acids in the liver. This autophagy of fat is termed “lipophagy” and follows the fundamental schema of autophagy. Lipophagy uses the same core proteins while featuring lipophagy-specific targeting and regulatory proteins³. Autophagy activation is a practical consequence of diet and exercise, and some of the benefit of these habits is mediated via activation and upregulation of autophagy^{4,5}. In short, autophagy plays a crucial role in standard cellular metabolism and physiology as the remodeling and trafficking arm of catabolism.

Evolution adapted this fundamental process to a wide variety of situations where some damaging agent needs to be isolated and degraded as soon as possible. The formation of the autophagosome allows rapid sequestration of the insult. Since these damaging agents might be robust to degradation—such as a whole bacterium⁶, or a non-soluble protein aggregate⁷—formation of an isolation body constitutes a key neutralizing step. Following this rapid isolation, the cell can leverage its most potent degradative enzymes in the lysosome to attempt to permanently neutralize the threat⁸. These forms of targeted autophagy include “xenophagy”, or autophagy of invading pathogens, “mitophagy”, or autophagy of damaged mitochondria, and “aggrephagy”, or autophagy of protein aggregates⁹.

The core proteins and mechanics of nonselective, or “bulk” autophagy are generally adapted to these newer targeted forms, while additional triggering and regulatory mechanisms are added on top of the existing autophagy machinery to generate specificity. In the most general sense, autophagy operates in three steps: Initiation, trafficking, and fusion, with regulatory proteins interacting with this process throughout (Figure 1).

Initiation begins via formation and activation of two crucial kinase complexes, the protein kinase ULK1 complex, and the lipid kinase complex PI3KC3-C1, and ends with lipidation of the membrane with the autophagosome marker LC3 and closure of the autophagosome. The ULK1 complex consists of the ULK1 catalytic subunit, as well as the obligate scaffolding subunits FIP200, ATG13, and ATG101. Knockout of any one of these factors renders cells wholly incapable of performing autophagy. The formation and activation of this complex is a crucial point of regulation in autophagy¹⁰. The complex contains multiple phosphorylation sites and receives multiple inputs from metabolic state sensors. Phosphorylation of Thr180 on ULK1, which occurs upon starvation, is necessary for kinase activity, and seems to be mediated by autophosphorylation upon complex formation. The starvation-active AMPK also phosphorylates ULK1 at multiple sites in its intrinsically disordered region (IDR), activating ULK1 and promoting autophagy initiation^{11,12}. In yeast, it was observed that the mTORC1 homologue, TORC1, phosphorylates ATG13 on its binding interface under rich conditions, preventing formation of the ULK1 complex¹³. Additionally, expression of these and other proteins responsible for autophagy initiation are governed by the transcription factor TFEB, which is phosphorylated, cytosolically retained, and therefore inactivated by mTORC1 under rich conditions^{14,15}. The early autophagosome structure is termed a “phagophore” and grows via direct contact with the endoplasmic reticulum and recruitment of vesicles positive for the lipid transfer protein ATG9^{10,16,17}.

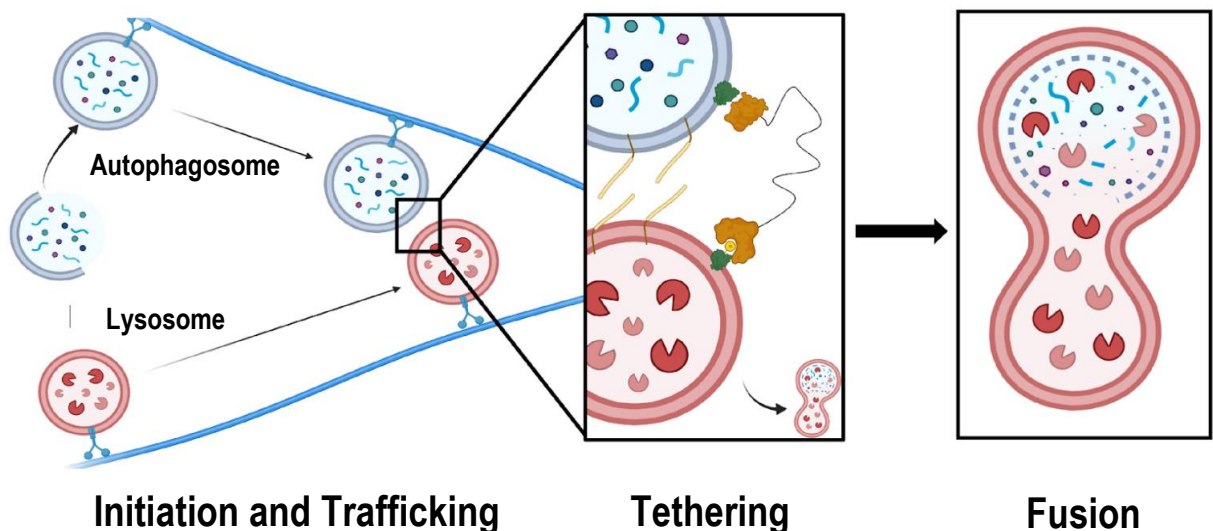


Figure 1. Basic autophagy mechanism

The most significant target of ULK1 is the PI3KC3-C1 complex, which is activated upon phosphorylation by ULK1. The PI3KC3-C1 complex consists of the lipid kinase domain VPS34, and the scaffolding/regulatory subunits VPS15, BECN1, and ATG14. There exists a variant of this complex, PI3KC3-C2, which substitutes ATG14 with UVRAG. PI3KC3-C2 is known to play a role in autophagosome and endosome maturation, while PI3KC3-C1 is responsible for autophagosome initiation. Activation and recruitment of PI3KC3-C1 to sites of autophagy initiation results in conversion of phosphatidylinositol (PI) lipids to Phosphatidylinositol 3-phosphate (PI3P). Formation of PI3P is a key marker of early autophagy, is a significant point of regulation, and is antagonized by regulatory lipid phosphatases. This regulation is particularly significant to cell types that are insensitive to starvation signaling, such as neurons^{10,18,19}.

Formation of PI3P results in direct binding and recruitment of WIPI family proteins, which coordinate the latter steps of autophagy initiation. Indispensable among this family is the isoform WIPI2. WIPI2 binds PI3P lipids directly, allowing it to recruit and coordinate the E3-like chemistry of the ATG12-5-16 complex, which covalently attaches the ubiquitin-like ATG8 family proteins to lipids on the surface of the autophagosome^{20,21}. This process is chemically analogous to the E1/E2/E3 reaction series that defines conjugation of Ubiquitin to substrate proteins. First, the ATG8 protein is proteolytically cleaved at the C-terminus by ATG4 family proteases to expose a glycine residue. This cleaved ATG8 is then activated via formation of a thioester bond with a cysteine of the E1-like protein ATG7. The LC3 is then transferred to the E2-like ATG3, and then interacts with the E3-like ATG-12-5-16 complex, which mediates lipidation of the ATG8 to phosphatidylethanolamine (PE) molecules on the phagophore. In humans, this family consists of 7 members (LC3A, LC3B, LC3B2, LC3C, GABARAP, GABARAPL1, and GABARAPL2), of which LC3B2 seems to be expressed only at very low levels^{22,23}.

For the rest of the lifetime of the autophagosome, a variety of proteins will recognize and bind to the autophagosome via the ATG8 molecules that decorate its surface. ATG8 family members vary in their binding affinity for various autophagy proteins and relative importance at different stages of autophagy. For instance, it was found that GABARAP proteins are particularly important for coordination of the tethering protein PLEKHM1 and bind this protein with a higher affinity than LC3²³. Consequently, GABARAP proteins hold specialized roles in mediating autophagosome-lysosome fusion. Recognition of LC3 via LC3-Interacting Regions (LIRs) is a conserved strategy by which autophagy can be targeted to specific cargos and forms the basis for targeted forms of autophagy. These *cargo receptors* consist of cargo-specific interacting regions and a LIR, which mediates attachment to growing phagophores²⁴. Cargo receptors lend specificity, but are not necessary for basic, bulk autophagy to occur. Notable cargo receptors include P62, NBR1, TAX1BP1, NDP52, CALCOCO1, and Optineurin, all united by their ability to bind Ubiquitin²⁵. Different forms of selective autophagy involve different sets of these cargo receptors, and the relative importance of each varies depending on cellular context.

As the phagophore grows it requires additional lipids to mediate this expansion. Formation of PI3P results in recruitment of WIPI4, which in turn mediates recruitment of the lipid-transfer protein ATG2, a rod-shaped lipid bridge^{26,27}. ATG2 tethers the phagophore to the endoplasmic reticulum and allows transfer of lipids to the growing phagophore^{28,29}. This process

of lipid acquisition is also mediated by the lipid scramblase ATG9, which both marks vesicles that provide an additional source of lipids and maintains the symmetry of the nascent phagophore as new lipid molecules are added^{17,30}.

Autophagosome initiation completes as the autophagosome begins to seal. This process is governed by curvature-sensing proteins as well as factors related to intrinsic membrane physics, and its final steps are mediated by ESCRT family membrane scission proteins. Specifically, the ESCRT-I subunit VPS37A is required for autophagosome closure and is followed by recruitment of the ESCRT-III component CHMP2A. Following this, autophagosomes associate with motor proteins and are trafficked towards the nucleus, followed by fusion with the lysosome^{31,32}. This maturation process is promoted by recruitment of the RAB family small GTPases RAB7A, RAB8B and RAB24, which become tethered to the autophagosome via their lipid-conjugated tails. The RAB family proteins RAB1, RAB5, and RAB7 have been shown to regulate autophagosome formation as well. Multiple studies have found that RAB7A is recruited to sites of mitochondrial damage and autophagy initiation and is required for robust mitophagy flux^{33,34}. As GTPases, these proteins cycle between an active (GTP-bound) and an inactive (GDP-bound) state, promoted via interaction with activating Guanine nucleotide Exchange Factor proteins (GEFs) and inactivating GTPase-activating proteins (GAPs)³⁵. Cycling of RAB family proteins between their active and inactive states regulates the timing of autophagosome formation and trafficking.

Autophagy completes when the autophagosome fuses with the lysosome. Following coarse colocalization of the autophagosome with the lysosome, a series of tethering proteins bridge the two vesicles. The HOPS complex as well as the Rubicon Homology (RH) protein PLEKHM1 are known to act as tethers³⁶⁻³⁸. The HOPS complex binds RAB7A, while PLEKHM1 has a putative RAB7A binding domain, and modestly interacts with RAB7 *in vitro* in my hands³⁹. These long-range interactions give way to much closer ones as SNARE proteins present on both the autophagosome and lysosome interact to mediate fusion⁴⁰.

Dysfunctions in autophagy can occur at any stage, and disease mutants have been identified in a wide variety of autophagy proteins, spanning the whole process. In addition to mutants that clearly ablate an important mechanistic protein in autophagy, it is likely that differential expression levels of a variety of autophagy factors can alter the frequency and flux of autophagy from person to person, and potentially underlies the sporadic nature of many neurodegenerative and metabolic diseases⁴¹⁻⁴³. While two organisms might both be capable of doing autophagy, they might yet vary in their frequency, and thus the degree to which cellular insults are caught and eliminated before damage can develop and spread. This argument is particularly compelling in the case of aggrephagy and protein aggregates, which likely increase in severity and difficulty of clearance the longer detection, isolation, and elimination takes to occur.

As a result, it is of paramount importance to understand the mechanistic and regulatory steps that underpin autophagy, and what auxiliary regulatory factors may set the rate of autophagy over the course of a lifetime. Many of the most damaging and intractable diseases of our time persist because most cases are sporadic in nature, and are thus resistant to traditional,

target-first approaches. Developing a clear model for how rates of flux through autophagy are set is the first step in designing therapeutic strategies that target the pathway as a whole.

Dysfunctions of Autophagy

Diseases of autophagy emerge because of impaired quality control over the course of a lifetime, and injury is often a result of consequences secondary to an accumulation of deleterious substances. Failure to dispose of cytotoxic aggregates often results in their export to the extracellular space, which in turn triggers immune cell infiltration and inflammatory damage; this process appears to contribute to some of the most common diseases of aging, including macular degeneration^{44,45} and chronic kidney disease⁴⁶. A similar model can be applied to neurodegenerative diseases, where a failure to properly dispose of damaging aggregates results in spread of these aggregates, cytotoxicity, activation of immune cells, and inflammation-triggered cell death. Over the last two decades, autophagy defects have been established as one of the key factors underlying a wide range of neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS), and Frontotemporal Dementia (FTD)^{47,48}. Evidence includes genetic studies, mechanistic work, mouse modelling, and patient-level expression data. While the relative importance of autophagy to the etiology of each of these diseases varies, studies largely point to autophagy as a neuroprotective process. In certain cases, autophagy defects have been definitively established as causing neurodegeneration.

The most notable example of this is the protective role of mitophagy in Parkinson's disease. Dysfunctions in mitochondrial damage sensing mapping to the PARKIN protein results in faulty mitophagy initiation and early onset, familial Parkinson's disease^{49,50}. This form of Parkinson's, termed PARK2 Parkinson's disease, comprises the largest fraction of patients with familial Parkinson's disease. Other mutations along this pathway are associated with sporadic cases of Parkinson's disease, further demonstrating the relevance of this pathway⁵¹. Other autophagy-relevant protein mutations result in different neurodegenerative conditions, potentially illustrating differential roles of autophagy by neuron and cell type. Mutations in RAB7A appear to result in defective autophagy closure and trafficking, and cause Charcot-Marie Tooth disease, a degenerative nerve condition⁵²⁻⁵⁴. Failure to clear SOD1 and TDP-43 protein aggregates typify ALS, and TBK1, a mitophagy initiating factor, is one of the most commonly mutated genes in familial ALS. Optineurin, a cargo receptor and substrate of TBK1, is also a commonly mutated gene in ALS, strongly indicating that the development of ALS in these instances is autophagy mediated⁵⁵. One of the key pathologies that characterize many neurodegenerative diseases is dysfunctional and deacidified lysosomes, affecting the final stages of autophagy⁴⁷. While autophagy appears to play many roles in neuronal homeostasis, its role in maintaining mitochondrial health has been the focus of intense research since the characterization of the PARKIN protein.

Mitochondrial homeostasis and mitophagy

The mitochondrion is the principal site of energy generation in eukaryotic cells and is particularly important to energetically demanding cells such as myocytes and neurons. Mitochondria can also pose risks to the surrounding cell when damaged or dysregulated. As the site of oxidative phosphorylation, mitochondrial metabolism results in the production of reactive oxygen species (ROS) that can irreversibly alter the chemical composition of molecules within the cell if not properly managed⁵⁶. Perforation of mitochondria and release of the protein cytochrome C is an initiating step in apoptosis⁵⁷. Mitochondria also contain their own, chemically distinct mitochondrial DNA (mtDNA) that potentially activates immune responses via the cGAS-STING pathway⁵⁸. This DNA comprises a separate mitochondrial genome, notably encoding for indispensable components of the electron transport chain. However, mitochondria lack much of the proofreading and repair enzymes that the nuclear genome possesses, resulting in elevated rates of mutation over a lifetime, and an increased risk of collapse as organisms age. Though not necessarily shown to be causative in all cases, mitochondrial dysfunction is a hallmark of neurodegeneration broadly speaking, potentially implicating poor mitochondrial DNA proofreading in the pathogenesis of neurodegeneration conditions⁵⁹. The most prominent marker of poor mitochondrial health is the inability for discrete mitochondria to maintain the integrity of the electron transport chain, and therefore their proton gradient. This depolarization serves as a signal for the cell to target mitophagy to the damaged mitochondrion^{49,60}.

The most well-characterized mitophagy pathway is the PINK-PARKIN system. This depolarization-sensing system targets mitochondria with depleted proton gradients for isolation and degradation. The kinase PINK1 acts as a mitochondrial polarity sensor; under basal conditions, it is constitutively transported into the mitochondrial lumen where it is degraded by resident proteases. Since mitochondrial transporters only function when the mitochondria are polarized, depletion of the proton gradient results in stabilization of PINK1 on the outer surface of mitochondria. There, it recruits, phosphorylates and activates the E3 ligase PARKIN, which assembles ubiquitin chains on the surface of the damaged mitochondrion⁶¹⁻⁶³. These ubiquitin chains act as substrates for the autophagy cargo receptors NDP52 and OPTN, which coordinate association between the mitochondrion and autophagic structures via their ubiquitin-binding domains and LIRs respectively. This process is further coordinated by the small GTPase RAB7A and its GTPase activating protein (GAP), TBC1D15. Cycling of RAB7A between these states plays a role in coordinating the expansion of the autophagosome around the target mitochondrion, and defects in this process result in malformed autophagosomes^{64,65}. Mitophagy is also promoted by the kinase TBK1, best known for its role in innate immunity signaling. Here, TBK1 phosphorylates and activates the cargo receptor Optineurin, promoting binding of Optineurin to ubiquitin chains. Optineurin also provides a scaffold for TBK1, positioning it to hetero-auto phosphorylate and activate^{66,67}. While some amount of mitophagy can still occur absent TBK1, the process is much less efficient. An additional role of TBK1 is to phosphorylate RAB7A at serine 72. This residue is located on a common binding interface of RAB family proteins in the Switch II region, providing a hint that this phosphorylation event modulates some RAB7A effector. While this, too, is dispensable for mitophagy, cells bearing non-phosphorylatable mutants of RAB7A suffer from a roughly 50 percent defect in mitophagy flux

upon treatment with mitochondrial depolarizers, indicating a significant impairment somewhere in the mitophagy process⁶⁸. The mechanism by which RAB7A phosphorylation promotes mitophagy was not known prior to this work.

It is intuitive that this process would be characterized by several auxiliary factors that improve the speed and sensitivity of mitophagy as a quality control system: neuron loss is highly damaging to the health of the organism, and there are no means of regenerating lost neurons. Furthermore, the ideal rate of mitophagy turnover likely varies from tissue to tissue, suggesting a role for these auxiliary enhancers of mitophagy. Differences in the frequency and speed of mitophagy, mediated by the pleiotropic effects of higher or lower expression of relevant proteins, provides an interesting explanation for the highly sporadic nature of neurodegenerative diseases. To illustrate, loss of function mutation in PARKIN results in a form of Parkinson's disease that accounts for less than 2% of all cases, with an onset of earlier than age 40. To contrast, most Parkinson's disease cases have no clear genetic basis, with an average age of onset of around 60⁵¹. This suggests that decreased efficiency of this pathway, rather than wholesale loss of mitophagy activity, may be a more realistic model for mitophagy defects. Given that Parkinson's disease is typified by a spreading phenotype, where early aggregation of the prion-like alpha synuclein spreads from cell to cell, and inflammatory damage can result in worsening damage over the course of the disease, it is reasonable that small lapses in mitochondrial damage detection can spiral into more extensive neurodegeneration.

Thus, it is important to understand not just the indispensable components of mitophagy, but also its regulators. As a result, I aimed to develop a better understanding of the mechanism by which phosphorylation of RAB7A promotes mitophagy. To better understand this process, I would have to find RAB7A binding proteins that act to promote or inhibit mitophagy, and then determine how this interaction might be modified by phosphorylation of RAB7 at serine 72. A promising family of RAB7 interactors that also regulate autophagy are the Rubicon Homology (RH) family of proteins.

Rubicon Homology Proteins

The Rubicon Homology proteins are a family of autophagy regulating proteins defined by their unique, L-shaped c-terminal domain, termed the "Rubicon Homology" (RH) domain. The most prominent and ancient representative of this family, present in all metazoans, is the autophagy inhibitor Run domain Beclin-1-interacting and cysteine-rich domain-containing protein, or more concisely, Rubicon. Rubicon consists of an N-terminal RUN domain, a small, helical PI3K binding domain, and its c-terminal RH domain, all linked together by an extensive Intrinsically Disordered Region (IDR)⁶⁹. The C-terminal RH domain has been demonstrated to bind RAB7A using biochemical, cell biological, and structural approaches. Mutations that ablate this binding interaction also inactivate Rubicon, promoting autophagy⁷⁰. The PI3K binding domain interacts with PI3KC3-C2 by binding to and restraining the BARA domain of the BECN1 subunit. This inhibits the ability of the BARA domain to associate the complex with membranes⁷¹. Since the PI3K binding domain is distal to the RH domain, it is unlikely that

RAB7 binding activates Rubicon via allostery. It is much more likely that binding to RAB7 localizes Rubicon, positioning it to inhibit autophagy. Rubicon is also believed to inhibit the fusion step of autophagy by some means^{69,72,73}.

The second family member, Protein associated with UVRAG as autophagy enhancer, or Pacer, is the inverse of Rubicon. Pacer consists of a C-terminal RH domain with no demonstrated binding activity prior to the start of this project, a long IDR, and a helical PI3K binding domain similar in structure to Rubicon's. Computational predictions of the structure of this PI3K binding domain generated via AlphaFold match those of Rubicon's. How one protein can be an activator while the other is an inhibitor despite having structurally identical interacting regions remains an open question. Pacer is a known activator of starvation autophagy in certain contexts, and overexpression of Pacer results in increased autophagosome initiation, as well as promotion of autophagosome-lysosome fusion. Pacer is known to bind to the SNARE protein STX17 via co-immunoprecipitation, further suggesting some role in fusion^{36,74}.

While direct evidence of binding between RAB7A and an RH family protein exists only in the case of Rubicon, sequence conservation across the domain and particularly on the binding interface of the RH domains suggest that the RH domain in Pacer may also be able to bind RAB7. Crystallographic data indicates that serine 72 of RAB7A is present on a loop that threads into the binding cleft of RUBICON, further supporting the idea that phosphorylation of RAB7 in the early stages of mitophagy could interact with this family of regulator proteins⁷⁰.

The final RH family member is the protein Pleckstrin homology and RUN domain containing M1 or PLEKHM1. This protein consists of a C-terminal RH domain of ambiguous binding activity, and an N-terminal RUN domain, and a long IDR between the two. PLEKHM1 appears to play two principal roles in autophagy: it acts as a molecular tether between

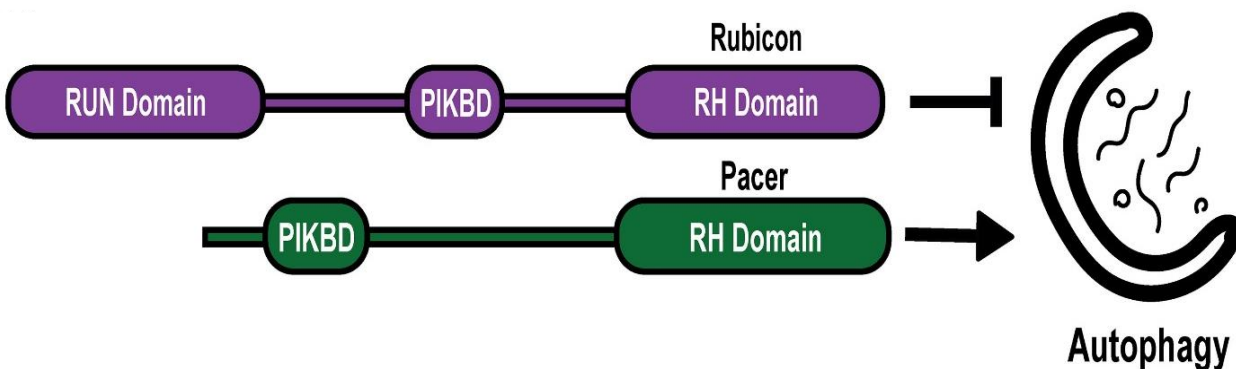


Figure 2. Domain maps of Rubicon and Pacer. Rubicon and Pacer both bind UVRAG via their PIK Binding Domains (PIKBD), and both possess Rubicon Homology (RH) domains. Rubicon is an autophagy inhibitor, while Pacer is an autophagy activator.

autophagosomes and lysosomes in the final, fusion steps of autophagy, and it acts as an adaptor between autophagosome surface proteins and the transport proteins that act to colocalize autophagosomes with lysosomes³⁹. In my hands, I find only weak binding of PLEKHM1 to both RAB7 and pS72 RAB7.

RAB7A phosphorylation

RAB7A is recruited to mitochondria following mitochondrial damage in a process regulated by RABGEF1 and TBC1D15/17, a RAB7A GEF and GAP respectively^{64,65}. RAB phosphorylation has emerged as a major theme in the regulation of membrane traffic, and in PD in particular⁷⁵. RAB7A is phosphorylated at Ser 72 by Tank-binding kinase 1 (TBK1) and leucine rich repeat kinase 1 (LRRK1)^{68,76,77}. An extensive body of data supports a central role for TBK1 in PINK1/Parkin-dependent mitophagy^{60,78-81}. Finally, and most directly, RAB7A Ser72 phosphorylation is necessary for efficient PINK1/Parkin-dependent mitophagy⁶⁸. However, the functional role of this phosphorylation event was not elucidated prior to this work.

Phosphorylation of RAB7 appears relevant to mitophagy only in the case of PINK-PARKIN mitophagy. Induction of mitochondrial damage via iron chelation and the subsequent adaptor-mediated mitophagy does not result in formation of pS72 RAB7. Phosphorylation of RAB7A appears to be an auxiliary mechanism by which pro-autophagy factors are activated, and anti-autophagy factors are disabled, potentially in order to accelerate autophagy in instances where mitochondrial damage is acute, and isolation needs to occur rapidly.

Here, I show that binding of RAB7A to the RH family proteins Rubicon and Pacer constitutes a bidirectional switch. Under basal conditions, Rubicon binds unphosphorylated RAB7A and antagonizes autophagy. When RAB7A is phosphorylated following mitochondrial depolarization, Rubicon is displaced, Pacer is recruited, and mitophagy is thereby accelerated. This switch is governed by structural differences in the RH domains of Rubicon and Pacer that have evolved to selectively bind dephospho-RAB7A and pSer72-RAB7A, respectively.

Chapter 2

Bidirectional regulation of mitochondrial autophagy by Rubicon and Pacer

Contents of this chapter have been previously published in:

Tudorica... Hurley et al. A RAB7A phosphoswitch coordinates Rubicon Homology protein regulation of Parkin-dependent mitophagy (2024). *Journal of Cell Biology*.

Rubicon inhibits Parkin-dependent mitophagy

The negative regulatory role of Rubicon in starvation-induced autophagy and xenophagy has been well-characterized^{73,82}, but it is unknown if Rubicon regulates PINK1/Parkin-dependent mitophagy. Rubicon KO HeLa cells were generated (Figure 4) and stably transfected with either LC3B-HaloTag to examine bulk autophagy or HA-Parkin and Su9-GFP-HaloTag to examine Parkin-dependent mitophagy. Activation of autophagy induces the engulfment of these reporters within autophagosomes, trafficking to lysosomes, and degradation. In order to initiate the assay, cells were pulsed with membrane permeable fluorescent HaloLigand, which binds HaloTag and renders it resistant to lysosomal processing⁸³ (Figure 3). Bulk autophagy was induced by incubation in Earle's Balanced Salt Solution (EBSS). Mitophagy was induced by treatment with the electron transport chain inhibitors Oligomycin and Antimycin A (OA). Autophagy flux was measured as the fraction of the pulsed reporter digested within a given interval.

Rubicon knockout strongly increased the extent of both starvation-mediated (by 55%) and basal autophagy (by 47%) (Figure 5A, B), consistent with past reports^{69,84}. We found that Rubicon knockout also increased mitophagy flux, though to a lesser degree than for starvation-induced autophagy (23% increase in mitophagy flux over 6 h of depolarization) (Figure 5C, D). The significantly smaller impact of Rubicon KO on mitophagy flux indicates that there is a mechanism that renders mitophagy more resistant to Rubicon inhibition than starvation-induced autophagy.

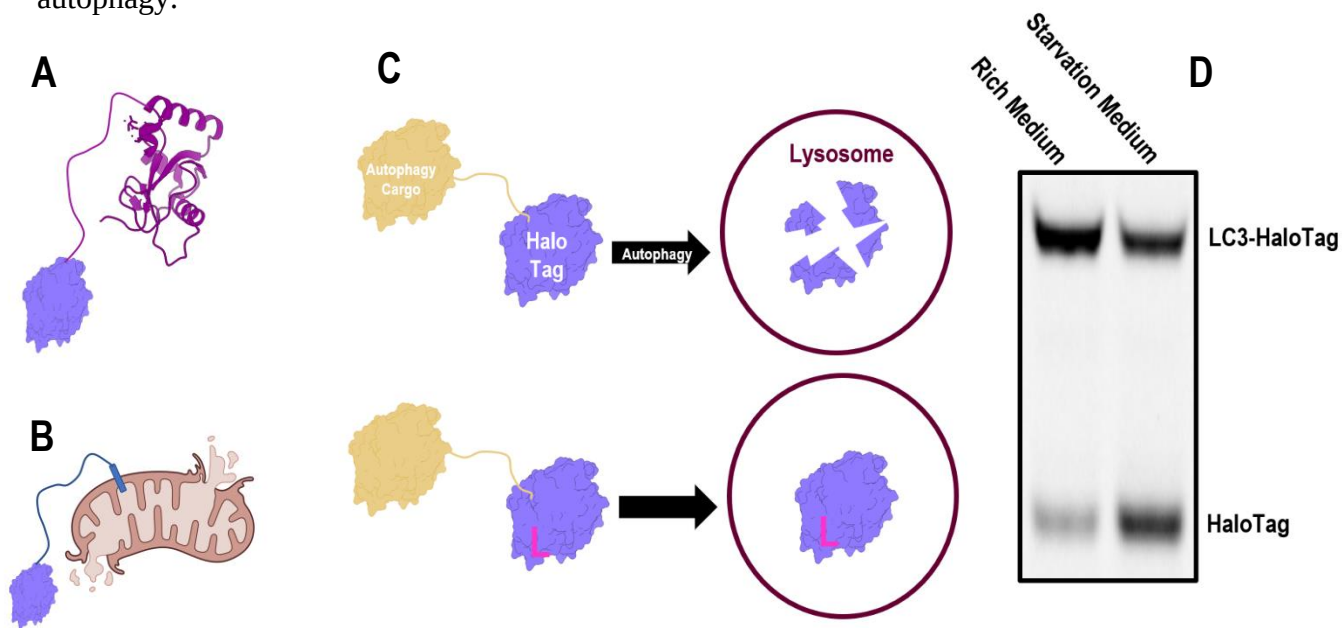


Figure 3. HaloTag Autophagy Flux Reporters. (A) LC3-HaloTag bulk autophagy and (B) mitochondrial ATP synthase Subunit 9 (Su9) HaloTag reporters. (C) Reporter mechanism. The reporter is cleaved upon delivery to the lysosome, resulting in formation of a lower MW band when lysates are ran on a gel. HaloTag resists rapid degradation only when bound by Halo Ligand, allowing for quantitative, pulse chase experiments, and lowering experimental background. (D) Sample flux gel. Autophagy activation results in a shift from the upper, unprocessed reporter band to the lower, cleaved HaloTag band.

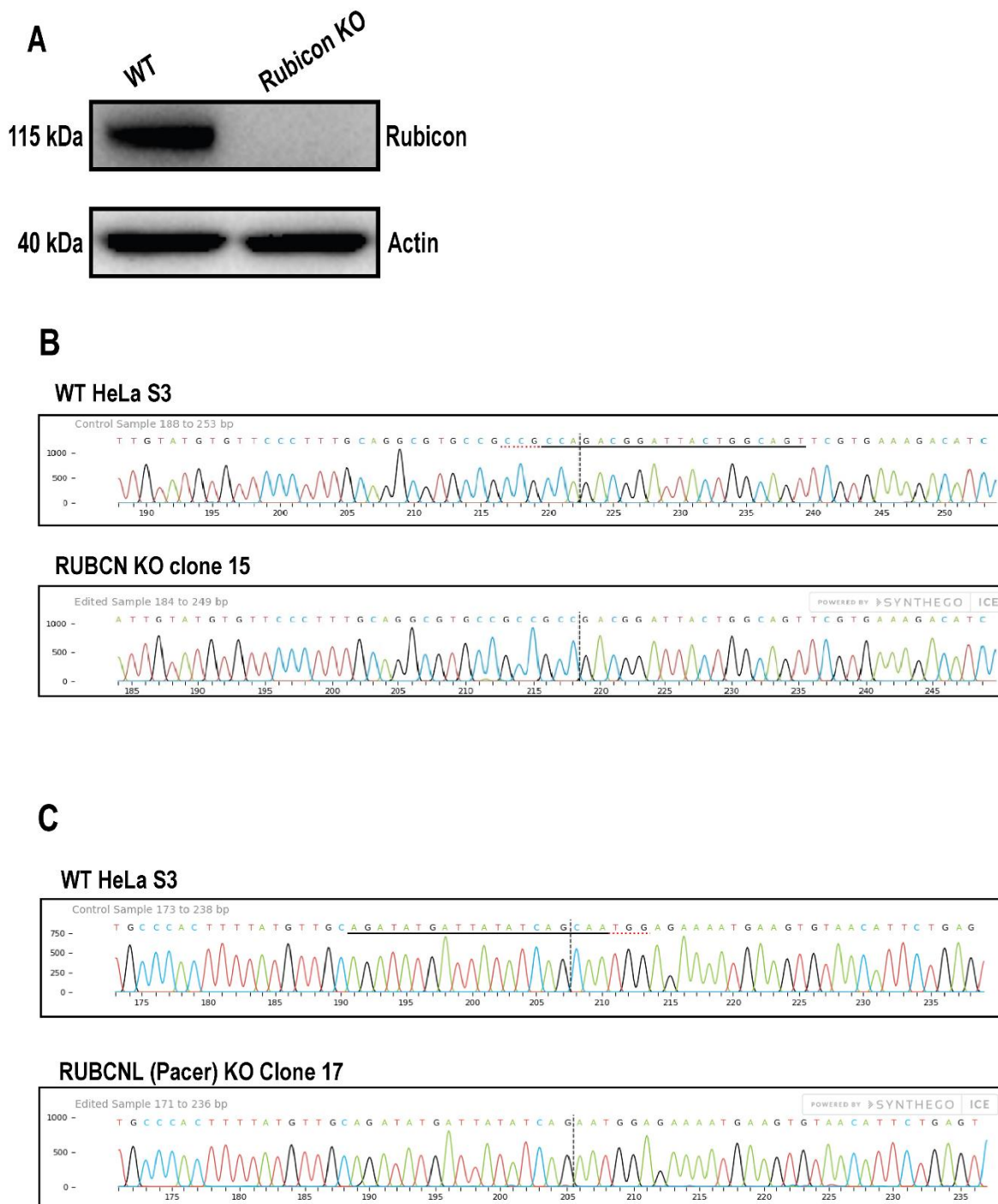


Figure 4. Validation of CRISPR KO of Rubicon and Pacer. (A) Immunoblot confirming knockout of Rubicon. Blot with molecular weight marker is depicted in Figure S4D. (B) Sanger sequence view showing Rubicon KO and WT sequences in the region around the guide sequence. The horizontal black line indicates the guide sequence. The horizontal red line is the PAM site. The vertical black dotted line represents the actual cut site. (C) Sequence view showing Pacer KO, represented as in (B).

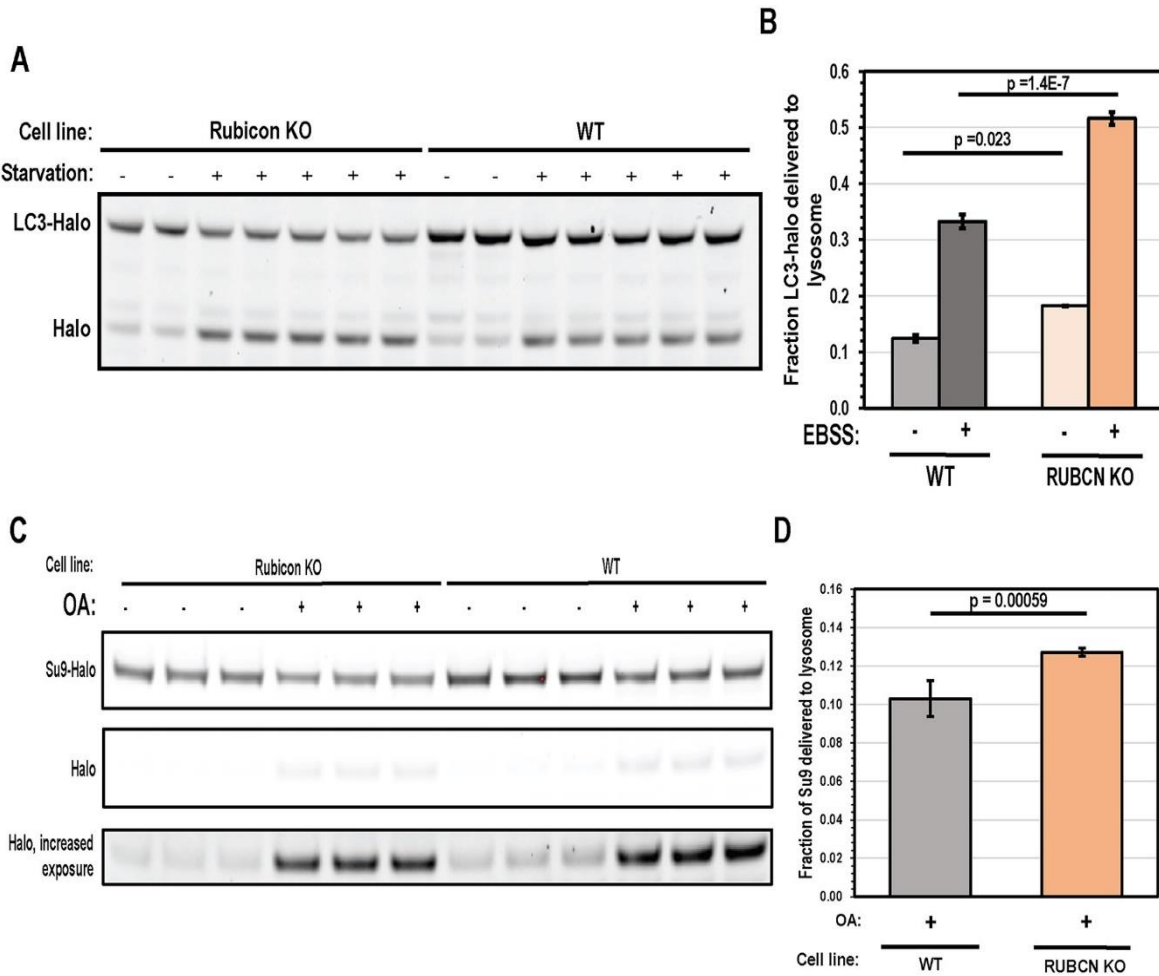


Figure 5. Rubicon depletion promotes bulk autophagy and Parkin mitophagy. (A) LC3-HaloTag processing assay used to assess starvation autophagy in WT and Rubicon KO HeLa cells. Cells incubated in either complete growth medium or EBSS for 3 hours prior to collection and analysis. (B) Quantification of starvation autophagy flux in panel A. Autophagy flux was quantified as the fraction of HaloTag signal in the lower, processed band relative to total signal ($n = 2$ for rich conditions, $n = 4$ for starvation conditions). Significance scores derived from a one-tailed T-test. Error bars indicate standard deviation. (C) Su9-GFP-HaloTag processing assay used to assess mitophagy flux in Parkin expressing WT and Rubicon KO HeLa cells. Cells were either incubated in rich medium, or rich medium supplemented with 10 μ M Oligomycin A and 5 μ M Antimycin A for 6 H. (D) Quantification of mitophagy flux in panel C. Mitophagy flux was quantified as the fraction of HaloTag signal in the lower, processed band relative to total signal ($n = 6$ for WT and Rubicon KO). Significance scores derived from a one-tailed T-test. Error bars indicate standard deviation. All statistical replicates were drawn from independent experiments, and each lane contains lysates from independent experiments.

Phosphorylation of RAB7A Ser72 controls binding to RH domains

The RH domain of Rubicon binds to RAB7A, and RAB7A phosphorylation at Ser72 is required for efficient mitophagy flux. We noticed that in the crystal structure of the Rubicon RH:RAB7A-GTP complex, Ser72, part of the GTP-dependent switch II region of RAB7A, is in direct contact with a sterically confined region of the RH domain⁷⁰. We therefore asked whether the phosphorylation state of RAB7A modulated RUBICON binding. We also considered the possibility that RAB7A phosphorylation might regulate Pacer, since Pacer also contains an RH domain (Figure 8). However, our structural modeling suggested that the analogous region of interaction with RAB7A is less sterically confined.

To probe these interactions, we expressed and purified recombinant Rubicon and Pacer RH domains, RAB7A, and one of the key kinases known to phosphorylate RAB7A, TBK1⁶⁸. Incubation of RAB7A with TBK1 in the presence of ATP resulted in stoichiometrically phosphorylated pSer72 RAB7A. Assay of this material using a PhosTag gel, which slows the migration of phosphorylated proteins and resolves distinct phosphorylated and unphosphorylated bands, confirmed that the kinase reaction proceeded essentially to completion (Figure 6B). To assay for RAB7A binding by purified RH domains, we used a confocal microscopy-based bead binding assay. Amylose beads were pre-loaded with MBP-RH domains, and incubated in a solution of the Alexa Fluor 647-labelled GTP-locked RAB7A Q67L (Figure 6A). The Rubicon RH domain robustly bound unphosphorylated RAB7A, but did not observably bind phosphorylated RAB7A. The opposite was true for the Pacer RH domain. Pacer-RH robustly bound only to Ser72 pRAB7A, with little to no binding observed to unphosphorylated RAB7A (Figure 6C). We followed up on these results, measuring the binding affinity of Rubicon RH for RAB7A via isothermal titration calorimetry (ITC). This binding interaction is characterized by a K_D of $1.7 \pm 0.3 \mu\text{M}$ (Figure 7). This represents a moderate affinity interaction, consistent with a regime where phosphorylation-induced affinity changes could effectively regulate the interaction between Rubicon and Pacer-binding competent states for RAB7A.

To attempt to explain why phosphorylation of RAB7A has opposing effects on binding to the Rubicon and Pacer RH domains, homology modelling was used to generate predictions for the Pacer-pRAB7A binding interface (Figure 8). These models were then compared to the crystal structure of the Rubicon RH:RAB7A complex⁷⁰. The Pacer RH domain structure has a prominent basic patch that is absent in the Rubicon RH domain and appears poised to bind the pSer72 phosphate. Basic residues Lys534, Arg623, and Arg628 within Pacer are all close enough to the negatively charged pSer72 phosphate to form direct salt bridges. In contrast, the Rubicon crystal structure shows that phosphorylation of Ser72 creates a steric clash by introducing the bulk of the phosphate group into a constricted site⁷⁰.

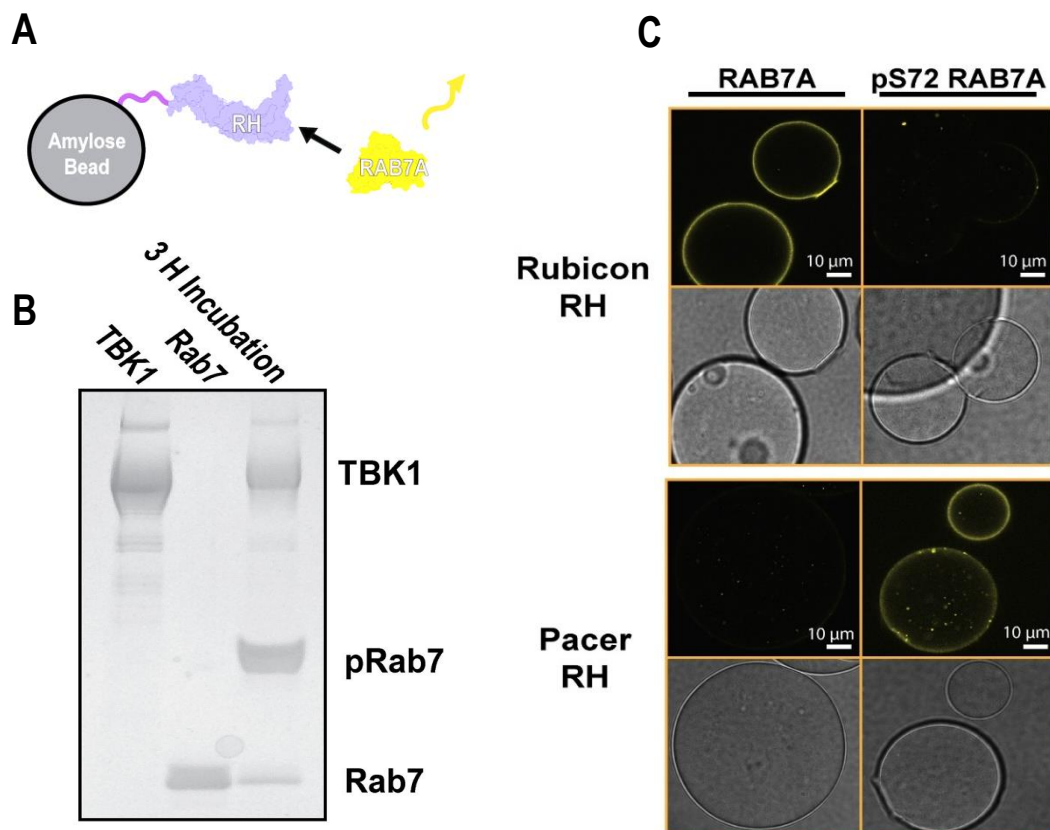


Figure 6. Confocal bead binding (A) Confocal based fluorescent bead-binding assay schematic. Amylose beads were functionalized with MBP-RH domain, and then incubated in solutions of either phosphorylated or unphosphorylated RAB7A chemically conjugated to a fluorescent dye. (B) PhosTag gel showing in vitro phosphorylation of RAB7A using recombinantly purified TBK1. Here, TBK1 was mixed with Q67L, GTP-locked RAB7A for 3h at room temperature, supplemented with ATP and Mg. PhosTag gels slow the migration of phosphorylated proteins. (C) Amylose beads were incubated in 500 nM MBP-RH domain and 2 μ M Alexa Fluor 647647-labelled RAB7A or pS72 RAB7A prior to washing and imaging on a confocal

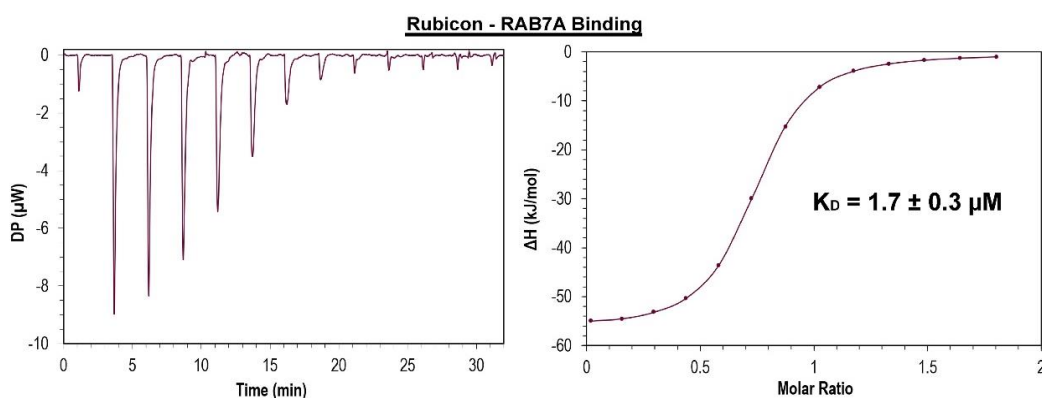


Figure 7. Isothermal Titration Calorimetry (ITC) of Rubicon binding to RAB7A. Injection plot (left) and binding curve (right) of Rubicon binding to RAB7A. 13 injections performed at 25 C with 150 second delays between injection. Data was fit to a binding curve assuming a simple, single binding site mechanism. Binding curve best fit suggest $n = 0.7$.

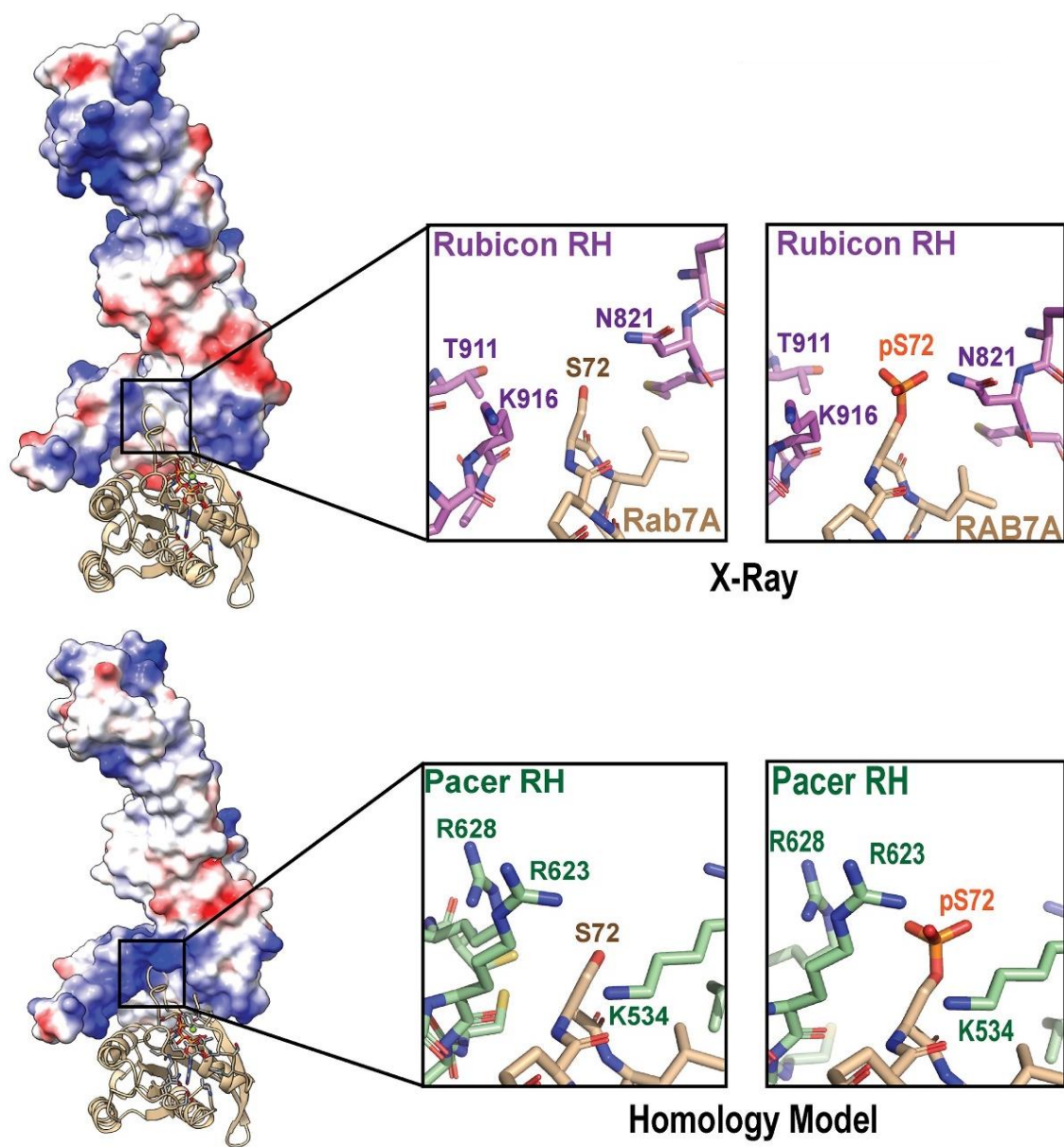


Figure 8. Crystal structure of the Rubicon RH domain in complex with RAB7A (top, PDB: 6wcw), and a homology model of the Pacer RH domain in complex with RAB7A (bottom, SwissModel). Coloration represents electrostatic potential (blue = positive charge, red = negative charge)

Phosphorylation of RAB7A Ser72 controls subcellular localization of RH proteins

In order to determine whether the *in vitro* binding preferences were correlated with subcellular localization, we transfected Parkin-expressing HeLa cells with mCherry-Rubicon, and performed immunofluorescence using anti-RAB7A and anti-pSer72-RAB7A (Abcam ab302494) (Figure 9A, B). In these cells, Rubicon clusters near the nucleus and co-localizes with RAB7A. In contrast, pSer72 RAB7A puncta were found throughout the cytoplasm and colocalized only sporadically with Rubicon. We found that Rubicon is more than three-fold more likely to colocalize with RAB7A than with pSer 72 RAB7A, consistent with the *in vitro* finding that RAB7A phosphorylation negatively regulates Rubicon binding (Figure 9C).

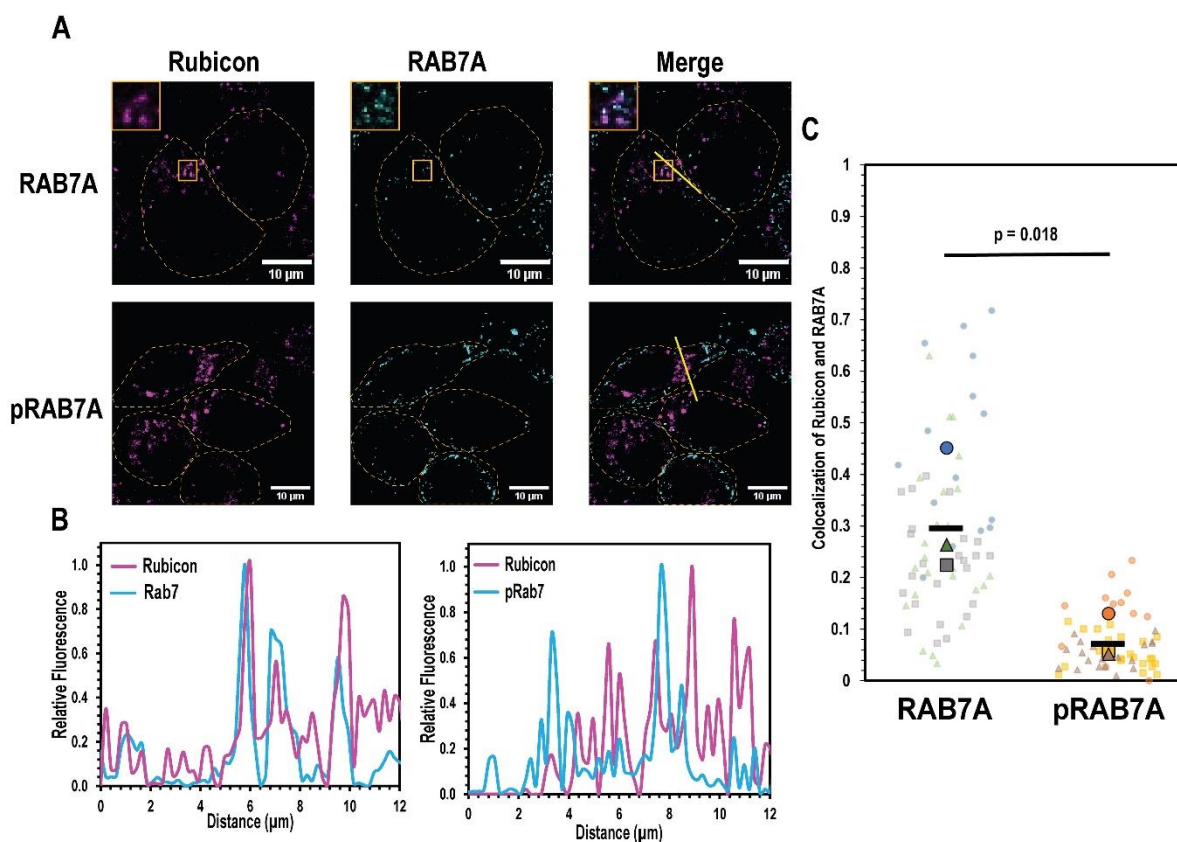


Figure 9. pRAB7A binding preferences dictate subcellular localization. (A) WT HeLa cells expressing untagged Parkin were transfected with mCherry labelled, full-length Rubicon, and then immunofluorescence was performed using general and pS72-specific RAB7A antibodies alongside an Alexa 488 secondary antibody. Rubicon-RAB7A colocalization was examined under rich conditions, and Rubicon-pRAB7A was quantified following 1 H depolarization with 10 μM CCCP. Insets are digitally expanded images, 3.7 μm wide. (B) Line scans indicated in RAB7A (left) and pRAB7A (right) imaging trials. Each point on the line scan was baseline subtracted, and then normalized to the maximum signal on the line scan for each channel. (C) Colocalization was calculated as the above-threshold Mander's colocalization coefficient representing the fraction of Rubicon-positive pixels that are also RAB7A/pRAB7A positive. For each experiment, ~20 cells were analyzed in this way (transparent markers), and then the average of these 20 measurements constitutes one biological replicate (solid markers). The average of 3 biological replicates is represented by the horizontal bar marker. The p statistic was calculated using a one-tailed, paired T test comparing average colocalization fractions measured on the same day.

A RH domain basic triad controls pRAB7A binding

To test the structural model for phosphorylation-dependent RAB7A binding, we generated a triple Rubicon RH mutant that contains the Pacer basic triad at the equivalent residues (Rubicon N821K, T911R, K916R), and performed the confocal bead binding assay using these mutants. The Rubicon mutant recruited pRAB7A to beads more effectively than it recruited RAB7A to beads (Figure 10A). Overall, it appears that this mutation enabled Rubicon to bind pRAB7A at the cost of a moderate decrease in its ability to bind RAB7A (Figure 10B). This suggests that the Pacer basic triad is responsible for conferring binding to pSer72 RAB7A. Reciprocally, mutants of the Pacer RH domain that swap these residues to their Rubicon equivalents (K534N, R623T, R628K) were generated, purified, and assayed in the fluorescent bead binding assay. The recruitment of pRAB7A was sharply diminished by even one of these mutations, while the double and triple mutants resulted in nearly complete loss of pRAB7A binding (Figure 11). These mutations did not, however, confer on Pacer the ability to bind dephosphorylated RAB7A.

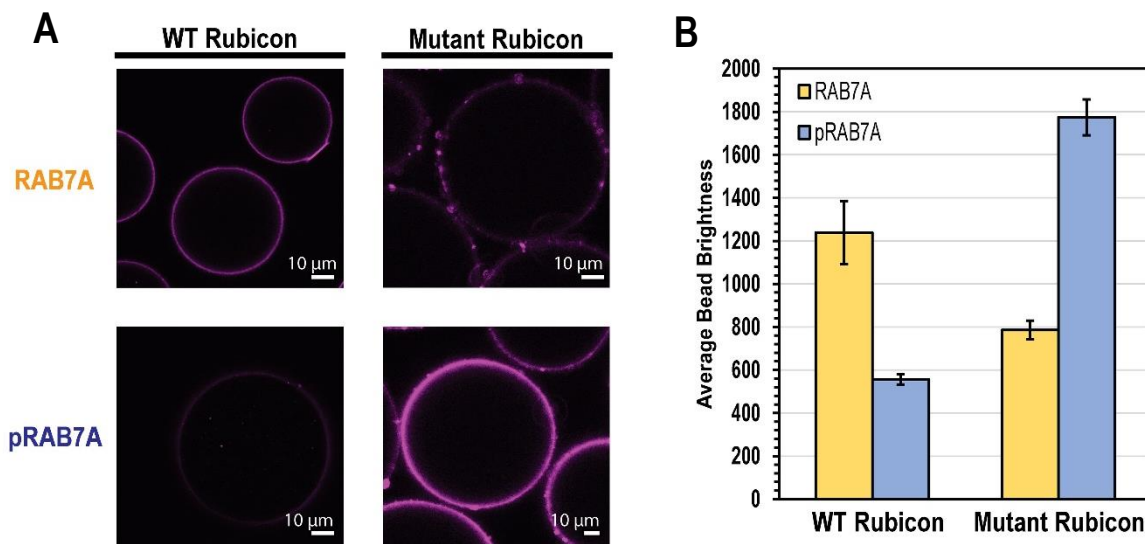


Figure 10. Confocal bead-binding assay of Rubicon mutant, conducted as in Figure 6C. (A) Amylose beads were incubated with either MBP-Rubicon RH or Mutant MBP-Rubicon RH N821K, T911R, K916R. Rab7/Rubicon Image derived from the same source image as Figure 2D. (B) Quantification of panel A. Briefly, an ROI was defined on the edge of a bead to be analyzed, and the maximum pixel value in the ROI was recorded as the brightness of the bead. Each condition consists of 3 independent experiments in which the brightness of ~20 beads was measured and averaged. Error bars indicate standard deviation of these independent experiments.

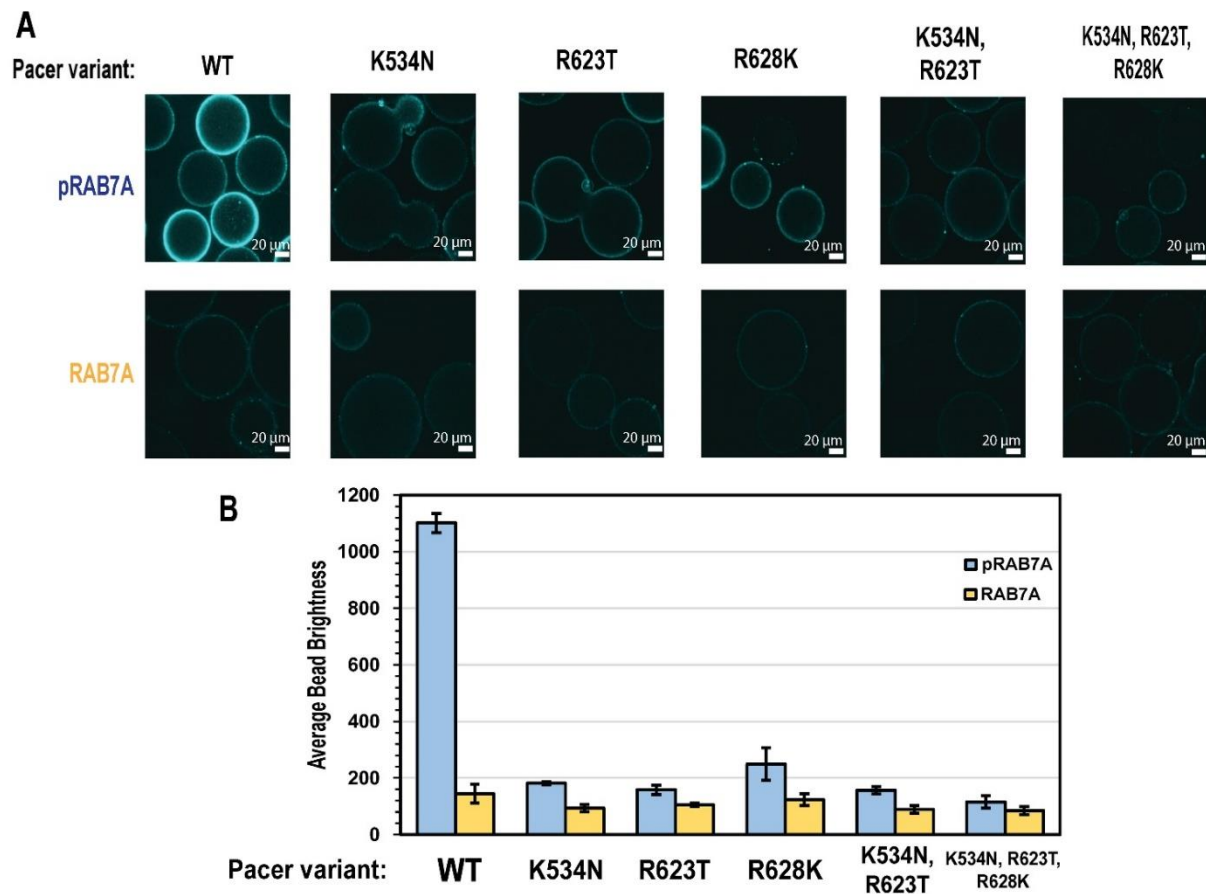


Figure 11. Fluorescent bead binding assay of Pacer mutant. (A) Experiment was performed and quantified as in Figure 7. Mutants of MBP-Pacer RH with the listed mutant were used in these experiments. (B) Quantification of panel A, conducted as in Figure 3E. Each experiment consists of ~20 different beads measured and averaged. This was repeated 3 times, and the mean of these biological replicates is plotted. Error bars indicate standard deviation of separate experiments

To determine the role of the RAB7A phosphoswitch on the localization of Pacer in cells, we compared the colocalization of wild-type and mutant Pacer with pRAB7A. Stable Parkin-expressing HeLa cells were transiently transfected with mCherry-tagged Pacer or K534N/R623T Pacer, fixed, and imaged by confocal microscopy (Figure 12). Pacer formed sparse punctate structures evenly distributed throughout the cell, in contrast to the perinuclear localization of Rubicon. Pacer co-localized extensively with pRAB7A (40%), while only about 10% of Pacer^{K534N/R623T} did so (Figure 12C), demonstrating that these residues are critical for proper Pacer localization. These data show that both Rubicon and Pacer binding to, and colocalization with, RAB7A is controlled by the phosphorylation state of RAB7A Ser72, which is in turn subject to induction by the same conditions that induce mitophagy.

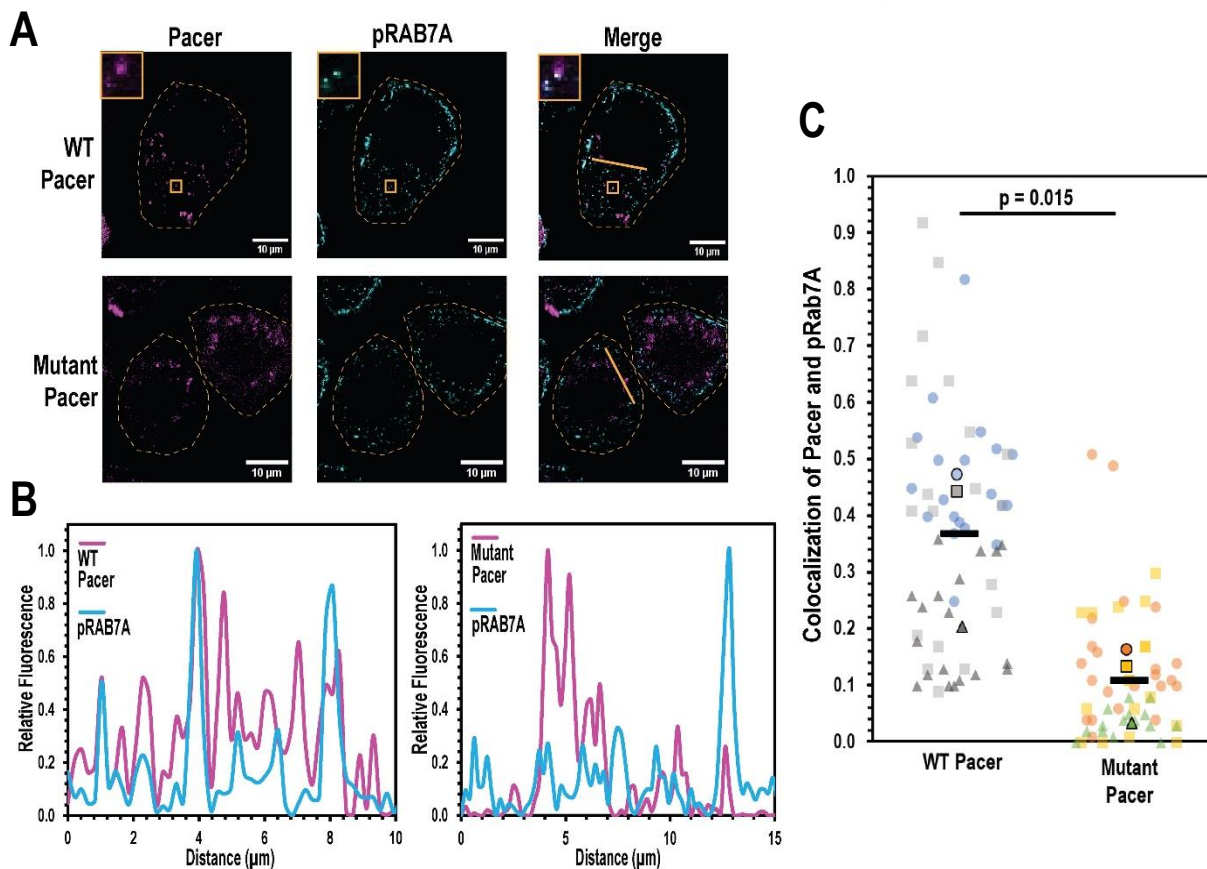


Figure 12. Colocalization of mCherry-wild-type Pacer and mCherry-Mutant (K534N, R623T) Pacer. Cells were transfected with the listed Pacer construct, and depolarized for 1 H using CCCP. Immunofluorescence was performed using the pS72 specific antibody of RAB7A, and the Alexa 488 secondary antibody was used to mark pRAB7A. Insets are digitally expanded images, 2.8 μm wide. (D) Linescans indicated in panel C, calculated as in Figure 3B. (E) Colocalization of WT and Mutant Pacer with pRAB7A, calculated as in Figure 3C.

Pacer regulates PINK/Parkin-dependent mitophagy

Pacer was reported to upregulate starvation-induced autophagy³⁶, but roles in selective autophagy have not been reported on in the literature. To compare the roles of Pacer in bulk autophagy and mitophagy, Pacer knockout HeLa cells were generated via CRISPR-mediated deletion (Figure 4), and the autophagy and mitophagy flux reporters LC3B-and Su9-GFP-HaloTag were stably transfected to probe bulk autophagy and mitophagy.

Pacer knockout HeLa cells displayed significantly reduced mitophagy flux following mitochondrial depolarization with OA, (Figure 13A, B). To probe the contribution of pRAB7A binding to Pacer activity, Parkin and mitophagy reporter-expressing cells were stably transfected with vector, wild-type Pacer, and Pacer^{K534N, R623T}. These cells were depolarized for 2 and 4 hours, and mitophagy flux was measured. Rescue with WT Pacer was evident after 2 hours, and mitophagy flux after 4 hours increased by up to 47% relative to KO cells. In contrast, the Pacer^{K534N, R623T} mutant was unable to promote mitophagy flux above the levels seen in Pacer KO cells, suggesting that pRAB7A binding is indispensable to the mitophagy-promoting activity of Pacer (Figure 13C, D).

To determine if Pacer might have a role in Parkin-independent mitophagy, these same cells were treated with the iron chelator deferiprone (DFP). DFP-mediated mitochondrial iron loss results in increased expression of mitophagy receptors including NIX and BNIP3 through HIF1a signaling, initiating mitophagy without activating the PINK-PARKIN pathway⁸⁵⁻⁸⁷. Consistent with this, treatment of cells with DFP does not result in an increase in pS72 Rab7 as measured via immunofluorescence (Figure 14). There was no discernible difference between Pacer KO cells with vector control or wild-type or mutant Pacer at 8, 16, or 24 H (Figure 13). This indicates that Pacer has a specialized, pS72 Rab7-dependent role in PINK1/Parkin-dependent mitophagy.

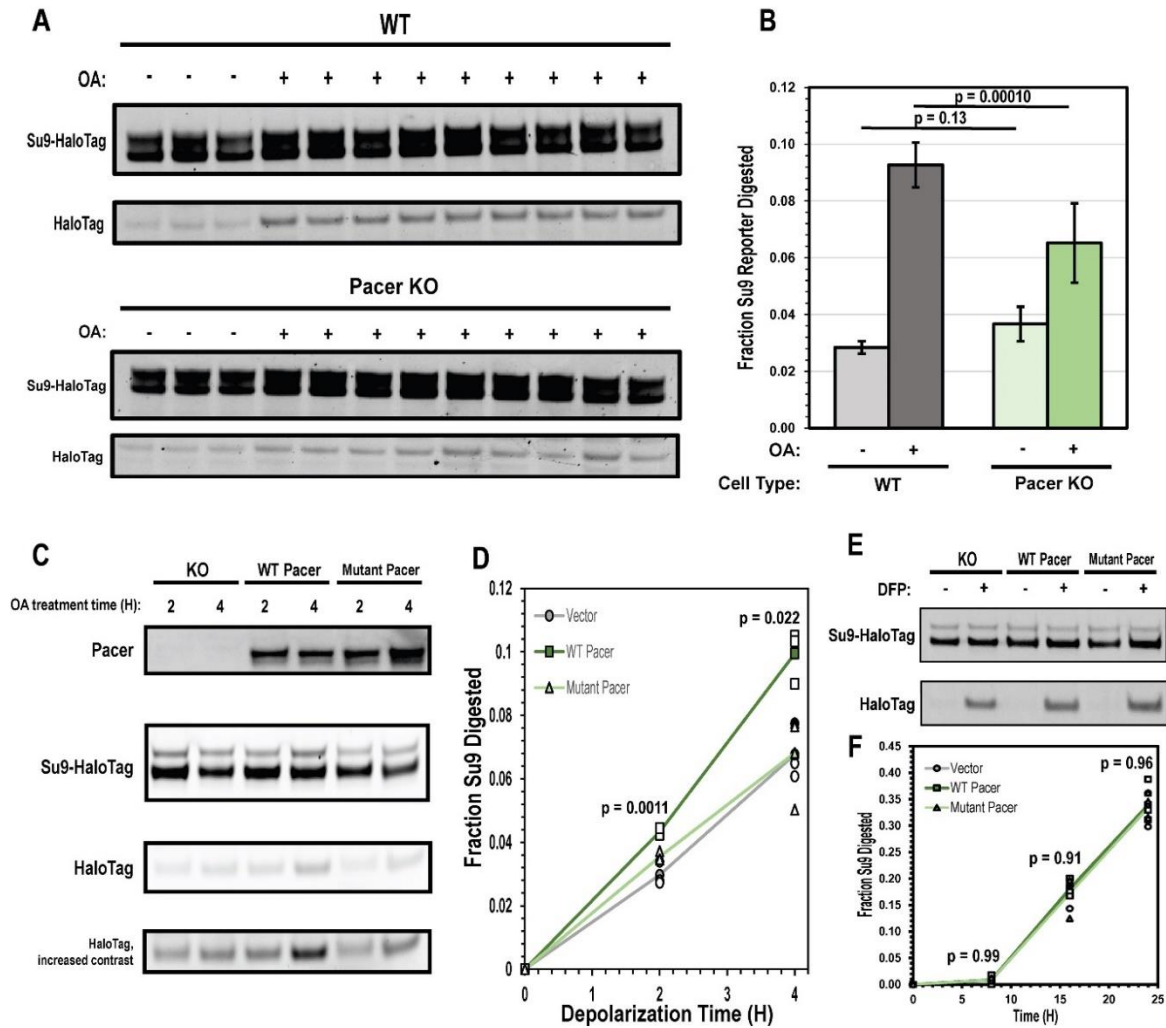


Figure 13 Pacer is a pS72 RAB7A-dependent activator of mitophagy. (A) Su9-HaloTag processing assay conducted as in Figure 1C. Parkin-expressing cells were treated with either complete medium, or complete medium supplemented with 10 μ M Oligomycin A, 5 μ M Antimycin A for 4 H. Each lane represents lysates from separate experiments (B) Quantification of panel A, conducted as in Figure 1D. $n = 3$ for the uninduced condition, $n = 9$ for the induced conditions. Error bars indicate standard deviation, and the p-value was calculated using a one-tailed T test. (C) Pacer KO cells expressing Parkin and Su9-HaloTag reporter were stably transfected with either Pacer, the pRAB7A binding mutant Pacer characterized in Figure 4C-E, or empty vector, and then depolarized for 2 or 4 H. $n = 3$ independent experiments. Pacer expression was probed via Western blot, while HaloTag processing was directly imaged using a fluorescent Halo ligand. (D) Quantification of panel C. P-value was calculated using a one-factor ANOVA at 2 and 4 H. (E) The same cells used in panel C were exposed to the non-Parkin mitophagy inducer DFP for 8, 16, and 24 H, and analyzed as with previous Su9-Halo processing blots. (F) Quantification of panel D. $n = 4$ independent experiments. P-value was calculated using a one factor ANOVA at each timepoint.

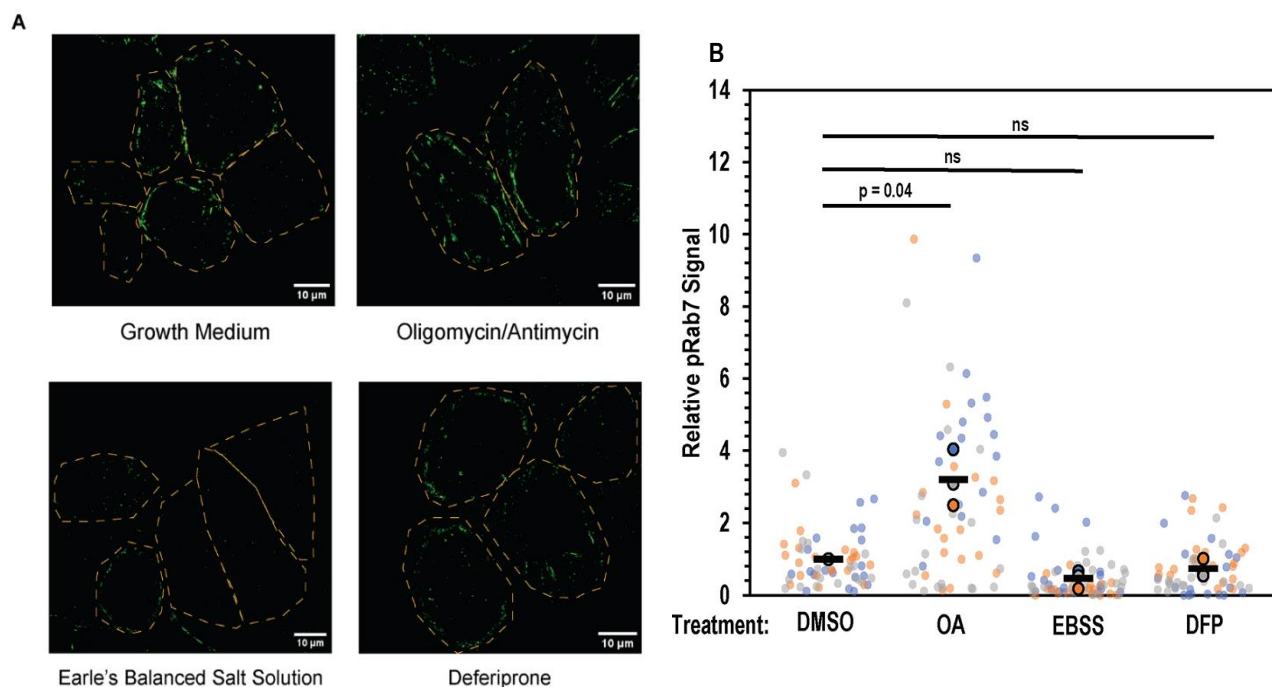


Figure 14. pS72 Rab7 formation following depolarization, starvation, and iron chelation. (A) Parkin-expressing HeLa cells were treated for 1 H with either growth medium, growth medium supplemented with oligomycin/antimycin, Earle's Balanced Salt Solution, or growth medium supplemented with deferiprone, fixed, and then labelled using an antibody specific for pS72 Rab7. (B) Quantification of pRab7 signal. Images of cells were backgrounded subtracted, cells were masked, and total fluorescence intensity was measured on a per cell basis. At least 15 cells were measured in this fashion, and averaged, constituting a single biological replicate (outlined, colored circles). This was repeated for a total of 3 independent replicates for each condition. Horizontal black bars indicate averages of the 3 replicates. Colors indicate replicates performed on the same day. P-value was determined using a paired, one-tailed T-test.

The impact of Pacer and pRAB7A-dependent action on starvation autophagy was also assayed by creating stable transfectants of wild-type Pacer and Pacer^{K534N, R623T} in Pacer KO HeLa cells expressing the LC3B-Halo bulk autophagy reporter. In contrast to the previous report of a Pacer role in starvation autophagy³⁶, these cells showed negligible change in the level of starvation-induced bulk autophagy flux at any time point tested (Figure 15). This indicates that, at least in HeLa cells, Pacer is principally an activator of PINK1/Parkin mitophagy rather than of autophagy generally.

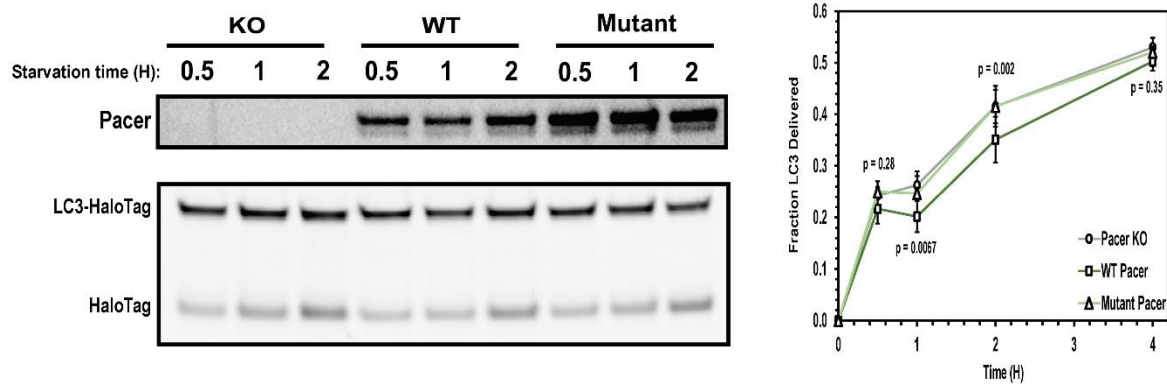


Figure 15. Role of Pacer in starvation autophagy. Pacer KO cells expressing LC3-Halo were stably transfected using either WT Pacer, pRAB7A-binding mutant Pacer, or empty vector, and the starvation autophagy flux assay was conducted as in Figure 1a. Between 3 and 9 independent experiments were conducted for each timepoint. P value was calculated using a one-factor ANOVA at each timepoint.

Pacer upregulates a pre-fusion stage of mitophagy

Pacer is thought to potentially promote autophagy via two mechanisms. First, Pacer is known to bind PI3KC3-C2³⁶ through a domain also present in Rubicon⁷¹. Second, Pacer has been proposed to promote recruitment of the HOPS complex to sites of autophagosome-lysosome fusion³⁶. HOPS complex recruitment to promote autophagosome-lysosome fusion is also dependent on RAB7A^{37,38}. While HOPS functions at the last stage in the progression of autophagy, PI3P production is essential throughout the initiation and expansion stages preceding fusion with lysosomes. To determine whether the pRAB7A-binding dependent action of Pacer occurs early or late in mitophagy, we imaged mCherry-tagged wild-type and mutant Pacer expressing cells using confocal microscopy. We quantified autophagosome formation and autophagosome/lysosome colocalization under nondepolarizing and depolarizing conditions using live-cell imaging. Autophagosomes were monitored with transfected HaloTag-LC3B and lysosomes were tracked via transfection with LAMP1-mNeon green (Figure 17A). Immunofluorescence of the mitochondrial marker HSP60 and LC3B in this system revealed that essentially all autophagosomes formed following depolarization contained mitochondria (Figure 16).

We found that the number of wild-type Pacer puncta per cell ranged from 2 to 10 puncta under nondepolarizing conditions, but increased as much as 3-fold following mitochondrial depolarization using CCCP. To contrast, the number of mutant Pacer puncta was insensitive to mitochondrial depolarization (Figure 17B). Pacer localized robustly with LC3B (Figure 17A). Expression of wild-type Pacer during OA treatment increased the total surface area positive for LC3B in the cell by 58% (Figure 17C). The total autophagosome-lysosome colocalized area also increased by 70% upon WT Pacer expression (Figure 17D), roughly proportionate to the increase in the LC3B positive area alone. This implies that the increased mitophagy flux that characterizes Pacer expression is primarily due to an increase in autophagosome formation rather than delivery to and fusion with lysosomes. If Pacer were principally a promoter of fusion in mitophagy, we would have expected the total LCB3-positive area to decrease because of Pacer expression due to accelerated fusion and degradation. Additionally, as an index of autophagosome-lysosome fusion efficiency, we quantified the fraction of the autophagosome area that is also positive for lysosomal markers on a per cell basis. Here, we found that there were only minor differences in fusion efficiency across the KO, wild-type, and mutant conditions. Wild-type Pacer trends towards significance in promoting fusion ($p = 0.07$), suggesting that Pacer may have fusion-promoting activity, but this likely plays only a minor role in mitophagy promotion in this system (Figure 17E).

Mitophagosome

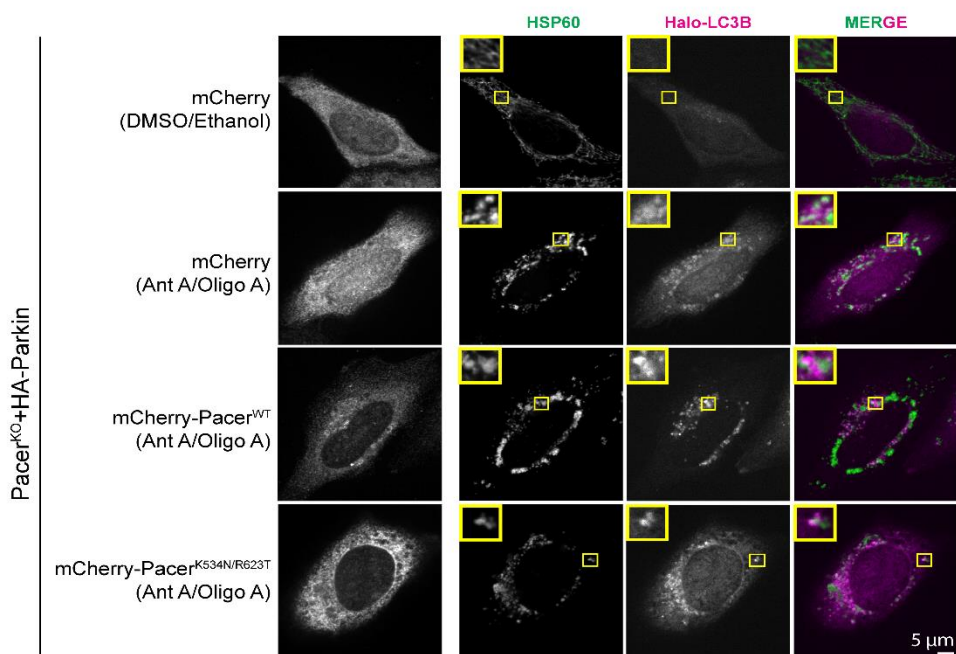


Figure 16. Immunofluorescence of mitochondria alongside LC3B. Cells were treated as in Figure 6A, fixed, and mitochondria were labelled using the mitochondrial marker HSP60. Under nondepolarizing conditions, LC3B is only diffuse. Treatment with antimycin/oligomycin causes formation of punctate autophagic structures that are also positive for mitochondria. Insets are 4 μm wide.

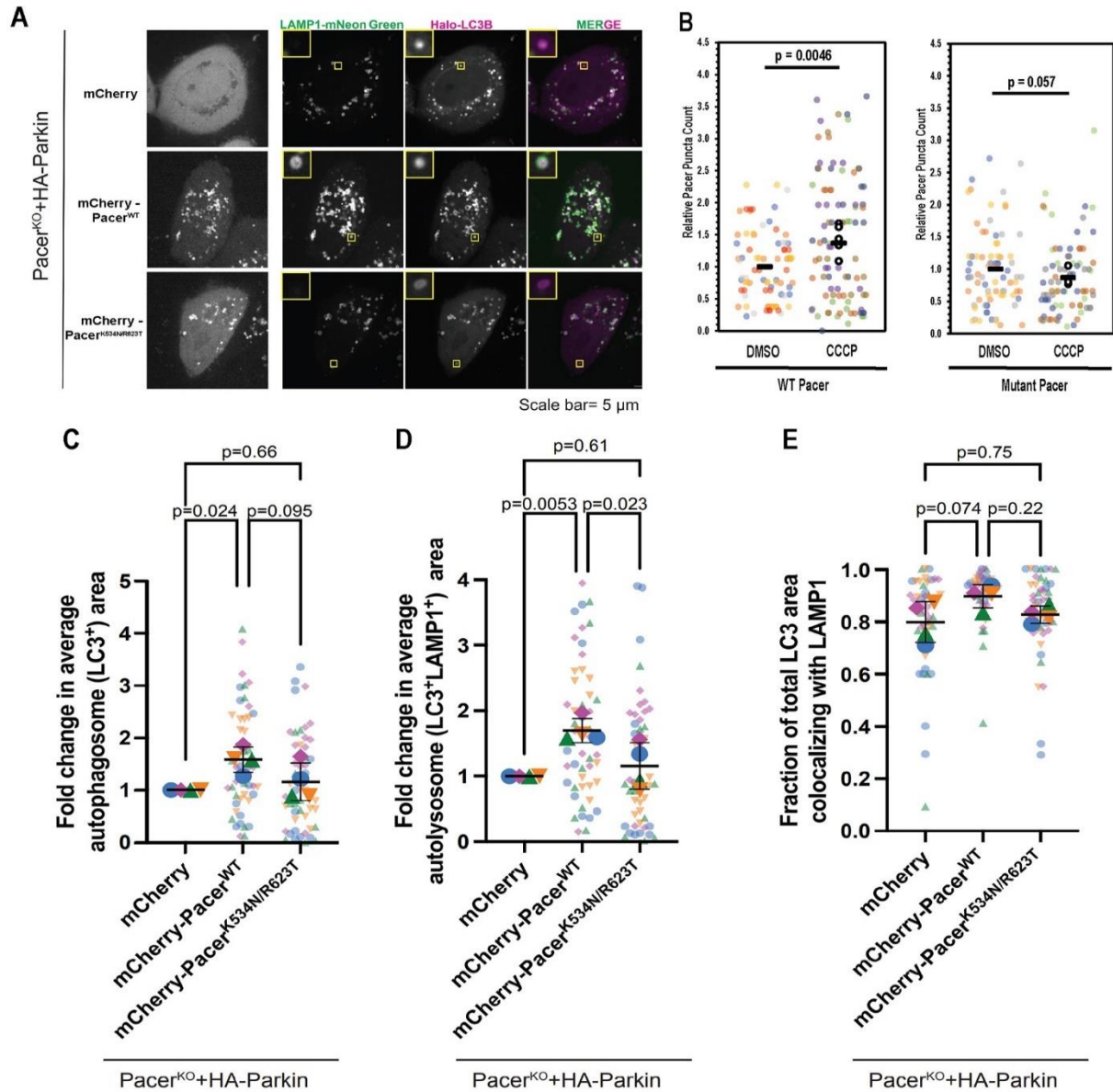


Figure 17. pRAB7A-dependent function of Pacer in mitophagosome expansion. (A) Pacer KO HeLa cells stably expressing Parkin were transiently transfected with mCherry-Pacer WT and the pRAB7A binding mutant of Pacer characterized in Figure 4C-E. Cells were cotransfected with LAMP1-mNeon as a lysosomal marker and Halo-LC3B as an autophagosome marker. Cells were depolarized for 4 H in 10 μ M Oligomycin A and 5 μ M Antimycin A prior to live imaging and quantification in panels C, D, and E. Insets are 4 μ m wide. (B) Pacer KO HeLa cells stably expressing Parkin and mCherry-WT Pacer/mCherry-Mutant Pacer were depolarized for 1 H in 10 μ M CCCP. Cells were fixed, and imaged. Pacer puncta were counted using an automated particle counting program. 20 cells were analyzed and averaged in each biological replicate, and each cell's puncta count was normalized to the average untreated puncta count for that day. 3-4 of these experiments were performed and averaged. Colored dots indicate technical replicates, black dots indicate averages for each experimental day, black horizontal line indicates averages of biological replicates. P-value calculated using a two-tailed T test of biological replicates. (C) Fold change in total autophagosome area. Cells imaged in panel A were masked and thresholded, and the total LC3 positive area was quantified. The total area for each cell was normalized to the average area for the mCherry transfected condition, and these values were averaged to calculate the value for a single biological replicate. This process was repeated in 4 independent experiments. P values were determined using Tukey's multiple comparison test. (D) Fold change in LC3+/LAMP1+ positive area, determined as in panel C. (E) Fraction of total LC3 area that is also positive for LAMP1 as a metric for autophagosome-lysosome fusion efficiency, analysis performed as in panel C.

To further probe the role of Pacer in mitophagy initiation, we co-transfected fluorescently labelled wild-type Pacer and the pRab7-binding deficient Pacer^{K534N, R623T} mutant with Halo-tagged WIPI2 and assessed the degree of WIPI2 puncta formation after treatment with the mitochondrial depolarizers oligomycin/antimycin (Figure 18A). WIPI2 is a marker of autophagosome expansion, and marks sites of autophagy initiation. WIPI2 is directly recruited by the lipid product of PI3KC3, PI3P, which is required for phagophore expansion. Transfection with wild-type Pacer more than doubled the WIPI2 punctate signal relative to the vector only treatment, while transfection with Pacer^{K534N, R623T} failed to increase WIPI2 puncta formation in a statistically significant manner (Figure 18B). This confirms that the role of Pacer in autophagy initiation activity is correlated with its ability to promote WIPI2 recruitment to sites of mitophagy initiation. This is consistent with the model that Pacer promotes PI3P production to promote mitophagy initiation. Finally, the number of WIPI2 puncta also positive for WT Pacer exceeded the number of puncta also positive for mutant Pacer more than twofold (Figure 18C), confirming that pRab7A binding allows Pacer to localize to sites of mitophagy initiation.

In contrast to wild-type Pacer, transfection with the Pacer^{K534N, R623T} did not significantly increase the LC3B-positive surface area nor the LC3B-LAMP1 colocalized area, and failed to promote formation of PI3P as measured via WIPI2 recruitment to nascent autophagosomes. Taken together, this indicates that the formation of pS72 Rab7 at sites of mitophagosome initiation acts to recruit Pacer, which in turn promotes formation of PI3P and recruitment of WIPI2 and the machinery for ATG8 protein lipidation and autophagosome expansion.

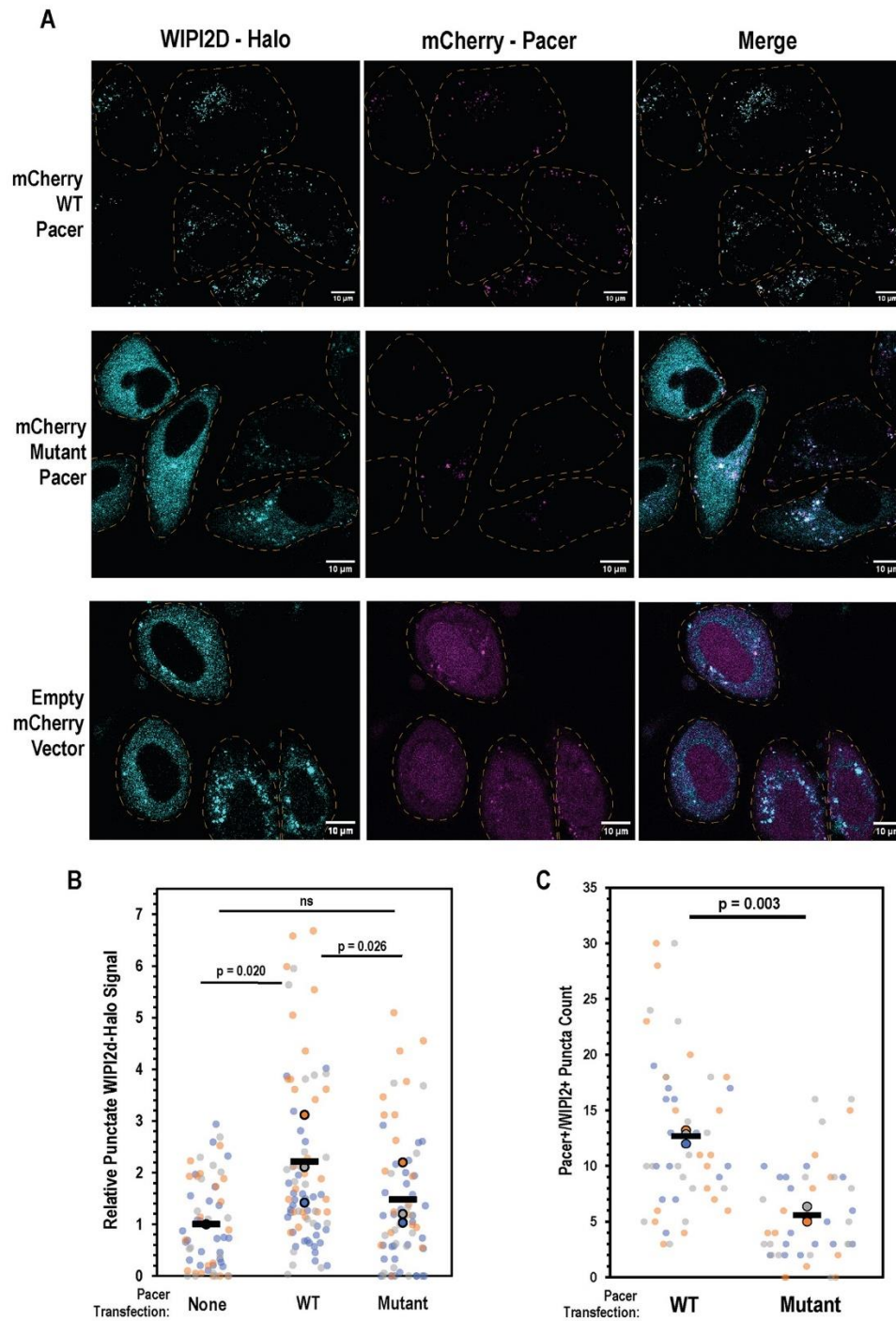


Figure 18. Pacer expression promotes formation of WIP12 puncta upon depolarization. (A) Pacer KO HeLa cells expressing Parkin and either wildtype Pacer, mutant PacerK534N, R623T, or empty vector, were depolarized for 1 H using 10 μ M Oligomycin A and 5 μ M Antimycin A following transient transfection with HaloTag-WIP12B. (B) For each independent trial, ~20 cells were imaged and the total above-cytosol background, or punctate, WIP12 signal was measured. Three trials were performed, and the trials performed in parallel are indicated by colors. Trial averages are indicated by bolded dots, and horizontal bars indicate averages across all three trials. P-value is the result of a one-tailed, paired T-test between the trial averages in each category. (C) Quantification of the number of puncta positive for both Pacer and WIP12 on a per cell basis. Replicates are indicated as in panel B, and P-value is also the result of a one-tailed, paired T-test.

Discussion

Here, we uncovered an unexpected and elegant mechanism whereby a single RAB phosphorylation event leads to dissociation of an inhibitor of mitophagy and recruitment of an activator. The roles of a variety of molecules in mitophagy, including RAB7A, Pacer, and Rubicon, have thus been put into new perspective. We found that RAB7A is not phosphorylated under starvation conditions. In the absence of RAB7A phosphorylation, Rubicon remains a stronger inhibitor of starvation-mediated autophagy. Pacer, however, appears to be a potent and specific PINK1/Parkin-dependent mitophagy accelerator, whilst having a minimal role in starvation-induced autophagy and DFP-induced mitophagy. This is consistent with the strong dependency of Pacer action on RAB7A phosphorylation. We found that PINK1/Parkin mitophagy increases less in response to Rubicon KO than starvation autophagy. The difference can be explained by the ability of RAB7A phosphorylation to abolish binding of Rubicon to RAB7A, which removes the Rubicon blockade upon mitophagy induction. Finally, we provide direct evidence that Pacer localizes to sites of mitophagy initiation to promote autophagosome elongation via promoting production of PI3P.

Phosphorylation of RAB proteins, especially in switch II at residues equivalent to RAB7A Ser72, is a frequent point of regulation in membrane trafficking^{75,88}. The discovery that the LRRK2 kinase mutated in PD acts by phosphorylating RAB proteins has spurred intense effort to characterize the downstream effects of RAB phosphorylation⁷⁵. RAB7A is not a known substrate of LRRK2, rather it appears to be a substrate of at least two other protein kinases, TBK1 and LRRK1. TBK1 is well-established as a central player in PINK1/Parkin mitophagy^{60,78,79}, and a mitophagy role for LRRK1 was more recently reported⁷⁷. The best explored function of RAB phosphorylation in autophagy is the regulation of JIP4-dependent transport of mature autophagosomes in axons by LRRK2 phosphorylation of RAB29⁸⁹. Here, we have discovered that RAB phosphorylation can also regulate the process of autophagosome biogenesis itself.

It was previously reported that pSer72 RAB7A preferentially recruits the FLCN:FNIP1 complex relative to unphosphorylated RAB7A⁶⁸. FLCN:FNIP1/2 is a GTPase activating protein complex (GAP) for the RagC/D GTPases involved in activating mTORC1 to phosphorylate and inactivate the lysosomal and autophagic transcriptional regulator TFEB⁹⁰⁻⁹². The FNIP1-containing FLCN:FNIP isocomplex is centrally involved in sensing and responding to changes in mitochondrial metabolism^{93,94}. FLCN KO does not phenocopy the effect of pRAB7A phosphorylation on recruitment of ATG9A to mitochondria⁶⁸, a key step in mitophagy initiation. Thus, it was postulated that RAB7A phosphorylation must have additional effects on the autophagy machinery. This concept is consistent with our own finding that purified FLCN:FNIP1 binds robustly to dephosphorylated RAB7A *in vitro* (unpublished data). While the possibility of yet other effects of RAB7A on the mitophagy machinery cannot be excluded, RAB7A Ser72 phosphoregulation of Rubicon and Pacer is, in principle, sufficient to account for the pRAB7A dependence of PINK1/Parkin mitophagy.

Most models for the regulation of selective autophagy focus on the first step, substrate recognition by cargo receptors^{25,95}. The assumption is often made that once initiated, the downstream cascade of protein interactions in autophagosome biogenesis is the same in bulk autophagy and the various forms of selective autophagy. Recent evidence shows that the situation is more complicated. In OPTN-dependent mitophagy, for example, the order of

recruitment of the core autophagy components ULK1 and PI3KC3-C1 is inverted compared to most other forms of autophagy⁸¹. Now we have uncovered a PINK1/Parkin mitophagy-specific mode of regulation of PI3KC3-C2 via Rubicon and Pacer, suggesting that selective autophagy subtype-specific regulator mechanisms may permeate the entire process of autophagosome biogenesis. The existence of these mechanisms may provide new opportunities for the modulation of specific types of autophagy for therapeutic benefit.

Methods

Structural modeling

In order to generate a homology model for the Pacer RH domain, the Pacer RH sequence (corresponding to residues 412 through 654) in FASTA format was downloaded from Uniprot (RRID:SCR_002380) (accession date: September 1st 2021) and uploaded to the Biozentrum Swiss-Model (RRID:SCR_018123). This sequence was aligned against the Rubicon sequence, and a model generated using a published crystal structure of the Rubicon RH domain in complex with RAB7A (PDB: 6wcw⁷⁰).

Plasmid Construction

In order to generate MBP-tagged RH domains for purification and subsequent *in vitro* binding experiments, the RH domain-encoding sequence of Rubicon and Pacer were amplified via PCR, purified via gel electrophoresis using a 1% agarose gel, and then subcloned into pET His10 MBP Asn10 TEV LIC cloning vector (2CT-10) via ligation independent cloning (LIC). XL10 Ultracompetent *E. coli* (Agilent technologies) were transformed with this plasmid, and then plated on ampicillin selective agar. Individual colonies were picked via a sterile loop and grown overnight in 5 mL of LB supplemented with ampicillin. Purified plasmid DNA was isolated via a commercial spin miniprep kit (Qiagen), and cloning was confirmed via Sanger sequencing.

To generate point mutants of the Pacer RH domain, ‘round-the-horn site-directed mutagenesis was employed. Briefly, non-overlapping primers, adjoining each other and “pointing in the opposite direction” are designed. One of the primers contains the desired mutations, and the primers are phosphorylated using polynucleotide kinase prior (NEB) to PCR such that each PCR product strand can be immediately ligated to itself. Following PCR using Q5 polymerase (NEB), the product is gel-purified on a 1% agarose gel, and ligated via incubation with T4 DNA ligase (NEB) prior to transformation into XL10 *E. coli*. The N821K, T911R, K916R mutant of the Rubicon RH domain was synthesized by Genscript in a pMAL-c5E vector.

In order to generate mCherry-tagged WT Rubicon and Pacer for use in confocal imaging and autophagy flux experiments, primers were designed to amplify the sequence encoding full-length Rubicon and Pacer respectively, using existing Rubicon and Pacer plasmids as templates⁷¹ while incorporating a BglII restriction site upstream of the open reading frame and a stop codon followed by a SalI restriction site downstream of the open reading frame. Following PCR amplification, the product, as well as a stock of pmCherryC1 mammalian expression vector (Clontech) was digested using BglII and SalI (NEB) according to manufacturer instructions. The digested PCR product and vector were purified via electrophoresis on a 1 and 0.5% agarose gel respectively. Subsequently, the Rubicon/Pacer full-length sequence was ligated into the pmCherry-C1 vector via an overnight incubation with T4 DNA ligase (NEB) at 16 °C.

RH domain mutants of full-length Pacer were generated via Gibson Assembly. Briefly, a fragment consisting of Pacer residues 1-412 was amplified via PCR, with a 20 base pair overlap with the pmCherryC1 sequence upstream of the amplified sequence via primer, and a 20 bp overlap with the RH domain included downstream of the fragment. Simultaneously, the mutant RH domains in the 2CT-10 cloning vector were amplified via PCR, with an additional 20 base pair overlap with pmCherryC1 included downstream of the Pacer RH sequence, and a 20 bp overlap with the Pacer 1-412 transcript included upstream. pmCherryC1 was linearized via incubation with BglII and SalI, and then the linearized vector, Pacer fragment 1-412 PCR product, and mutant Pacer RH domain PCR product were mixed with Gibson Assembly master mix (NEB) in a 1:3:3 molar ratio prior to incubation at 50 °C for 15 minutes and transformation into XL10 *E. coli*. Plasmids were subsequently purified via overnight culture followed by purification via a commercial endotoxin-free spin midi prep kit (Qiagen).

To prepare for stable cell-line generation, mCherry-tagged full-length Pacer, and Pacer pRAB7A binding mutant were subcloned in a pCDH lentiviral vector. Briefly, both the pCHD1 vector and pmCherry-RH protein mammalian expression plasmids were digested using NheI and BamHI before being purified via gel electrophoresis. Bands corresponding to mCherry - RH protein were isolated and purified before being ligated into the pCDH backbone via T4 DNA polymerase. Subsequently, the final pCDH – RH protein construct was transformed into One Shot Stbl3 Chemically Competent *E. Coli* (Thermo Fisher) before being selected via antibiotic, and grown in an overnight culture. A maxiprep was performed to purify the plasmid (Qiagen), and the plasmid subsequently used for stable cell line generation via lentiviral transduction.

E. coli expressible constructs were verified via sequencing and SDS-PAGE of target proteins. Mammalian expressible constructs were verified via sequencing, visibility of fluorescent tag, and western blot against construct protein in a knockout background.

Protein Expression and Purification

Plasmids were transformed into BL21 star (DE3) *E. coli* (Agilent technologies), and individual colonies picked. For purification, 20 mL of overnight culture was inoculated, and then diluted into 4 L of lysogeny broth supplemented with maintenance antibiotics. When purifying MBP-Rubicon RH or MBP-Pacer RH, cultures were also supplemented with 150 μ M ZnCl₂. Cultures were grown at 37 C until OD₆₀₀ = 0.55 was reached, and then cultures were chilled in ice water baths for 15 minutes before being induced with 350 μ M isopropyl- β -D-thiogalactoside (IPTG). Induced cultures expressed protein at 18 C for 16-20 H before being harvested via centrifugation. Harvested cells were resuspended in a buffer consisting of 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM MgCl₂, and 10 mM TCEP, and a tablet of cOmplete protease inhibitor cocktail (Roche). Cells were lysed via sonication, and the lysate was clarified by centrifugation at 34,500 g for 1 h at 4 °C.

MBP-tagged RH domain was isolated via passing cleared lysate over a gravity amylose resin column containing 5 mL of settled resin (NEB). Column was washed with 150 mL of buffer before eluting protein with wash buffer supplemented with 20 mM maltose. As a final polishing step, protein was concentrated down to 500 μ L before being loaded onto a Superdex 75

Increase 10/300 GL size-exclusion column (Cytiva). Fractions were analyzed via SDS-PAGE, and pure fractions were pooled and concentrated before being concentrated and snap-frozen in liquid nitrogen

GST-tagged RAB7A was similarly isolated via passing cleared lysate over a gravity column packed with 5 mL of Glutathione Sepharose 4B (GE Healthcare), followed by elution using 10 mM reduced glutathione dissolved in 50 mM Tris-HCl pH 8.0. To cleave the GST tag, purified TEV protease was added in a 1:20 protease: RAB7A ratio by mass, and the solution was dialyzed overnight into a 50 mM HEPES 7.5, 150 mM NaCl, 2 mM MgCl₂, 10 mM TCEP buffer. Protease and cleaved GST was removed from solution by passing the dialyzed and cleaved protein solution over a 10 mL Hispur Ni-NTA gravity column (Thermo Fisher) three times. Subsequently, the cleaved RAB7A was concentrated down to 500 μ L, and further purified via size exclusion as with the purified RH domains.

Bead-binding

A 40 μ L solution of 25% (v/v) amylose resin (NEB) was co-incubated with 500 nM MBP-RH domain and 4 μ M Alexa Fluor 647-labeled RAB7A (life technologies, DOL = 0.9) in a buffer consisting of 50 mM HEPES 7.5, 150 mM NaCl, 2 mM MgCl₂, 10 mM TCEP. Samples were incubated on rocker at room temperature for 1 H. Resin was collected using a tabletop centrifuge, supernatant was aspirated off, and resin was washed 3 times with 1 mL of room temperature buffer. Beads were resuspended in 200 μ L buffer and transferred to an 8-well confocal imaging chamber (LabTek). Images were collected on a Ti2 Nikon confocal microscope with a 63x Plan Apochromat 1.4 NA objective, and a ti2 eclipse camera, using Nis elements capture software (RRID:SCR_014329). Binding was quantified via the Alexa Fluor 647 channel. Bead fluorescence intensity was assessed in ImageJ (RRID:SCR_003070) via selecting an in-focus bead, defining an ROI that includes the bead edge, and recording the maximum pixel intensity. Any changes in image brightness performed for Figure legibility were applied uniformly across images in a Figure ure.

Phosphorylation reactions

Purified RAB7A was phosphorylated via incubation with purified strep-tagged TBK1. Briefly, a solution consisting of 4 μ M RAB7A, 0.4 μ M TBK1, 50 mM HEPES 7.5, 150 mM NaCl, 10 mM MgCl₂, and 200 μ M ATP was incubated at room temperature for 3 H. Strep-tagged TBK1 was removed from solution via incubation with Strep-Tactin Sepharose (Cytiva life sciences). Resin was collected via a filtered gravity column yielding pure, phosphorylated RAB7A.

To assess degree of phosphorylation, a 15 μ g pRAB7A sample was then buffer-exchanged into MQ water via dilution and concentration in a centrifugal concentrator, and samples were subsequently analyzed via electrophoresis on a SuperSep Phos-tag Precast gel (Wako Fujifilm). Complete band shift of the RAB7A band post phosphorylation confirms stoichiometric phosphorylation of the protein.

Isothermal titration calorimetry

To prepare proteins for isothermal titration calorimetry (ITC) binding studies, recombinantly purified RAB7A and MBP-Rubicon RH were co-dialyzed overnight at 4 °C into a degassed buffer containing 50 mM HEPES 7.5, 150 mM NaCl, 2 mM MgCl₂, and 10 mM TCEP. The next morning, RAB7A was concentrated to 800 µM, and MBP-Rubicon RH to 80 µM using a centrifugal concentrator. The concentrated protein solutions were again degassed via exposure to vacuum, allowed to come to room temperature, and loaded onto a Microcal PEAQ-ITC (Malvern Panalytical), with the MBP-Rubicon RH contained in the ITC chamber, and the RAB7A solution in the syringe. The titration was conducted, and the resulting binding curve fit using the PEAQ-ITC's native curve-fitting and analysis software.

Tissue culture

All cell lines were grown and maintained in Dulbecco's Modified Eagle Medium (Corning) supplemented with 10% fetal bovine serum (Gibco) and 1x Penicillin-Streptomycin (Thermo Fisher). To depolarize cells for mitophagy induction, growth medium was supplemented with 10 µM Oligomycin and 5 µM Antimycin A (Sigma-Aldrich), or with 10 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Medchem Express). To induce starvation autophagy, cells were washed into Earle's Balanced Salt Solution (EBSS) (Sigma Aldrich).

Generation of knockout lines using CRISPR/Cas9 gene editing

RUBICON and PACER KO lines in this study were generated using CRISPR/Cas9 gene editing. CRISPR guide RNAs (gRNAs) targeting a common exon of splicing variants of each gene of interest were cloned into BBsI-linearised pSpCas9(BB)-2A-GFP vector (Addgene #48138) using NEBuilder® HiFi DNA Assembly (New England Biolabs) as described in the following protocol: <https://doi.org/10.17504/protocols.io.j8nlkkzo6l5r/v1>. gRNA constructs were transfected into HeLa S3 cells (<https://www.atcc.org/products/ccl-2.2>) with X-tremeGENE 9 (Roche) overnight. With fluorescence activated cell sorting (FACS), GFP-positive single cells were sorted into individual wells of 96-well plates. To identify knockout cell lines, single cell colonies were expanded and initially screened via western blot. In the absence of an antibody, the PACER KO line was confirmed via sequencing alone. The CRISPR targeted regions of the knockout clones and wild-type cells were amplified via PCR and sequenced using primers that annealed to the amplified product. Synthego ICE v2 CRISPR Analysis Tool (<https://synthego.com/products/bioinformatics/crispr-analysis>) (RRID:SCR_024508) was used to compare the sequencing data of the control and knockout cell lines to confirm the presence of indels.

Generation of stable cell lines

To prepare the retrovirus transduction solution, HEK293T cells were transfected for 6 H with pMRX-IP-based or, pMRX-IB-based retroviral plasmids containing HaloTag autophagy reporters or HA-tagged Parkin, pCMVR8.74, and pCG-VSV-G using TransIT-LT-1 transfection reagent (Mirus). Subsequently, the medium was replaced with DMEM, and the HEK cells were allowed to produce pseudotyped retrovirus for 3 days. The medium was harvested, cleared via centrifugation, and concentrated 10-fold via Lenti-X Concentrator solution (Takara). Retroviral solution was added to WT, Pacer knockout, and Rubicon knockout HeLa cells in a 12-well plate at 50% confluence. Cells were left to incubate for 24 H before swapping medium to fresh DMEM, and assessing transfection efficiency via fluorescence. Transfected cells were selected via incubation with puromycin or blasticidin depending on the transduction plasmid, and uniform express of autophagy reporters across cell types was confirmed via western blot and fluorescence microscopy

Immunofluorescence Imaging and Colocalization

Cells were seeded two days in advance on chambered glass slides treated with poly-L-lysine. Cells were allowed to grow to 80% confluence, and then transfected with mCherry-labeled RH proteins using lipofectamine 3000 (Thermo Fisher). Cells were allowed to recover for 48 H before being depolarized using CCCP or Oligomycin A/Antimycin A for 1 H or starved via incubation in EBSS, then washed with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized via a 15 min incubation in Streptolysin O from *Streptococcus pyogenes* (Sigma Aldrich), washed three times with PBS, then blocked via a 30 min incubation in 2% BSA dissolved in PBS. Cells were washed and incubated for 1 H in a 1:200 primary solution of Rab7 (ab137029, Abcam) or pRAB7A primary antibody (ab302494, Abcam). The pRAB7A antibody was previously demonstrated to be specific to pS72 RAB7A⁹⁶. Cells were washed 3 times with PBS, and then incubated in a 1:1000 solution of anti Rabbit secondary antibody conjugated to Alexa 488 for 1 H (A-11034, Thermo Fisher). Cells were again washed 3 times, and imaged immediately. Images were captured using the same setup as in the bead-binding experiments.

To analyze and quantify colocalization, images were opened in FIJI (RRID:SCR_002285), an open-source distribution of ImageJ. To subtract the background of each channel, a portion of the image expected to be dark, such as a nucleus, is defined and the average signal intensity measured. This average signal intensity is subtracted from the image as a whole using FIJI's *Math>Subtract* function. Subsequently, lasso ROIs are used to define individual cell boundaries, and the Mander's colocalization coefficient with a Costes' threshold regression is calculated using the *BIOP JaCoP* plugin (version 2.11, <https://github.com/radsz/jacop>, doi:10.1111/j.1365-2818.2006.01706.x, RRID: SCR_025164). When images are brightened for clarity, gamma adjustments are made uniformly across compared images.

For the LC3 puncta formation assays, Pacer KO+ HA-Parkin HeLa cells were transfected with Halo-LC3 and either mCherry, mCherry-PacerWT or PacerK534N, R623T using Lipofectamine 2000 (Invitrogen Cat#: 11668030). Post 18-24 hrs of transfection, cells were

treated with Ethanol and DMSO or Antimycin A and Oligomycin A for 4 hrs. During the last hour of treatment, cells were labeled with Janelia Fluor 646-Halo ligand (Cat#GA1120; Promega) for 15 mins followed by a wash and incubation in fresh treatment media for another 45 mins. Cells were then fixed and permeabilized with ice-cold methanol at -20°C for 8 mins. Post this, cells were blocked at RT in blocking buffer [5% goat serum and 1% BSA in 1X Phosphate Buffer Saline (PBS) buffer] for 90 mins. Following blocking, cells were incubated overnight at 4°C with primary antibodies diluted in blocking buffer (HSP60: Rabbit polyclonal sigma-Aldrich Cat#SAB4501464 IF: 1:100; mCherry: chicken polyclonal Abcam, Cat#ab205402, IF: 1:200). Post 14-16 hrs of incubation, cells were washed thrice with 1X PBS. Cells were then incubated for 1 hr at RT with blocking buffer containing the corresponding secondary antibody [Alexa Fluor 488 goat anti-rabbit IgG (H + L) Invitrogen Cat#: A11034; Alexa Fluor 546 goat anti-chicken IgG (H + L) Invitrogen Cat#: A11040; dilution: 1:1000] and Janelia Fluor 646-Halo ligand. Samples were washed thrice in 1XPBS and then mounted in Prolong Gold (Life technologies P36930). Images were acquired on a PerkinElmer UltraView Vox spinning disk confocal on a Nikon Eclipse Ti Microscope with an Apochromat 100x 1.49 N.A. oil-immersion objective and a Hamamatsu CMOS ORCA Fusion (C11440) camera with VisiView (Visitron).

HaloTag processing assay

Cells stably expressing LC3-Halo or Su9-Halo were seeded for 80% confluence on the day of the assay in 12-well tissue culture plates. To pulse-label the reporter, cells were incubated for 20 min in 100 nM cell-permeable Halotag conjugated to Janelia Fluor 549 dye (Promega) before washing twice with PBS. Cells were incubated in growth medium, or in growth medium supplemented with 10 μ M oligomycin and 5 μ M antimycin, or in growth medium supplemented with 1 mM deferiprone (Millipore Sigma), or in Earle's Balanced Salt Solution before harvesting cells via scraping. Incubation times are listed in the respective Figure caption. Cells were lysed, and the protein concentration was quantified in a plate reader using a Pierce BCA protein quantification kit (Thermo Fisher). For each condition, 20 μ g of protein was loaded onto a gel for SDS-PAGE, and then the gels were imaged using a ChemiDoc fluorescent imager (Biorad).

Images of gels (Figure S4) were imported into FIJI, and analyzed using the *Gel Analyzer* tool. Briefly, selections were defined that contained the bands of interest, and the integrated signal for each band was recorded. The intensity of the processed HaloTag band as a fraction of the total Halo signal was calculated.

Western blotting

Adherent cells to be analyzed were washed twice with PBS, and then scraped into a microcentrifuge tube. Cells were pelleted, lysed, and their concentration measured as performed for the HaloTag processing assay. 20 μ g of protein was loaded into each lane. Protein was transferred to a nitrocellulose membrane (GE Healthcare) using a TransBlot Turbo transfer device (Biorad) and the device's default protocol. Post transfer, the membrane was cut lengthwise into strips to isolate bands of particular molecular weights before being washed with

TBST and incubated at room temperature for 1 H in a 5% blocking buffer solution (Biorad). The western blot strips were then incubated overnight at 4 C in a solution consisting of the primary antibody diluted into blocking buffer. The next morning, the western blot strips were washed in TBST and incubated in a solution of secondary antibody diluted in blocking buffer for 1 H at room temperature. The strips were rigorously washed, and prepared for chemiluminescent imaging via incubation in SuperSignal West Femto ECL detection substrate (Thermo Fisher) for 5 minutes prior to imaging on a ChemiDoc (BioRad).

Autophagosome fluorescence microscopy live imaging

For live-cell imaging Pacer^{KO}+HA-Parkin HeLa cells were first co-transfected with pCIG2-LAMP1-mNeon Green and pHaloTag-LC3B and either pmCherry, pmCherry-Pacer^{WT} or pmCherry-Pacer^{K534N/R623T}. Post 16-18 hr of transfection, cells were treated with Antimycin A and Oligomycin A for 4 hrs. For imaging, the culture media was replaced with Leibovitz's L-15 medium (Gibco 11415064) supplemented with 10% fetal bovine serum and 1% Glutamax along with Antimycin A and Oligomycin A. Imaging was performed on a PerkinElmer UltraView Vox spinning disk confocal on a Nikon Eclipse Ti Microscope with an Apochromat 100x 1.49 N.A. oil-immersion objective and a Hamamatsu CMOS ORCA Fusion (C11440-20UP) camera with VisiView (Visitron). mCherry was expressed to a higher level than either pmCherry-Pacer^{WT} or pmCherry-Pacer^{K534N/R623T}, resulting in higher fluorescent intensities per cell. Thus, the red channel for mCherry-expressing cells was acquired at a lower laser power and shorter exposure time than those used for both mCherry-Pacer^{WT} and mCherry-Pacer^{K534N/R623T} expressing cells; however, the LAMP1 and LC3B channels were imaged at the same laser power and exposure time across all three constructs. Imaging parameters were kept constant across biological replicates. Quantification was performed on individual slices from a Z-stack. Images from the LC3 and LAMP1 channels were thresholded and binarized using Yen thresholding in ImageJ. To quantify area of colocalization the 'AND' function from Image Calculator was used. Area of colocalization was then quantified using Analyze Particles.

WIPI2 puncta formation assay

Hela cells expressing Parkin were seeded two days in advance on chambered glass slides treated with poly-L-lysine. Cells were allowed to grow to 80% confluence, and then transfected with HaloTag-WIPI2B and mCherry-Pacer using lipofectamine 3000 (Thermo Fisher). Cells were allowed to recover for 48 H before being depolarized using Oligomycin A/Antimycin A for 45 min. After this incubation, cells were labelled using 1 μ M cell-permeable Oregon Green Halo Ligand (Promega) diluted in depolarization medium. Cells were incubated for an additional 15 minutes, washed twice with PBS, and immediately fixed with 4% paraformaldehyde for 15 min. Cells were immediately imaged on a confocal microscope.

To analyze and quantify WIPI2 puncta formation, images were opened in FIJI, an open-source distribution of ImageJ. To subtract the background of each channel, a portion of the image containing only cytosolic WIPI2 signal is defined, and the average signal intensity is measured. This average signal intensity is subtracted from the image as a whole using FIJI's

math > subtract function. Subsequently, lasso ROIs are used to define individual cell boundaries, and the *measure* function is used to calculate the total above-background, punctate WIP12 signal.

To measure colocalization, ROIs are defined around cells, then images are processed using FIJI's *auto threshold > max entropy* automatic thresholding function. This is performed on both WIP12 and Pacer channels. Then, images are multiplied together using FIJI's *image calculator > multiply* function. The resulting image contains only pixels positive for both WIP12 and Pacer. Then, *analyze > analyze particles* is used on each ROI with a size of 0-infinity and a circularity of 0-1 to automatically count the number of particles.

Chapter 3

Development of a Rubicon degrader

Introduction

Autophagy has been implicated in a variety of diseases, ranging from developmental diseases to metabolic conditions to diseases of aging. The characterization of rapamycin, and the identification of the mammalian target of rapamycin (mTORC1) complex, was a watershed moment in the effort to pharmacologically target autophagy. Under nutrient replete conditions, mTORC1 phosphorylates the autophagy initiating ATG13, preventing it from being recruited to the crucial ULK1 complex^{15,91}. Additionally, mTORC1 phosphorylates the pro-autophagy transcription factor TFEB, retaining it in the cytoplasm and limiting transcription of a host of autophagy proteins. As a result, mTORC1 is a potent regulator of autophagy¹⁴. Rapamycin is a macrolide that inhibits the mTORC1 complex, which in turn activates autophagy.

Rapamycin is relatively well-tolerated in humans, providing proof of concept for pharmacological enhancement of autophagy⁹⁷. In mice, treatment with rapamycin provides significant geriatric benefits, extending lifespan and reducing the prevalence of age-related phenotypes⁹⁸⁻¹⁰⁰. However, mTORC1 is a central regulator of many cellular processes other than autophagy, resulting in a variety of off-target effects, notably suppression of immune function. As a result, rapamycin has found limited clinical use, largely being used as an immunosuppressant and in a small set of cancers, rather than as a broad-spectrum activator of autophagy^{101,102}. Thus, while rapamycin demonstrates the feasibility of autophagy modulation as a therapeutic strategy, inhibition of mTORC1 is likely too blunt an instrument to target autophagy-related diseases in a clinically viable way.

To avoid the pleiotropic effects of mTORC1 inhibition, it is necessary to develop agents that act on autophagy directly. This, however, encounters a similar problem: many of the regulators of autophagy are interconnected with other cellular processes, and are entangled with the broader nutrient-sensing apparatus of the cells. Here, Rubicon provides a special example of a protein that appears to be a pure inhibitor of autophagy in essentially all contexts. Rubicon seems to be ubiquitously expressed across all tissues and is leveraged by cells in a variety of circumstances to act as a blockade on autophagy at the expression and stability level. In fat cells, elevated Rubicon expression suppresses autophagy of lipid droplets, termed lipophagy, and diminishes release of fatty acids into the bloodstream¹⁰³. In liver cells, Rubicon similarly suppresses lipophagy, and therefore metabolism of fatty acids¹⁰⁴. Constitutively higher expression of Rubicon, mediated via stability of its mRNA, sets the rate of autophagy in liver cells, and is thought to influence susceptibility to Non-alcoholic Fatty Liver Disease (NAFLD)¹⁰⁵. Neurons decrease their Rubicon population via ubiquitin-proteasome degradation to promote mitophagy following mitochondrial damage¹⁰⁶. Following heart attacks, cardiac cells will increase their expression of Rubicon to halt autophagy in the short term. However, Rubicon expression remains high in the days following the heart attack, resulting in post-ischemic injury mediated by constitutively blocked autophagy^{107,108}. Notably, expression of Rubicon in patient samples appears to correlate well with susceptibility to disease; for instance, Rubicon expression is elevated in the livers of patients with NAFLD¹⁰⁴, in the kidneys of patients with Chronic Kidney Disease¹⁰⁹, and in the spinal cords of patients with ALS⁷⁴. Thus, the expression level of Rubicon appears to be a clinically relevant, generalized brake on autophagy. Developing a

pharmaceutical that can inhibit or degrade Rubicon thus seems like a promising means by which to target autophagy for therapeutic benefit, for disease specific as well as generalized goals.

Since Rubicon has no known catalytic activity and relies entirely on protein-protein interactions to exert its block on autophagy, traditional small molecule inhibitors would likely struggle to effectively interact with Rubicon. In contrast, a heterobifunctional degrader of Rubicon would be perfectly suited given the structural and biochemical makeup of this protein. These molecules, often referred to as proteolysis-targeting chimeras, or “PROTACs,” seek to promote ubiquitin-proteasome degradation of their targets. They consist of three general chemical moieties: a protein binding “warhead,” a flexible linker, and an E3-recruiting group. Mechanistically, these molecules act by tethering an E3 ligase to a target protein. This induced proximity allows the E3 to ubiquitinate the target protein even if it is not its natural substrate (Figure 19). Ubiquitinylation of these “neosubstrates” results in degradation of the protein of interest, and release of the degrader molecule to bind to and mark another copy of the protein of interest for degradation. This mechanism results in several advantages relative to traditional small molecule inhibitors. Firstly, the action of the molecule is catalytic, since the degrader is released as the protein becomes degraded by the proteasome. Secondly, since the dwell time of the degrader on the protein of interest only needs to be long enough to engage formation of a ubiquitin chain, it does not need to have the kind of binding affinities that are necessary for traditional small molecule inhibitors. This makes degraders a promising target for proteins that may not have well-defined binding pockets or catalytic sites¹¹⁰. Finally, pharmacological degradation of Rubicon via the proteasome mimics already existing mechanisms by which the cells promote autophagy, suggesting that this strategy would be effective and well-tolerated^{105,106}.

This Targeted Protein Degradation (TPD) field emerged following observations that viruses and plants produce naturally occurring PROTAC-like molecules to modulate the lifetime of target proteins¹¹¹. At least two dozen viruses leverage the human proteasome to antagonize host immune responses and promote their own survival. Notably, the HIV-1 genome encodes two PROTAC-like proteins, Vpr and Vpx, that recruit DDB1 and CUL4-associated factor 1 (DCAF1) to induce degradation of host proteins^{112,113}. The seminal, naturally occurring small molecule TPD inducer is auxin (indole-3-acetic acid), which is synthesized as a hormone during plant development and maturation. Auxin stabilizes the interaction between Aux/IAA proteins and the plant E3 ligase Tir1. Auxin is the founding member of a wider class of organic plant hormones that function by inducing proteasomal degradation and serves as the inspiration for the development of “molecular glue” degraders¹¹⁴. Molecular glues are low molecular weight, rigid, small molecules that stabilize the interaction between proteasome substrates and their respective E3 proteins without the presence of a flexible linker. These molecules have the advantage of being more druglike but depend on very specific structural fits between their target proteins and relevant E3 ligase¹¹⁵.

The first fully synthetic PROTAC, termed Protac-1, was conceived in a collaboration between Raymond Deshaies and Craig Crews, and targeted methionyl aminopeptidase 2 (METAP2). This PROTAC was constructed using ovalicin, an angiogenesis inhibitor and known binder of METAP2, and a 10-residue phosphopeptide from nuclear factor- κ B inhibitor- α (NF-

κ BI α) that binds the E3 ligase β -transducin repeat-containing E3 ubiquitin–protein ligase (β -TRCP). In 2001, it was shown that Protac-1 mediates ubiquitination and degradation of its target in *Xenopus* egg extracts¹¹⁶. These early PROTACs were superseded by true, cell-penetrant molecules with the discovery of synthetic mimics of hypoxia-inducible factor 1 subunit- α (HIF1 α), a peptide that binds the E3 ligase von Hippel–Lindau tumour suppressor (VHL)^{117,118}. Soon after, the E3 ligase Cereblon (CRBN) was identified as the target of the small molecule drug thalidomide¹¹⁹.

The discovery of these small organic molecule binders of Cereblon and VHL established these ligases as the two mainstay PROTAC ligases. Of the two, Cereblon-based degraders appear to have been more effective at reaching clinical stage studies¹¹⁰. These degraders recruit Cereblon using either thalidomide, or chemical analogs of thalidomide, like pomalidomide or lenalidomide. Recent structural and biochemical work, enabled by cryo-electron microscopy studies, indicates that Cereblon features an open “active” conformation and a closed, “inactive” conformation. Binding of thalidomide and its derivatives to Cereblon biases the structure towards the open conformation, increasing its propensity to ubiquitinylate neosubstrates^{120,121}. Due to this, treatment with these thalidomide derivatives absent any additional chemical moiety is sufficient to cause degradation of the immune-related transcription factors IKAROS (IKZF1) and AIOLOS (IKZF3) as neosubstrates. As a result, these thalidomide derivatives are used clinically as “immunomodulator imide drugs (IMiDs),” notably in the treatment of multiple myeloma^{122,123}. These facts, however, immediately suggest a concern about thalidomide-based degraders: will these degraders have the same pharmacological activity as thalidomide on its own, since the degraders incorporate a thalidomide derivative? While this is a reasonable concern, safety and efficacy studies indicate that this is not the case, largely because of the catalytic nature of the degrader, and the naturally low bioavailability of these bulky, heterobifunctional degraders. Work performed in industry indicates that in clinical stage degraders, less than 1% of the cell’s Cereblon pool is engaged by degraders at a given time, minimizing the odds of undesired neosubstrate degradation. However, off-target binding of the degrader molecule itself remains a concern, and limiting this is a major point of safety and efficacy testing.

While these two E3 ligases make up the lion’s share of all functional degraders, there are reports in the literature of other E3 ligases proving effective, and there is significant interest in targeting E3 ligases that are enriched in particular tissues to confer tissue specificity to the degrader¹²⁴. Our goal, however, is to create a general-use activator of autophagy. This makes Cereblon the ideal E3 ligase for my purposes, and earmarks the -imide molecules as preferred E3 ligase-binding moieties for my degraders

Following the identification of synthetic E3 ligase binders, the TPD field entered a phase of first gradual and then exponential growth, culminating in the start of PROTAC clinical trials under the aegis of Arvinas in 2019. These clinical trials constituted the first evidence that PROTACs can be well tolerated by patients and efficacious as chemotherapeutic agents¹¹⁰.

Bifunctional degraders are not, however, without their weaknesses. Notably these molecules tend to be quite large, often approaching 1 kDa, are generally poorly soluble, and have

bioavailability profiles that would traditionally be disqualifying for small molecule drugs. However, because of the catalytic nature of these compounds, even a small amount of active degrader is often sufficient to diminish the cellular pool of their target proteins by significant amounts. These physical properties also make it more challenging for these molecules to cross biological membranes and partitions, including the blood brain barrier. On the other hand, the large and flexible nature of these molecules allows them to fold in on themselves, reducing their effective surface area and promoting membrane partitioning in ways that often flout traditional small molecule “rules”. Thus, these molecules often prove surprisingly effective at inducing degradation in hard-to-reach tissues^{125,126}. Two companies have recently reported success in developing brain-penetrant degraders. Arvinas reports that their LRRK2 degraders are active in deep brain tissues in primate studies, and Nurix reports that their BTK degraders have proven effective in treating a set of brain cancers in a small-scale study. As a result, it seems like protein degraders may be useful in more cases than was previously anticipated.

These facts taken together suggest that degraders are the most promising modality by which to drug Rubicon. The discovery stage work for a Rubicon degrader followed roughly three stages: (1) identification of Rubicon-binding small molecules, (2) construction and optimization of a bifunctional Rubicon degraders using these molecules, and (3) demonstration of *in cellulo* efficacy of these degraders to lower the level of Rubicon in cells and induce autophagy.

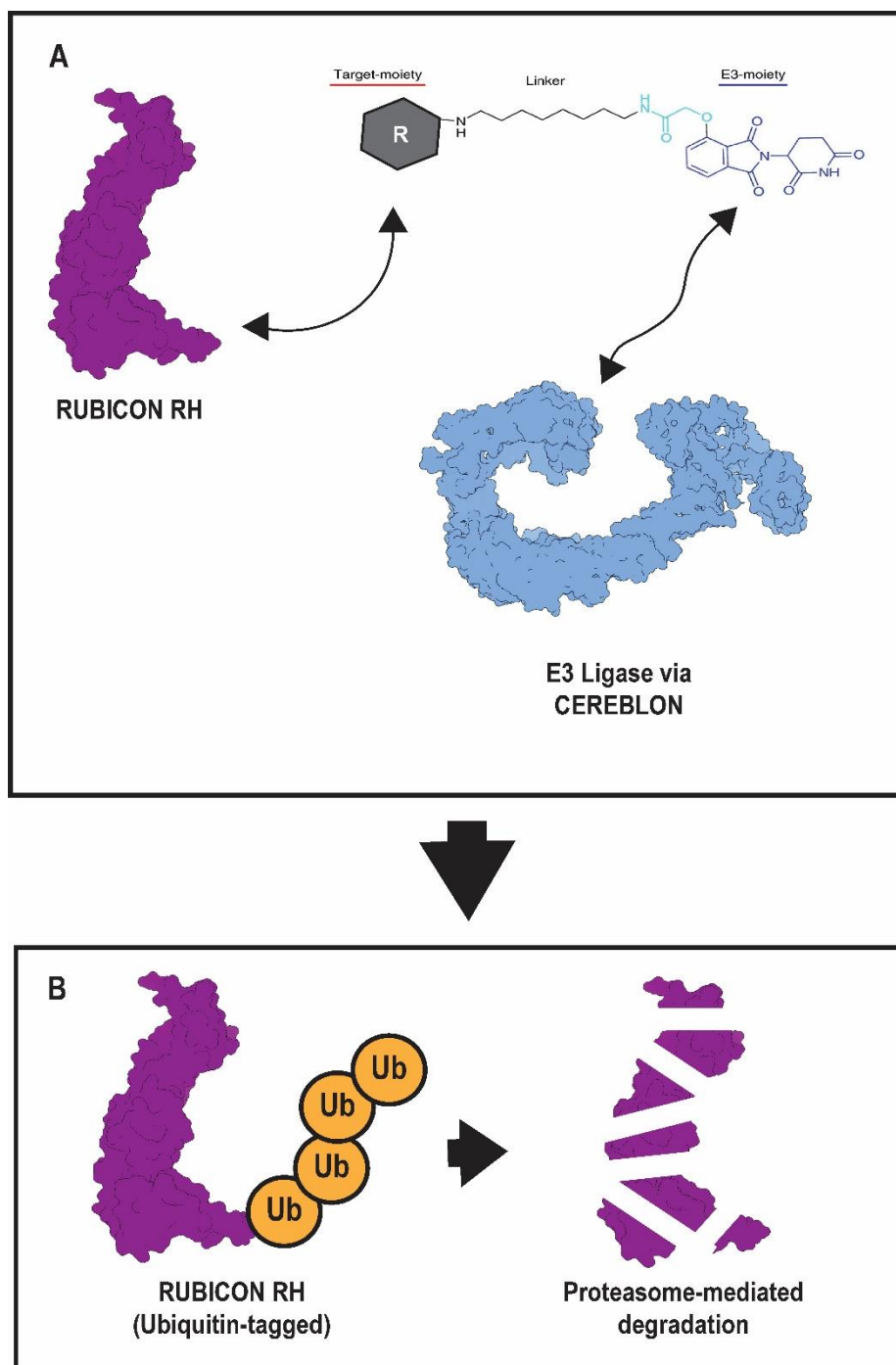


Figure 19. Mechanism of bifunctional degrader activity. (A) A degrader tethers a target protein via some small molecule moiety (R) to an E3 ligase, in this case CEREBLON, recruited via a thalidomide-like molecule. (B) The induced proximity of the ligase with the target protein results in ubiquitination of the target protein, and subsequent proteasome-mediated degradation.

Identification of Rubicon-binding small molecules

The primary goal of this screening step is to identify a small molecule that binds Rubicon with at least micromolar-range affinity. While such a binder would be a poor hit in a traditional small molecule drug development pipeline, micromolar affinity degraders have been developed and demonstrated to be efficacious in the past. Such a hit would be a sufficiently strong binder to quantify under a variety of biophysical methods and would likely be able to be optimized further under a dedicated Structure-Activity Relationship (SAR) study. To find such a hit, I used a DNA-Encoded Library (DEL), a relatively recent development in small molecule screening.

The DNA encoded library consists of a synthetic scheme in which a chemically labile scaffold is reacted with a series of three “building blocks,” termed R1, R2, and R3. Each of these blocks describes a library of molecules with chemistries allowing them to conjugate to the scaffold at specific sites, but which vary structurally. Following incubation with a pool of “R1” building block molecules, the resulting products are exposed to a pool of “R2” building block molecules, resulting in formation of combinatorial products. This procedure continues until all building block pools have been added to the scaffold, generally for three cycles. Because of this, the total number of synthesized molecules increases exponentially with the number of cycles used to generate the library, resulting in roughly 10^8 distinct molecules in a single, pooled library. This procedure is often repeated with a different type of scaffold and a different set of building blocks to create additional libraries, which are then mixed to form a “library of libraries.” To track this massive population of distinct molecules, each building block is tagged by a unique oligonucleotide. When the building block is chemically bound to the scaffold, the DNA tag is added to that of the scaffold. This process repeats with the second and third building block, resulting in each molecule having a unique DNA identifier¹²⁷.

The concept of DNA-encoding was first established in 1992 in a theoretical paper by Brenner and Lerner, who proposed tagging each synthesized entity with a unique oligonucleotide tag¹²⁸. The first practical implementation of this idea was achieved in the following year by J Nielsen, S. Brenner and K. Janda and similarly by the group of M.A. Gallop^{129,130}. Early efforts at DEL library design were hampered by the relatively narrow range of organic synthetic reaction conditions that unprotected DNA can tolerate without being destroyed or otherwise compromised. Advances in chemistry allowing the protection of the DNA tag, as well as novel methods of library construction, have greatly improved the size and quality of DELs in the last two decades. In the modern day, DELs are routinely used in the pharmaceutical and biotech industries.

A lab scientist performing a DEL screen gets a single test tube populated with 10^8 to 10^9 distinct compounds, each one labelled with a unique DNA tag. To screen for binders, the user binds their protein of interest to a resin via standard affinity methods, and then incubates this resin with the library. The resin is washed, and the bound DEL molecules are eluted via incubation at 95 C. This population of molecules contains binders for the protein of interest, but likely has many nonspecific binders as well. To further enrich for true, specific binders, this sample is applied to another, identically prepared affinity resin with the protein of interest, washed, and eluted via heat (Figure 20). This cycle of binding, washing, and eluting enriches the

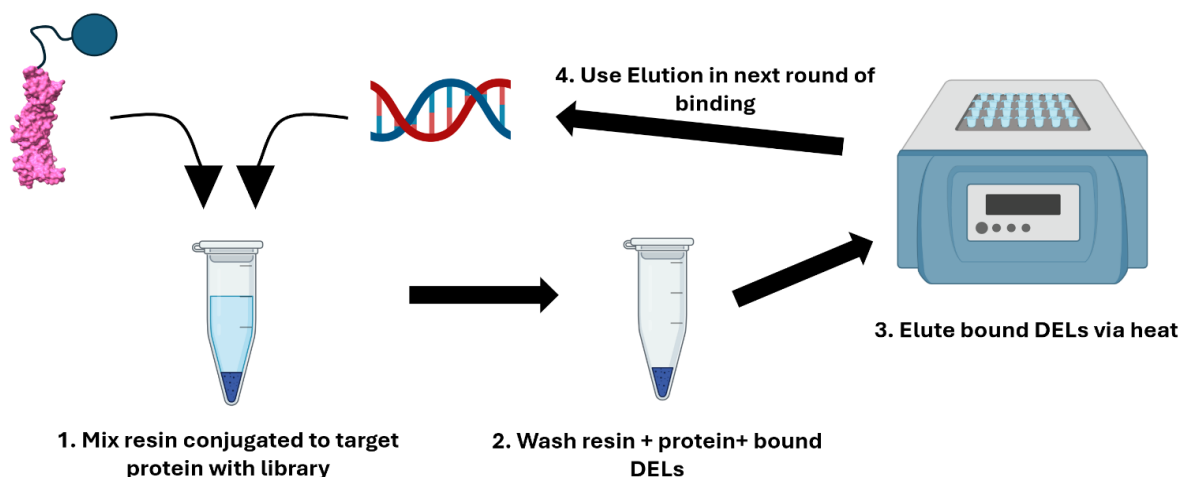


Figure 20. DEL screening cycle. Images generated via BioRender

sample with specific binders¹³¹. The rigor of the washing is tracked by quantifying the total amount of DNA in each sample via standard qPCR methods. The more rigorous the washing, the lower the total amount of DNA left in solution, because nonspecific binders (along with their tags) are being washed out. Once the DNA tag count drops beneath a certain threshold, the sample is assumed to be relatively specific and rigorously selected and is ready for decoding. To identify the compounds present in the final elution, the DNA tags are amplified via PCR, and then read using next generation sequencing (NGS). This allows the user to rapidly decode the diverse and complex pool constituted by the DEL library. The final output of this process is a list of each molecule present in the solution, identified by its scaffold, R1/2/3 building block identity, and the “counts” of molecule present in the final elution.

Of course, each molecule in this list is not completely unique—since every combination of building block is, in theory, present in the DEL, there are many molecules in the set that might vary only by a single chemical group. Furthermore, every molecule in each sublibrary has the same scaffold as other members. To better understand the similarities between identified compounds, and better grasp relationships and trends, the DEL screening results are plotted as a series of 3D spaces, each one corresponding to a sublibrary/particular scaffold. Each axis represents the index of “building block 1/2/3,” and each plotted point corresponds to a hit identified by its position in the space (ie, the x coordinate gives the identity of building block 1, the y coordinate gives the identity of building block 2, the z coordinate gives the identity of building block 3). In this space, the number of “counts,” or the relative strength of each hit, is represented by the size of the dot (Figure 21, 22).

Two considerations should be made when analyzing DEL data in this way. Firstly, while every combination of building block is expected to be present in the DEL, the relative frequency of each is not necessarily known; building blocks may vary in how reactive they are with regards to the scaffold, so the absolute amount of each molecule going into the screening is not known. As a result, a molecule that has many counts may indeed be a strong binder of the protein of interest, or it may happen to just be a molecule that is easily synthesized, and thus is

overrepresented in the library. As a result, decisions regarding which hits to advance to the next stage of the drug development pipeline should not be made on the basis of the count of each molecule, but rather on the basis of “trends” observed in the 3D plot. These trends indicate that binding occurred even as one or two of the building blocks varied in the DEL, giving confidence that the molecules are not present simply because they were readily synthesized. Visually, this can appear in the 3D plot as “planes” (which correspond to only one of the building blocks being necessary for binding), “lines” (in which two of the building blocks contribute to binding in some way, and the third is dispensable), or “points” (in which all three of the building blocks need to have a particular identity in order for—putative—binding to occur)^{127,132}. This last situation is considered more suspect, since there is no replication of binding across a variety of structurally similar compounds.

The unique nature of DELs as relatively uncontrolled yet data-rich screening methods have several implications for the hit validation stage and other downstream optimization approaches, including ones relevant to degrader design. Firstly, users should expect an elevated rate of false positives compared to traditional small molecule screening approaches. Balancing this, the screening results themselves come structure-activity relationship information: one can quickly check the “lines and planes” of the 3D plot to determine which chemical groups in the hit molecules are necessary, and which ones are disposable, allowing users to quickly pare down the molecule to its active, minimal chemical groups. This is particularly important in the case of bifunctional degraders, which are large molecules to begin with; minimizing the size of the warhead affords greater flexibility in the design of the degrader farther down the pipeline. Additionally, the data-rich nature of the DEL lends itself well to machine learning methods, which can be leveraged to propose hits to validate within the library itself and can potentially point to chemical matter outside of the library but bearing similarity to the validated hits^{127,132}.

I screened three samples using a commercially available DEL library: one consisted of MBP-Rubicon RH, which represents the only domain of Rubicon that was tractable in my hands. The second consisted of MBP alone and served as a blank trial. The third sample consisted of MBP-Rubicon RH, and a saturating amount of RAB7 was included in all the buffers. The goal of this final sample was twofold: firstly, to ensure that identified binders can associate with Rubicon RH even in the presence of RAB7, its binding partner. Secondly, if a hit is abolished by addition of RAB7, this indicates that RAB7 occludes the binding interface between these two proteins. Since Rubicon needs to bind to RAB7 to be fully active, such a molecule could serve as an activator of autophagy even absent any other chemical features.

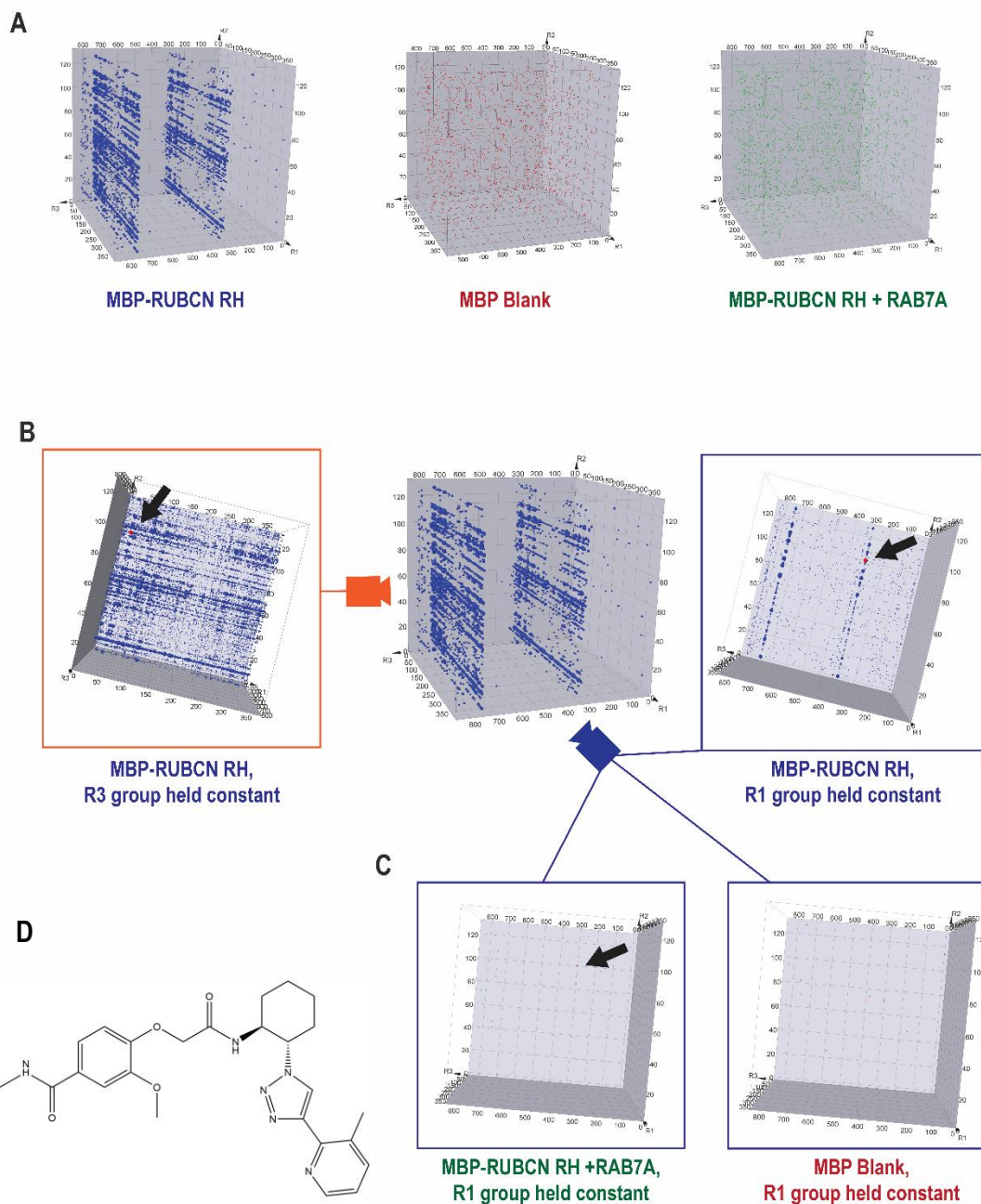


Figure 21. DEL Identification of DAT-12. (A) Overviews of DEL results in this sublibrary by sample, with the MBP-RUBCN RH sample thresholded to only include hits with counts >4, and MBP blank and MBP-RUBCN RH/RAB7A sample representing all hits regardless of count. (B) Slices of MBP-RUBCN RH hit space with either R3 group or R1 group held constant. Arrows indicate DAT-12. (C) R1 constant slice depicting RUBCN/RAB7A and MBP Blank samples. (D) Chemical structure of DAT-12.

DAT-2

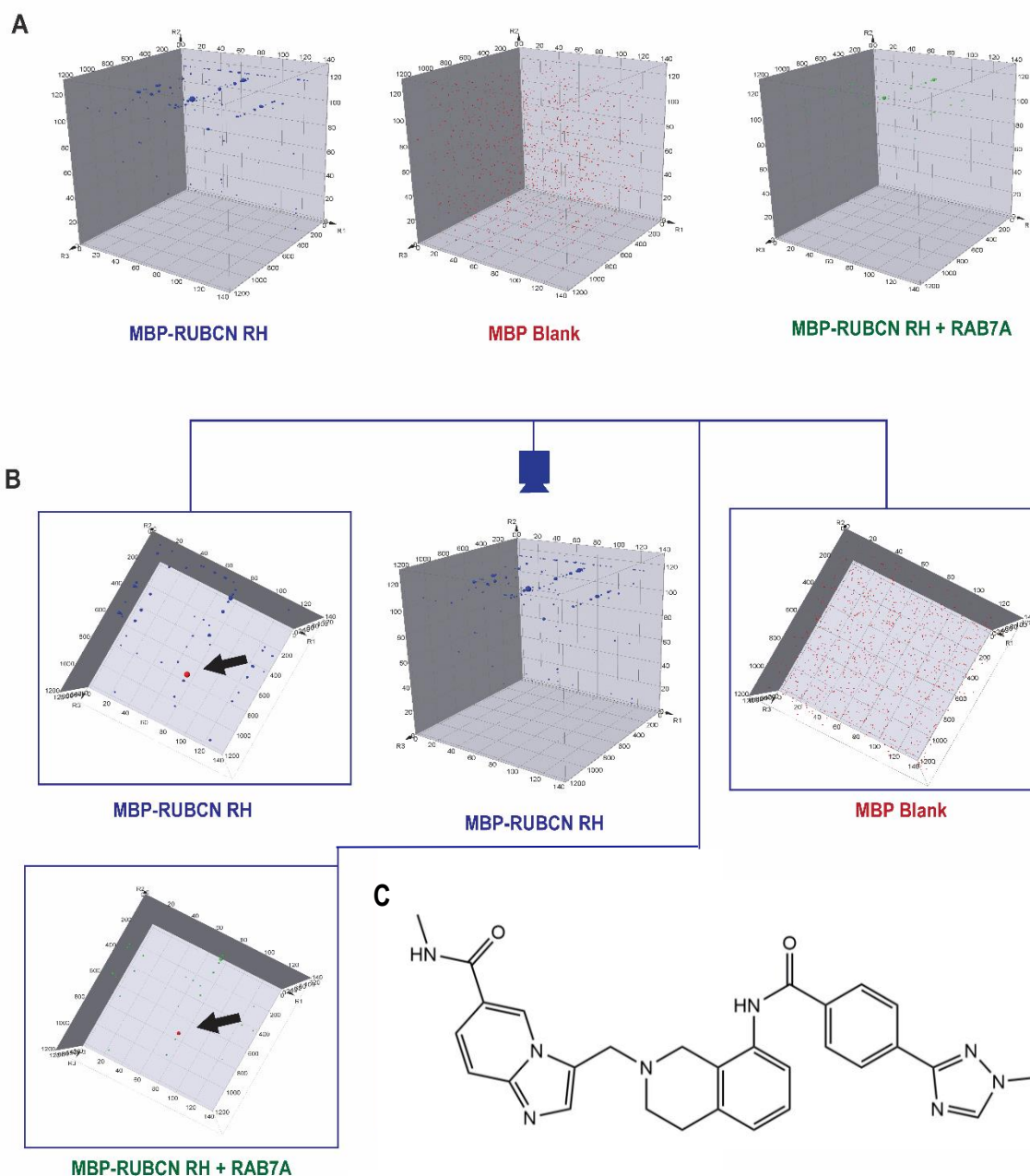


Figure 22. DEL Identification of DAT-2. (A) Overviews of DEL results in this sublibrary by sample, with the MBP-RUBCN RH sample thresholded to only include hits with counts >4, and MBP blank and MBP-RUBCN RH/RAB7A sample representing all hits regardless of count. (B) Slices of MBP-RUBCN RH hit space with either R2 group held constant. Arrows indicate DAT-2. (C) Structure of DAT-2.

From this effort, I identified two Rubicon binding leads, which I term “DAT-2” and “DAT-12.” DAT-12 was identified from a plane, indicating that a single building block comprised the active binding unit (Figure 21). This turned out to be the case—synthesis of the highest count hit in this plane, including the scaffold and two additional building blocks, yielded a molecule with a $K_D = \sim 15 \mu\text{M}$. A custom synthesized molecule representing only building block 3 (DAT-38), however, had a K_D of $\sim 10 \mu\text{M}$ (Figure 24). DAT-2 features a distinct scaffold and set of building blocks and appears to bind MBP-Rubicon RH with a much higher affinity than DAT-12 ($K_D = \sim 1 \mu\text{M}$ for DAT-2 compared to $>10 \mu\text{M}$ for DAT-12) (Figure 22, 23). However, some low amount of binding to MBP was observed. Following an SAR campaign, I was able to identify a much smaller truncation of this molecule, labelled, DAT-39, which binds Rubicon with a K_D of 500 nM, and has essentially no binding to MBP (Figure 23). Between these two hits, DAT-12 showed poorer binding in the +RAB7A conditions than did DAT-2, suggesting that DAT-12 may compete with RAB7A while DAT-2 does not.

To validate these hits, resynthesized compounds were tested for binding using Surface Plasmon Resonance (SPR), and positive hits were double-checked using ITC to rule out any contribution to binding from the SPR chip or immobilization chemistry used to tether MBP-Rubicon to the surface of the chip. With two Rubicon-binding small molecules in hand, we developed a set of “first generation” degraders using these moderate affinity binders, while also attempting to improve binding affinity via generation of structural analogs to the initial two leads.

This strategy was chosen because the relationship between the binding affinity of the bifunctional degrader for its protein target does not directly relate to its ability to degrade this protein. While unintuitive, this fact reflects the unique nature of degraders as mediating a transient interaction between E3 ligases and their target proteins. In fact, a binding affinity that is too high may hamper the ability of the degrader to dissociate from its target protein once a ubiquitin chain has been built and thereby impair the catalytic nature of the degrader molecule. This is a significant difference between degraders and small molecule inhibitors, where increased binding affinity for the target directly relates to increased potency. Nonetheless, it is still useful to identify the minimal binding group of the protein warheads, as well as to improve the binding affinity to generate a wider space of degraders to test *in cellulo*. Thus, creation and optimization of “full” degrader molecules can be performed in parallel with more traditional small molecule binding optimization studies.

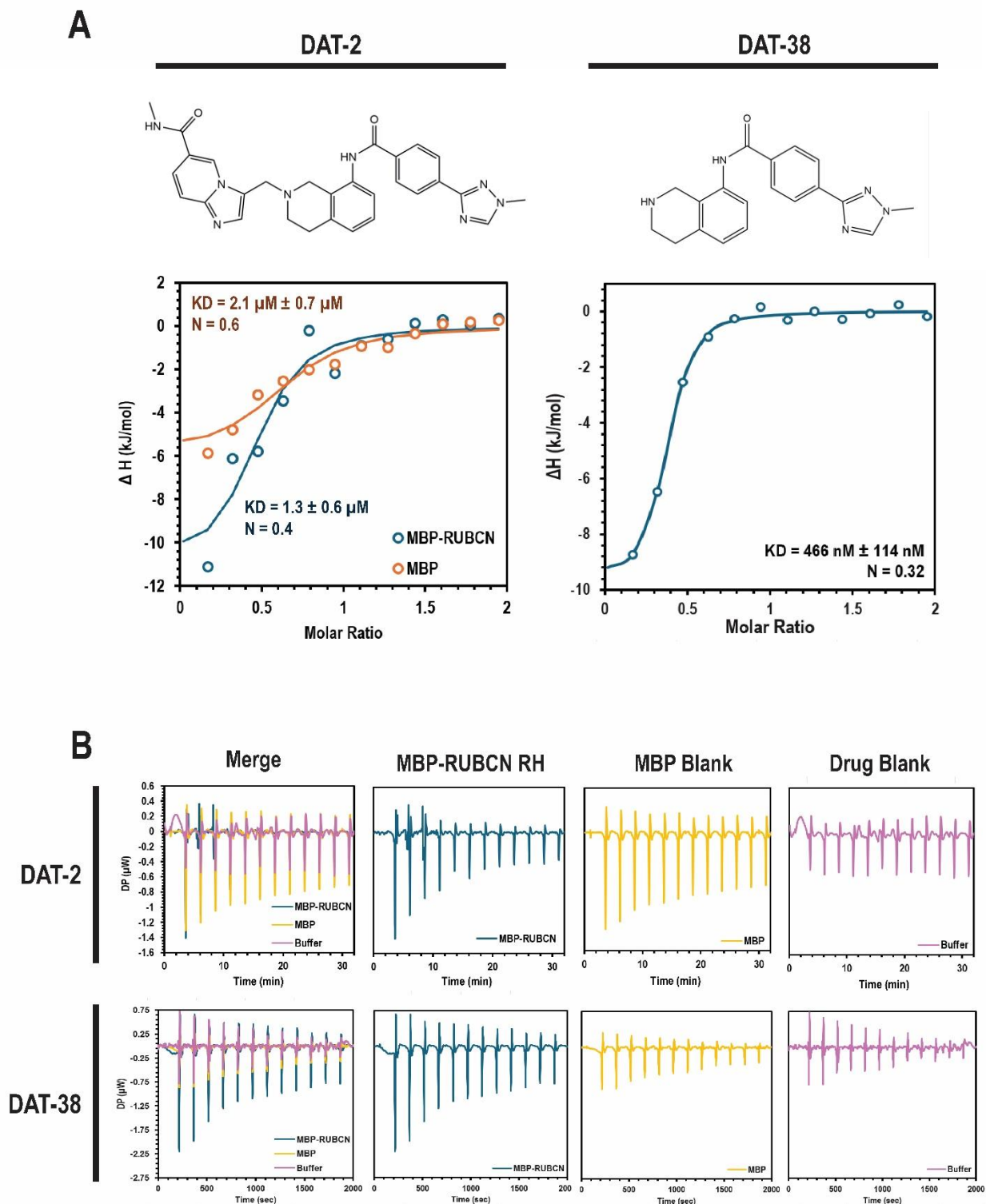


Figure 23. ITC determination of DAT-2 and DAT-38 binding. (A) Binding curves representing molar heats evolved from each injection in the titration. The DAT-2 binding curve depicts MBP-RUBICON (lower curve, blue) as well as MBP (upper curve, orange). The DAT-38 binding curve shows only binding to MBP-RUBICON RH, as no appreciable binding to MBP was observed. K_D and N values are derived from fitting the data to a single site binding model. (B) ITC traces representing titrations of DAT-2 or DAT-38 injected into samples of either MBP-RUBCN RH, MBP, or buffer (“drug blank”). Traces are a 13 injection series, where the chamber is kept at a [protein] of 25 μ M, and the binder is injected at a concentration of 250 μ M.

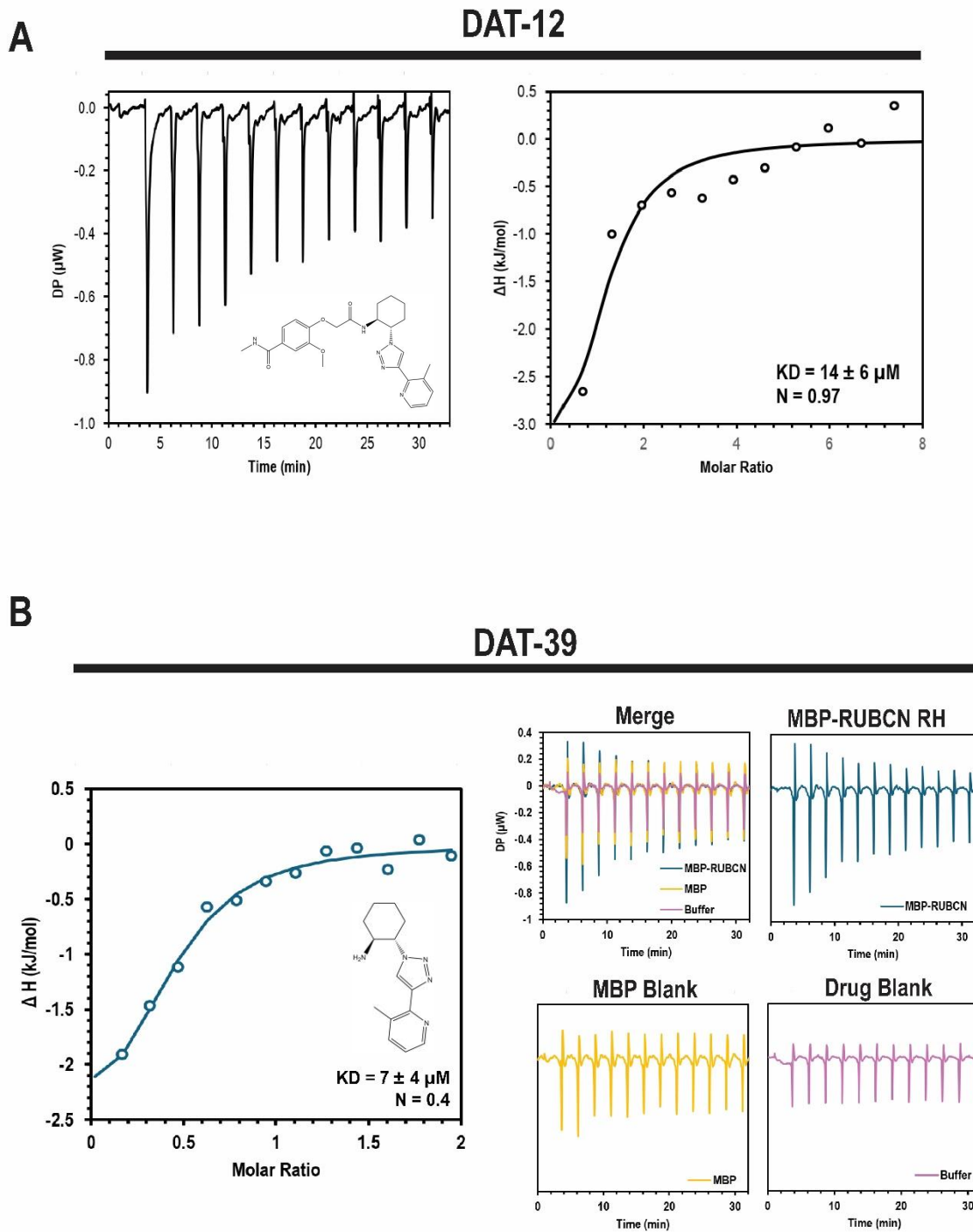


Figure 24. ITC determination of DAT-12 and DAT-39 binding. Data collected and processed as in previous Figure. (A) DAT-12 ITC trace and binding curve. (B) DAT-39 ITC trace and binding curve.

The DEL libraries used in this study were designed to be relatively “drug-like” out of the box. In other words, the median member of this DEL library is sufficiently small and lipophilic to be able to cross membranes and engage target proteins within the cell. However, not all members of the library share these qualities, and many may be incapable of binding their target proteins when incubated alongside cultured cells. To verify that DAT-2 and DAT-12 can cross cell membranes, I performed a Cellular Thermal Shift Assay (CETSA)¹³³. In this assay, cultured HEK-293 cells expressing mCherry tagged Rubicon are incubated in medium containing DAT-2 or DAT-12 for a half hour before being resuspended and exposed to high temperatures. Quantification of soluble Rubicon via fluorescence is made possible by the fact that mCherry is a highly robust protein, with an unfolding temperature (T_m) of 95 C, allowing it to track the presence of Rubicon without being affected by the denaturing temperatures used. After exposure to high temperatures, the cells are lysed via freeze-thaw in liquid nitrogen, and then pelleted at high speeds. Unfolded Rubicon would be retained in the pellet, while stable, soluble Rubicon would remain in the supernatant. The soluble fraction is run on a gel, and the prevalence of mCherry-Rubicon is assessed via in-gel fluorescence. Performing this experiment at several temperatures using a thermocycler allows construction of a denaturation curve. Positive binding of a drug to Rubicon would be represented by a rightward shift in the degradation curve, as binding of a drug to Rubicon results in the formation of stabilizing drug-protein interactions, and consequently a higher denaturation temperature.

Indeed, both DAT-2 and DAT-12 stabilize Rubicon under this assay. Rubicon has a denaturation temperature of 45 C, which is enhanced to 48.5 C in the presence of DAT-12, and 46.9 C in the presence of DAT-2. While this effect may be small, it is clear to the naked eye, and reflects the relatively weak binding affinities of these initial hits (Figure 25). Having shown that these Rubicon binders are able to cross cellular membranes, we were free to construct full size, degradation-capable PROTACs.

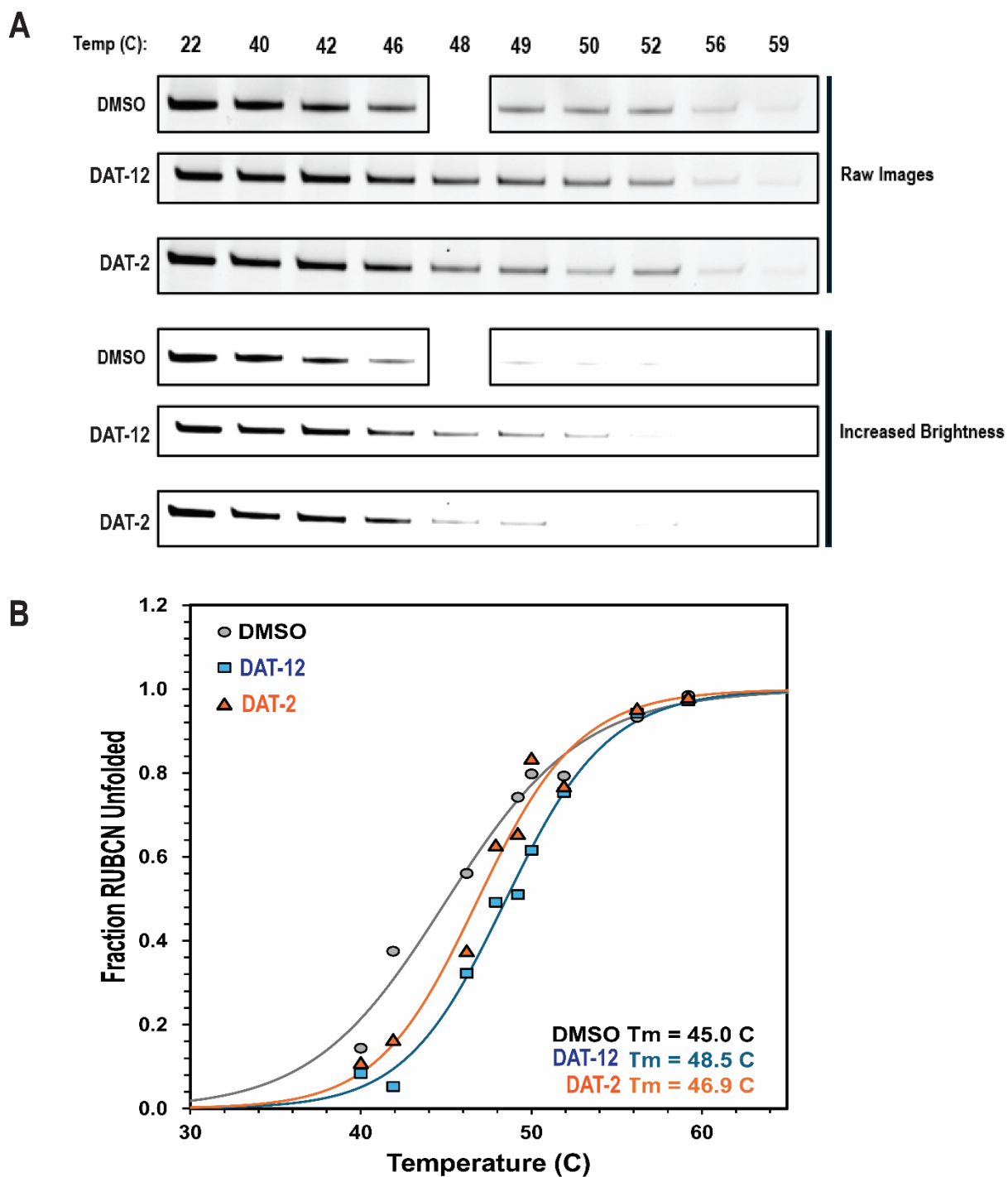


Figure 25. CETSA of DAT-2 and DAT-12 with mCherry-Rubicon. (A) in-gel, mCherry-Rubicon fluorescence. (B) Thermal Denaturation curve fit to the fluorescence intensity in panel A. Fit performed using the Solver plugin in Microsoft Excel.

Construction and optimization of a bifunctional Rubicon degrader

A full, functional PROTAC consists of a protein binding warhead, a flexible linker, and an E3-recruiting moiety. The design of the flexible linker, meaning its chemical composition and length, can have a massive impact on the ability of the degrader to coordinate both the target protein and the E3 ligase. There is only a limited set of “rules” that define what a good flexible linker looks like: 70% of published degrader linkers consist of either alkyl chains or polyethylene glycol (PEG) polymers, and typically range from 5 to 20 heavy atoms, with a median of 12. PEG is hydrophilic, while alkyl chains are hydrophobic^{134,135}. As a result, the choice of linker can be informed by the desired physicochemical characteristics of the molecule, and the properties of the chosen warhead.

In the case of more mature, well-studied PROTAC-protein interactions, there is significant interest in cyclizing, shortening, or otherwise rigidifying the PEG linker, which is expected to improve target engagement and reduce the entropic penalty of a linker associating with or adhering to a protein’s surface. However, these more sophisticated approaches require high quality structural data, and depend on the existence of highly cooperative ligase-protein interaction that can form stable and tractable tertiary complexes¹³⁴. There is no guarantee that such a complex could be formed with the Rubicon RH domain but may provide an interesting optimization avenue in the future.

As a first panel of possible degraders, we linked both our initial Rubicon warheads, DAT-2 ($K_D = 1 \mu\text{M}$) and DAT-12 ($K_D = 15 \mu\text{M}$), to a pomalidomide (a thalidomide derivative) moiety via linkers consisting of either PEG or alkyl groups, ranging from 4 to 16 heavy atoms. To test their degradative capacity, dilutions of these molecules ranging from 10 μM to 100 nM were made, and HEK-293 cells were incubated with these compounds for 6 and 16 hours. All the DAT-2 derivatives showed some amount of degradation, ranging from 10 to 80% depending on the linker types and compound concentration as assessed via Western blot (Figure 26). To contrast, the DAT-12 derivatives showed weak or inconsistent degradation, in keeping with the poor affinity of DAT-12 for Rubicon (Figure 23). This degradation was completely blocked by treatment with the proteasomal inhibitor MG-132; it was also characterized by a “hook effect,” where treatment with high concentrations of these degraders prove less effective than treatment with moderate amounts of degrader as binding sites become titrated out and binary complexes become favored over tertiary complexes. These two phenomena demonstrate that these molecules are degrading Rubicon via Cereblon recruitment and subsequent proteasomal degradation. The most potent degrader in this first panel was defined by a D_{max} of at least 80%, with a coarse half-degradative concentration (DC_{50}) estimate in the low hundreds of micromolar. This molecule is a fully functional PROTAC, performing well compared to the set of published PROTACS (across all published PROTACS, the median DC_{50} is in the hundreds of micromolar, and the median D_{max} is 70%).

DAT-2 warhead degraders

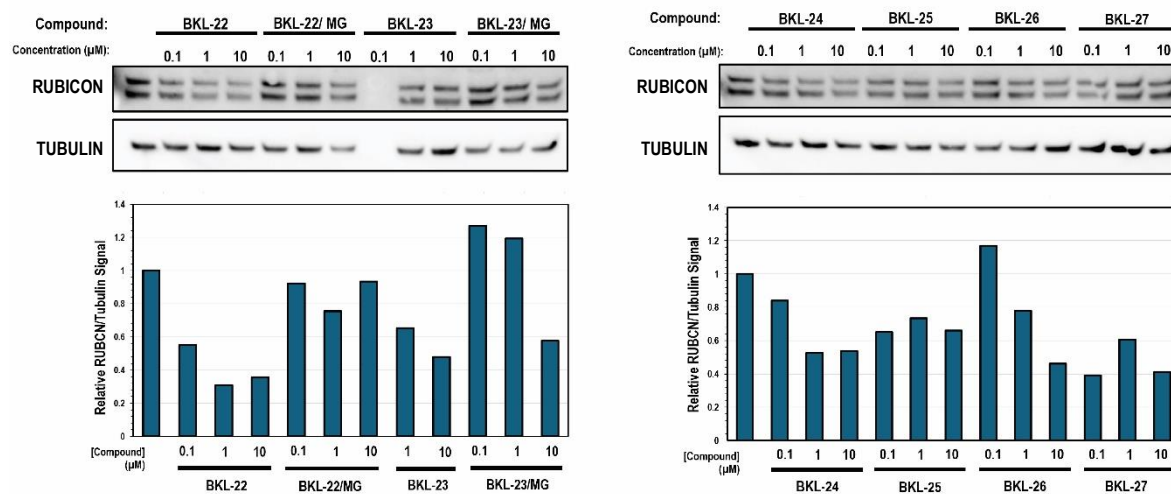


Figure 26. Western blot showing Rubicon degradation after a 16 H incubation with DAT-2 derivative degraders. with each indicated degrader candidate. HEK-293 cells were grown to be 80% confluent at time of harvest. Bar graph indicates normalized Rubicon/Tubulin loading control lanes. Conditions labelled with “MG” included 1 μM MG-132 alongside the indicated PROTAC, representing a proteasome inhibition control.

DAT-12 warhead degraders

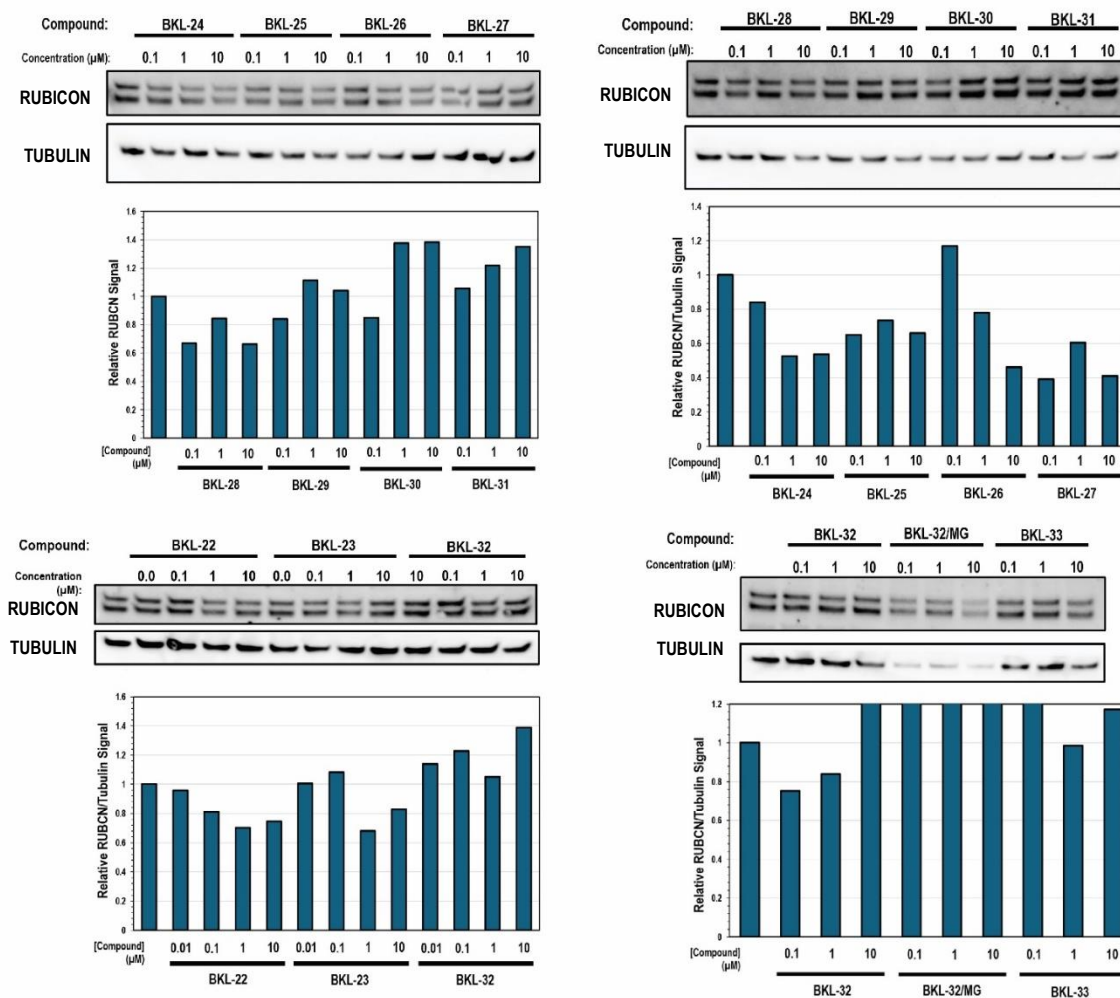
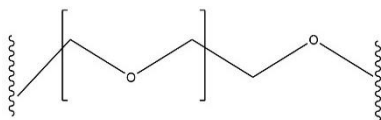


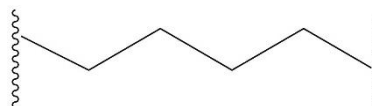
Figure 27. Western blot showing Rubicon degradation after a 16 H incubation with DAT-12 derivative degraders. Experiment performed and data presented as in previous figure.

Table 1. First Generation Rubicon Degradable Designs

Compound	Warhead	Linker Composition	Linker Length (# heavy atoms)	Apparent D _{max} (%)
BKL-22	DAT-2	PEG	5	69.2
BKL-23	DAT-2	PEG	8	52.1
BKL-24	DAT-2	PEG	11	47.5
BKL-25	DAT-2	Alkyl	5	34.8
BKL-26	DAT-2	Alkyl	8	53.9
BKL-27	DAT-2	Alkyl	11	60.8
BKL-28	DAT-12	PEG	5	20.1
BKL-29	DAT-12	PEG	8	0.0
BKL-30	DAT-12	PEG	11	0.0
BKL-31	DAT-12	Alkyl	5	0.0
BKL-32	DAT-12	Alkyl	8	24.7
BKL-33	DAT-12	Alkyl	11	0.0



PEG Linker Composition



Alkyl Linker Composition

Future direction: ternary complex optimization studies

With an effective degrader in hand, the goal then becomes to optimize the molecule to improve its potency and maximum extent of degradation. Degradators often have very poor pharmacological profiles due to their large size and complex topology and are rendered effective only by their catalytic nature and consequently their high “per molecule” potency¹²⁶. The key biochemical event that this effort should be optimized around is the formation of the “ternary complex” featuring the protein of interest, the degrader molecule, and the E3 ligase in question (in our case, Rubicon, our degrader, and Cereblon). In contrast to traditional inhibitors, which generally rely on the formation of a binary complex between a target protein and its small molecule inhibitor, it is possible to “over titrate” the degrader. Generally, as the concentration of degrader is increased, formation of the ternary complex is promoted up to a certain concentration. Past this point, the kinetics of complex formation favor the creation of binary complexes between the degrader and the POI, or between the degrader and the E3, which are nonproductive interactions. This “hook effect” alters the way in which optimization of the degrader is conducted, since simple improvement of binding affinity may be insufficient to overcome the hook effect and can even exacerbate it by favoring formation of the binary over the tertiary complex. The hook effect imposes a ceiling on the effectiveness of a given degrader, and contributes to the existence of a $D_{\max}$ parameter, or maximum extent of target degradation achievable¹³⁶. Given these facts, optimization efforts should be focused on studying and promoting formation of the ternary complex. In pursuit of this, there is a toolbox of assays consisting of *in vitro* and *in cellulo* experiments.

The most commonly used experiment in studying formation of the ternary complex is the NanoBRET assay, patented and sold by Promega. This is an *in cellulo*, Bioluminescence Resonance Energy Transfer (BRET) assay. Briefly, the protein of interest is expressed with a luciferase tag, alongside Cereblon bearing a fluorescent ligand-containing HaloTag. Colocalization of the protein of interest with Cereblon within cells results in energy transfer from the POI-luciferase to the Cereblon-HaloTag. The relative extent of ternary complex formation can be measured as the ratio of the HaloTag fluorescence over the brightness of the luciferase, providing a quantitative method of observing complex formation in the context of cells. This approach can also be scaled up to 96-well plates, allowing for effective screening of many different degrader analogs. This experiment can also be run kinetically if the correct conditions are observed, allowing the formation of tertiary complex and the degradation of the target protein to be followed in real time. NanoBRET assays allow users to quantify formation of a tertiary complex in the native environment of the cell but come with several notable weaknesses. Firstly, these experiments rely on transient transfection to expression tagged versions of your protein of interest and your target ligase. As a result, the expression level of these proteins can be much higher than would otherwise be expected, potentially resulting in kinetic data that is drastically different compared to experiments run using endogenous levels of protein expression¹³⁷. This can be ameliorated somewhat by using cell lines where the protein and ligase of interest are tagged at the endogenous genetic loci with BRET tags, but this can incur significant additional time and expense, to the tune of tens of thousands of dollars in extra costs and months of development time.

A lower throughput, *in vitro* approach is to examine formation of the tertiary complex via biophysical binding approaches such as SPR and ITC. Here, you would perform an experiment where you measure the binding of your compound to your target protein with and without the E3 ligase included in the experiment. We would expect inclusion of the ligase to increase the apparent affinity of the degrader for its target, since the E3 would also be recruited and would form additional, stabilizing contacts with the protein of interest. These assays are limited by their throughput, since they are not amenable to 96 well formats, and require more sophisticated automation to screen many compounds efficiently. Thus, this approach is generally applied to a small number of key molecules in a typical drug development effort. Bilayer interferometry (BLI), an alternative to SPR better suited for measuring protein-protein interactions, can also be leveraged in these circumstances. While the sensitivity of BLI is generally insufficient for small molecule experiments, its high-throughput, 96 well setup makes it preferable for protein-scale experiments. Since this experiment tracks formation of a ternary complex and therefore recruitment of a protein rather than a small molecule, BLI may be a preferable alternative to more traditional biophysical approaches¹³⁸.

Special care must be taken in these experiments to avoid the hook effect. Generally, this involves keeping one member of the ternary complex at a high concentration—generally ten times the KD of the binary interaction—to ensure that every degrader molecule is always bound to at least one member of the ternary complex. Subsequently, the second protein member of the ternary complex is added, and the resulting binding affinity or kinetics is measured via whichever biophysical method is chosen, tracking either the released heats of binding in the case of ITC or recruitment to the chip in the case of SPR. Since every copy of the degrader molecule is assumed to already be bound to the “high concentration” member of the tertiary complex, any observed attribute of the binding interaction reflects recruitment of the protein and therefore formation of the ternary complex. The parameter determined from an experiment like this is termed the “cooperativity,” or α , and is calculated by dividing the KD of binary complex binding by the KD of tertiary complex binding ($\alpha = \text{KD}_{\text{binary}}/\text{KD}_{\text{tertiary}}$, or $\alpha = \text{KA}_{\text{tertiary}}/\text{KA}_{\text{binary}}$). Simply put, this parameter describes how much more stable the catalytically active tertiary complex is relative to the intermediate binary complex, and therefore how likely binding of the degrader to the target protein is to result in recruitment of an E3 ligase and ubiquitination/degradation¹³⁸. While binary affinity between the degrader and target protein has very poor predictive power for the D_{max} or DC_{50} of the degrader *in cellulo*, this cooperativity parameter is able to reliably determine which candidates will be more effective in cells, albeit in a coarse way. As a result, determination of the cooperativity of a given degrader can be done at key decision-making junctions and could inform which candidates are the best to advance further down the drug development pipeline.

Future direction: Phenotypic experiments – enhancement of autophagy *in cellulo*

There is a wealth of experimental evidence in both tissue culture and mouse model settings that indicates that deletion of Rubicon promotes autophagy flux and ameliorates several disease related phenotypes^{84,103-105,107,108}. However, it is important to replicate these results using

a Rubicon degrader. Pharmacological degradation of Rubicon is likely to display a different, and perhaps more potent phenotype than knockout experiments. Autophagy is intimately involved in broader metabolic regulatory schemes^{1,2,94}, which are known to have “thermostat” like capacities to restore flux to an equilibrium when perturbed in some way. Knockout of Rubicon via CRISPR is likely to result in clones that have constitutively lower levels of autophagy to compensate for their lack of Rubicon. In mice, it was found that deletion of key mitophagy factors at birth did not result in Parkinsonism, as expected, while an identical deletion in rats did¹³⁹. Gene expression profiling found that during development, these mice experienced a change in gene expression relative to wildtype mice, resulting in altered levels of mitophagy regulators, compensating for loss of key mitophagy proteins. As a result, much of the literature may understate the ability of Rubicon degradation to promote autophagy flux.

The first *in cellulo* experiments are intended to demonstrate that Rubicon degradation does promote autophagy. This can be performed with the same HaloTag flux assays used chapter two of this thesis. In brief, a protein expected to be delivered to the lysosome during autophagy is recombinantly expressed with a HaloTag in cultured mammalian cells. HaloTag is resistant to lysosomal processing when it is bound to its HaloLigand, but not in its apo form. As a result, addition of HaloLigand to cell culture medium begins a pulse-chase experiment where a higher molecular weight HaloTag fusion protein is processed down to only HaloTag upon delivery to the lysosome. Practically, this data is visualized as a downward shift in a protein band, as the higher MW, unprocessed band is converted to the processed, lower molecular weight Halo band. The relative intensity of each band is a quantitative measurement of the extent of autophagy flux that has occurred in a given time (Figure 3).

Once it has been validated that pharmacological degradation of Rubicon induces autophagy, some of the more classical neurodegenerative phenotypes can be examined—such as aggregation of the prion-like proteins Alpha-Synuclein and Tau. Alpha-synuclein aggregation and spread is intimately connected with the progression of Parkinson’s disease, though it does not perfectly track progression¹⁴⁰. Tau aggregates are similarly associated with the development of Alzheimer’s disease. It is well-appreciated in the literature that upregulation of autophagy tends to ameliorate these hallmarks of neurodegeneration¹⁴¹. In the case of Alpha-synuclein, it is believed that encapsulation and delivery of aggregates to the lysosomes promotes their proteolytic degradation and can limit or prevent nucleation of additional aggregates or spreading from cell to cell. As a result, dysfunctions in lysosomal function, including deacidification, are known to exacerbate the progression of Parkinson’s disease, presumably because they inhibit this process^{142,143}. In addition to this aggregate-clearing activity, autophagy may also play an important surveillance role, preventing small aggregates from growing into larger ones, or reducing the lifetime of aggregation-susceptible proteins.

Tau is known to form membrane-penetrant fibrils, which are suspected to perforate lysosomes, resulting in their deacidification and subsequent loss of degradative capacity^{144,145}. Similarly, Synuclein fibrils can cross membranes, but only fibrils of a certain size and structure¹⁴⁶. Both Tau and Synuclein need to “mature” from their native, soluble form into these pathogenic fibrils, and this transition is believed to be a key trigger for the development of these

neurodegenerative diseases¹⁴⁷. In this setting, autophagy could act as a “guard” mechanism, isolating and delivering monomers and smaller aggregates to the lysosomes before they are able to form the truly pathogenic, membrane-penetrant fibrils. It stands to reason that higher rates of autophagy flux could more effectively capture and degrade these future fibrils before they are able to damage the lysosomal system and begin spread from cell to cell. Consequently, defects in autophagy will likely exacerbate this, making it more likely that a neuron will “lapse” and fail to “catch” a nascent fibril. This could help explain the largely sporadic and stochastic nature of neurodegenerative diseases.

To directly address this, we will express aggregation-prone variants of Tau and Synuclein in neuron-like SH-SY5Y cells, and measure formation of aggregates over the course of days either with or without our Rubicon degrader. We expect that enhancing autophagy via Rubicon degradation will increase the time to aggregation onset, reduce the extent of aggregation, and decrease the time it takes for these pathogenic aggregates to dissipate.

Conclusion and significance

One of the most significant focus areas in contemporary drug development is the targeting of “undruggable proteins.” These are proteins that do not have deep or chemically favorable pockets, preventing development of a binder, or which lack catalytic activity entirely, preventing development of a small molecule inhibitor. Rubicon qualifies as one of these proteins, since it is not an enzyme, and its well-folded domains are quite dynamic and are characterized by relatively shallow pockets. Degraders are an effective strategy for drugging such proteins because their catalytic activity allows them to be potent even when their binding affinity is quite weak. In my hands, this allowed a Rubicon binder with a K_D of only 1 μM to form the basis for an effective degrader.

This Rubicon degrader represents the first time that this protein has been effectively targeted in a drug development campaign, and in fact represents a totally novel approach to pharmacologically increasing autophagy. Degradation of Rubicon is likely to be a universal strategy for increasing autophagy flux, since both Rubicon and Cereblon are ubiquitously expressed proteins, and Rubicon regulates autophagy in essentially all tissues of the human body. A similar ubiquitous enhancement of autophagy can be achieved via inactivation of the mTORC1 complex using rapamycin or rapamycin analogs. Treatment with rapamycin ameliorates many age-related phenotypes, and many of these effects are autophagy dependent. However, the mTORC1 complex is a central regulator of a wide variety of biological processes, resulting in many off-target effects. Degradation of Rubicon allows us to more selectively enhance autophagy, opening the door for therapeutic interventions that seek to correct the autophagy defects that cause or promote the development of age-related diseases.

Methods

Plasmids construction, protein expression, protein purification, Western blotting, and isothermal titration calorimetry were conducted as in the chapter 2 methods section.

DEL Screen

A commercial DEL library screening kit was acquired from a vendor. This kit consisted of the complete DEL library, that was subsequently reconstituted in screening buffer, as well as salmon sperm screening “additives” to reduce nonspecific DNA binding to target proteins. 6xhis-RUBCN RH, 6xhis-MBP, and RAB7A were purified for screening using this kit. Briefly, hundreds of pmol of each protein were immobilized on Ni-charged MagBeads (Genscript) during a 30 minute incubation, and excess, unbound protein was washed off. The beads decorated with our target proteins were then incubated in the reconstituted DEL library for 1 H on a rotator before the solution was collected and the beads washed 3 times with screening buffer. The beads were resuspended in an elution buffer, then heated to 95 C for 5 minutes to elute the bound DEL members. The beads were pelleted, and the eluted DEL components were recovered from the MagBeads via aspiration of the supernatant. 3 Samples were screened via this method: a sample consisting of 6xhis-MBP-Rubicon RH, a blank consisting of 6xhis-MBP, and a sample consisting of 6xhis-MBP-Rubicon RH in the presence of 10 μ M RAB7A (a saturating concentration of RAB7A) during all screening and washing steps.

A small sample of each elution was collected, and qPCR was performed to estimate the number of DNA tags in the sample, and therefore the number of molecules present. This number was compared to expected yields provided by the manufacturer, and the molecule count fell within usual ranges. qPCR Primers were provided as a part of the kit.

A fresh set of protein-bound MagBeads was prepared and incubated with the elution from the previous cycle. The incubation, washing, elution, and DNA quantification steps are repeated until the total DNA count falls beneath a certain threshold. The MBP-Rubicon sample fell within this target threshold after 2 washes, while the MBP blank and MBP-Rubicon + RAB7A condition fell within after 3 washes.

Subsequently, the final elution was lyophilized, and then shipped to the kit manufacturer to decode and quantify the hits. Data was visualized using the open-source software Osiris DataWarrior (v06.01.00: January 2024 Thomas Sander, Joel Freyss, Modest von Korff, Christian Rufener. DataWarrior: An Open-Source Program For Chemistry Aware Data Visualization And Analysis. J Chem Inf Model 2015, 55, 460-473, doi 10.1021/ci500588j)

Surface Plasmon Resonance (SPR)

All experiments were performed on a benchtop P4SPR (Affinite Instruments). The instrument was loaded with a Ni-NTA gold sensor chip (Affinite Instruments), and washed with water and then PBS. A 0.3 mg/mL solution of 6xhis-MBP-Rubicon RH was injected into the chamber, and allowed to bind for 30 minutes. During this time, binding of Rubicon to the chip was followed as a change in the SPR response, and expected to achieve a response of ~3,000 response units (RU) over the water baseline. The chip was then washed with PBS + 0.1% DMSO (DMSO content matched to that of the Rubicon binder samples to be analyzed). To prepare samples for SPR, Rubicon binders were diluted in DMSO to 1,000x the concentration in the final sample, and 1 μ L of binder in DMSO was added to 999 μ L of PBS. The DMSO content from sample to sample, as well as in the wash buffer, was a uniform 0.1%.

For each sample, ~300 μ L was injected manually using a syringe, and allowed to bind for a minimum of 5 minutes, or until the reading stabilized. Each binder was then washed off with a 500 μ L wash of PBS + 0.1% DMSO, also manually injected.

To analyze data, samples were run at increasing concentration until a maximum response value, $R_{\max}$, was acquired. Fraction of protein bound was calculated as the response value at a particular concentration divided by the $R_{\max}$. In order to determine a K_D , a binding curve was constructed plotting the fraction of protein bound at each concentration of binder, and this data was fit to the Hill Equation with $n=1$ using Microsoft Excel's Solver plugin (RRID:SCR_016137).

Cellular Thermal Shift Assay (CETSA)

HEK-293 cells were cultured to confluence and then transiently transfected with pCMV-mCherry-Rubicon using lipofectamine 3,000. Cells were allowed to recover for 24 H, and then detached and resuspended in a buffer consisting of PBS supplemented with 50 μ M putative Rubicon-binding molecule. Cells were allowed to incubate for 30 min at 37 C under occasional agitation, and then exposed to the temperatures indicated in the Figure caption for 3 minutes. Cells were then lysed via 3 cycles of freezing and thawing, and the cells were pelleted, clearing cellular debris as well as aggregated mCherry-Rubicon. The supernatant was collected, and the concentration of protein in each sample was determined using a BCA assay. Using this information, 20 μ g of protein was loaded into each lane of an SDS-PAGE gel (under reducing condition, in loading buffer, but without additional boiling). The gel was run at 150 V for 70 minutes, and then collected and imaged using the "Alexa 594" preset on a fluorescent visualizer (Chemidoc, Bio-Rad). The image was exported to FIJI, and the intensity of the mCherry-Rubicon band was measured using the gel analyzer tool. The unfolded fraction at each temperature was determined by dividing the mCherry-Rubicon signal at each temperature by that of a 4 C control (fraction of Rubicon in its native state), and then subtracting that value from 1. The data was fit to a protein denaturation curve (fraction unfolded = $1/(1+2.7182^{(\text{sample temperature} - T_m)})$) using Microsoft Excel's Solver plugin.

References

1. Kim, J., Kundu, M., Viollet, B., and Guan, K.-L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biology* 13, 132-141. 10.1038/ncb2152.
2. Park, J.-M., Lee, D.-H., and Kim, D.-H. (2023). Redefining the role of AMPK in autophagy and the energy stress response. *Nature Communications* 14. 10.1038/s41467-023-38401-z.
3. Zhang, S., Peng, X., Yang, S., Li, X., Huang, M., Wei, S., Liu, J., He, G., Zheng, H., Yang, L., et al. (2022). The regulation, function, and role of lipophagy, a form of selective autophagy, in metabolic disorders. *Cell Death & Disease* 13. 10.1038/s41419-022-04593-3.
4. Halling, J.F., and Pilegaard, H. (2017). Autophagy-Dependent Beneficial Effects of Exercise. *Cold Spring Harbor Perspectives in Medicine* 7, a029777. 10.1101/cshperspect.a029777.
5. He, C., Sumpter, J.R., and Levine, B. (2012). Exercise induces autophagy in peripheral tissues and in the brain. *Autophagy* 8, 1548-1551. 10.4161/auto.21327.
6. Mao, K., and Klionsky, D.J. (2017). Xenophagy: A battlefield between host and microbe, and a possible avenue for cancer treatment. *Autophagy* 13, 223-224. 10.1080/15548627.2016.1267075.
7. Lamark, T., and Johansen, T. (2012). Aggrephagy: Selective Disposal of Protein Aggregates by Macroautophagy. *International Journal of Cell Biology* 2012, 1-21. 10.1155/2012/736905.
8. Yu, L., Chen, Y., and Tooze, S.A. (2018). Autophagy pathway: Cellular and molecular mechanisms. *Autophagy* 14, 207-215. 10.1080/15548627.2017.1378838.
9. Vargas, J.N.S., Hamasaki, M., Kawabata, T., Youle, R.J., and Yoshimori, T. (2023). The mechanisms and roles of selective autophagy in mammals. *Nature Reviews Molecular Cell Biology* 24, 167-185. 10.1038/s41580-022-00542-2.
10. Chang, C., Jensen, L.E., and Hurley, J.H. (2021). Autophagosome biogenesis comes out of the black box. *Nature Cell Biology* 23, 450-456. 10.1038/s41556-021-00669-y.
11. Wong, P.-M., Puente, C., Ganley, I.G., and Jiang, X. (2013). The ULK1 complex. *Autophagy* 9, 124-137. 10.4161/auto.23323.
12. Bach, M., Larance, M., James, D.E., and Ramm, G. (2011). The serine/threonine kinase ULK1 is a target of multiple phosphorylation events. *Biochemical Journal* 440, 283-291. 10.1042/bj20101894.
13. Jung, C.H., Jun, C.B., Ro, S.-H., Kim, Y.-M., Otto, N.M., Cao, J., Kundu, M., and Kim, D.-H. (2009). ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Molecular Biology of the Cell* 20, 1992-2003. 10.1091/mbc.e08-12-1249.
14. Martina, J.A., Chen, Y., Gucsek, M., and Puertollano, R. (2012). MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy* 8, 903-914. 10.4161/auto.19653.
15. Noda, T. (2017). Regulation of Autophagy through TORC1 and mTORC1. *Biomolecules* 7, 52. 10.3390/biom7030052.
16. Reggiori, F., and Ungermann, C. (2017). Autophagosome Maturation and Fusion. *Journal of Molecular Biology* 429, 486-496. 10.1016/j.jmb.2017.01.002.
17. Mailler, E., Guardia, C.M., Bai, X., Jarnik, M., Williamson, C.D., Li, Y., Maio, N., Golden, A., and Bonifacino, J.S. (2021). The autophagy protein ATG9A enables lipid mobilization from lipid droplets. *Nature Communications* 12. 10.1038/s41467-021-26999-x.
18. Hurley, J.H., and Young, L.N. (2017). Mechanisms of Autophagy Initiation. *Annual Review of Biochemistry* 86, 225-244. 10.1146/annurev-biochem-061516-044820.
19. Chen, M., Ren, X., Nguyen, T.N., Khuu, G., Cook, A.S.I., Lazarou, M., and Hurley, J.H. (2023). Structure and activation of the human autophagy-initiating ULK1C:PI3KC3-C1 supercomplex. Cold Spring Harbor Laboratory.
20. Fracchiolla, D., Chang, C., Hurley, J.H., and Martens, S. (2020). A PI3K-WIPI2 positive feedback loop allosterically activates LC3 lipidation in autophagy. *Journal of Cell Biology* 219. 10.1083/jcb.201912098.

21. Strong, L.M., Chang, C., Riley, J.F., Boecker, C.A., Flower, T.G., Buffalo, C.Z., Ren, X., Stavoe, A.K., Holzbaur, E.L., and Hurley, J.H. (2021). Structural basis for membrane recruitment of ATG16L1 by WIPI2 in autophagy. *eLife* *10*. 10.7554/elife.70372.
22. Scheffner, M., Nuber, U., and Huibregtse, J.M. (1995). Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. *Nature* *373*, 81–83. 10.1038/373081a0.
23. Martens, S., and Fracchiolla, D. (2020). Activation and targeting of ATG8 protein lipidation. *Cell Discovery* *6*. 10.1038/s41421-020-0155-1.
24. Wirth, M., Zhang, W., Razi, M., Nyoni, L., Joshi, D., O'Reilly, N., Johansen, T., Tooze, S.A., and Mouilleron, S. (2019). Molecular determinants regulating selective binding of autophagy adapters and receptors to ATG8 proteins. *Nature Communications* *10*. 10.1038/s41467-019-10059-6.
25. Adriaenssens, E., Ferrari, L., and Martens, S. (2022). Orchestration of selective autophagy by cargo receptors. *Current Biology* *32*, R1357–R1371. 10.1016/j.cub.2022.11.002.
26. Chowdhury, S., Otomo, C., Leitner, A., Ohashi, K., Aebersold, R., Lander, G.C., and Otomo, T. (2018). Insights into autophagosome biogenesis from structural and biochemical analyses of the ATG2A-WIPI4 complex. *Proceedings of the National Academy of Sciences* *115*, E9792–E9801. 10.1073/pnas.1811874115.
27. Otomo, T., Chowdhury, S., and Lander, G.C. (2018). The Rod-Shaped ATG2A-WIPI4 Complex Tethers Membranes In Vitro. *Contact* *1*, 251525641881993. 10.1177/2515256418819936.
28. Maeda, S., Otomo, C., and Otomo, T. (2019). The autophagic membrane tether ATG2A transfers lipids between membranes. *eLife* *8*. 10.7554/elife.45777.
29. Osawa, T., Kotani, T., Kawaoka, T., Hirata, E., Suzuki, K., Nakatogawa, H., Ohsumi, Y., and Noda, N.N. (2019). Atg2 mediates direct lipid transfer between membranes for autophagosome formation. *Nature Structural & Molecular Biology* *26*, 281–288. 10.1038/s41594-019-0203-4.
30. Matoba, K., Kotani, T., Tsutsumi, A., Tsuji, T., Mori, T., Noshiro, D., Sugita, Y., Nomura, N., Iwata, S., Ohsumi, Y., et al. (2020). Atg9 is a lipid scramblase that mediates autophagosomal membrane expansion. *Nature Structural & Molecular Biology* *27*, 1185–1193. 10.1038/s41594-020-00518-w.
31. Jiang, W., Chen, X., Ji, C., Zhang, W., Song, J., Li, J., and Wang, J. (2021). Key Regulators of Autophagosome Closure. *Cells* *10*, 2814. 10.3390/cells10112814.
32. Takahashi, Y., He, H., Tang, Z., Hattori, T., Liu, Y., Young, M.M., Serfass, J.M., Chen, L., Gebru, M., Chen, C., et al. (2018). An autophagy assay reveals the ESCRT-III component CHMP2A as a regulator of phagophore closure. *Nature Communications* *9*. 10.1038/s41467-018-05254-w.
33. Zhou, F., Wu, Z., Zhao, M., Murtazina, R., Cai, J., Zhang, A., Li, R., Sun, D., Li, W., Zhao, L., et al. (2019). Rab5-dependent autophagosome closure by ESCRT. *Journal of Cell Biology* *218*, 1908–1927. 10.1083/jcb.201811173.
34. Ao, X., Zou, L., and Wu, Y. (2014). Regulation of autophagy by the Rab GTPase network. *Cell Death & Differentiation* *21*, 348–358. 10.1038/cdd.2013.187.
35. Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nature Reviews Molecular Cell Biology* *10*, 513–525. 10.1038/nrm2728.
36. Cheng, X., Ma, X., Ding, X., Li, L., Jiang, X., Shen, Z., Chen, S., Liu, W., Gong, W., and Sun, Q. (2017). Pacer Mediates the Function of Class III PI3K and HOPS Complexes in Autophagosome Maturation by Engaging Stx17. *Molecular Cell* *65*, 1029–1043.e1025. 10.1016/j.molcel.2017.02.010.
37. Jiang, P., Nishimura, T., Sakamaki, Y., Itakura, E., Hatta, T., Natsume, T., and Mizushima, N. (2014). The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Mol Biol Cell* *25*, 1327–1337. 10.1091/mbc.E13-08-0447.
38. Takáts, S., Pircs, K., Nagy, P., Varga, Á., Kárpáti, M., Hegedűs, K., Kramer, H., Kovács, A.L., Sass, M., and Juhász, G. (2014). Interaction of the HOPS complex with Syntaxin 17 mediates autophagosome clearance in *Drosophila*. *Mol Biol Cell* *25*, 1338–1354. 10.1091/mbc.E13-08-0449.

39. Marwaha, R., Arya, S.B., Jagga, D., Kaur, H., Tuli, A., and Sharma, M. (2017). The Rab7 effector PLEKHM1 binds Arl8b to promote cargo traffic to lysosomes. *Journal of Cell Biology* 216, 1051-1070. 10.1083/jcb.201607085.
40. Wang, Y., Li, L., Hou, C., Lai, Y., Long, J., Liu, J., Zhong, Q., and Diao, J. (2016). SNARE-mediated membrane fusion in autophagy. *Seminars in Cell & Developmental Biology* 60, 97-104. 10.1016/j.semcdb.2016.07.009.
41. Yang, Y., and Klionsky, D.J. (2020). Autophagy and disease: unanswered questions. *Cell Death & Differentiation* 27, 858-871. 10.1038/s41418-019-0480-9.
42. Levine, B., and Kroemer, G. (2008). Autophagy in the Pathogenesis of Disease. *Cell* 132, 27-42. 10.1016/j.cell.2007.12.018.
43. Aman, Y., Schmauck-Medina, T., Hansen, M., Morimoto, R.I., Simon, A.K., Bjedov, I., Palikaras, K., Simonsen, A., Johansen, T., Tavernarakis, N., et al. (2021). Autophagy in healthy aging and disease. *Nature Aging* 1, 634-650. 10.1038/s43587-021-00098-4.
44. Mitter, S.K., Rao, H.V., Qi, X., Cai, J., Sugrue, A., Dunn, W.A., Grant, M.B., and Boulton, M.E. (2012). Autophagy in the Retina: A Potential Role in Age-Related Macular Degeneration. In (Springer US), pp. 83-90. 10.1007/978-1-4614-0631-0_12.
45. Kaarniranta, K., Blasiak, J., Liton, P., Boulton, M., Klionsky, D.J., and Sinha, D. (2023). Autophagy in age-related macular degeneration. *Autophagy* 19, 388-400. 10.1080/15548627.2022.2069437.
46. Lin, T.-A., Wu, V.C.-C., and Wang, C.-Y. (2019). Autophagy in Chronic Kidney Diseases. *Cells* 8, 61. 10.3390/cells8010061.
47. Nixon, R.A. (2013). The role of autophagy in neurodegenerative disease. *Nature Medicine* 19, 983-997. 10.1038/nm.3232.
48. Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441, 885-889. 10.1038/nature04724.
49. Arkinson, C., and Walden, H. (2018). Parkin function in Parkinson's disease. *Science* 360, 267-268. 10.1126/science.aar6606.
50. Dawson, T.M., and Dawson, V.L. (2010). The role of parkin in familial and sporadic Parkinson's disease. *Movement Disorders* 25, S32-S39. 10.1002/mds.22798.
51. Poewe, W., Seppi, K., Tanner, C.M., Halliday, G.M., Brundin, P., Volkmann, J., Schrag, A.-E., and Lang, A.E. (2017). Parkinson disease. *Nature Reviews Disease Primers* 3, 17013. 10.1038/nrdp.2017.13.
52. Zhan, L., Chen, S., Li, K., Liang, D., Zhu, X., Liu, L., Lu, Z., Sun, W., and Xu, E. (2017). Autophagosome maturation mediated by Rab7 contributes to neuroprotection of hypoxic preconditioning against global cerebral ischemia in rats. *Cell Death & Disease* 8, e2949-e2949. 10.1038/cddis.2017.330.
53. Saveri, P., De Luca, M., Nisi, V., Pisciotto, C., Romano, R., Piscosquito, G., Reilly, M.M., Polke, J.M., Cavallaro, T., Fabrizi, G.M., et al. (2020). Charcot-Marie-Tooth Type 2B: A New Phenotype Associated with a Novel RAB7A Mutation and Inhibited EGFR Degradation. *Cells* 9, 1028. 10.3390/cells9041028.
54. Wong, Y.C., Jayaraj, N.D., Belton, T.B., Shum, G.C., Ball, H.E., Ren, D., Tadenev, A.L.D., Krainc, D., Burgess, R.W., and Menichella, D.M. (2023). Misregulation of mitochondria-lysosome contact dynamics in Charcot-Marie-Tooth Type 2B disease Rab7 mutant sensory peripheral neurons. *Proceedings of the National Academy of Sciences* 120. 10.1073/pnas.2313010120.
55. Hardiman, O., Al-Chalabi, A., Chio, A., Corr, E.M., Logroscino, G., Robberecht, W., Shaw, P.J., Simmons, Z., and Van Den Berg, L.H. (2017). Amyotrophic lateral sclerosis. *Nature Reviews Disease Primers* 3, 17071. 10.1038/nrdp.2017.71.

56. Zorov, D.B., Juhaszova, M., and Sollott, S.J. (2014). Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. *Physiological Reviews* 94, 909-950. 10.1152/physrev.00026.2013.
57. Bock, F.J., and Tait, S.W.G. (2020). Mitochondria as multifaceted regulators of cell death. *Nature Reviews Molecular Cell Biology* 21, 85-100. 10.1038/s41580-019-0173-8.
58. Kim, J., Kim, H.-S., and Chung, J.H. (2023). Molecular mechanisms of mitochondrial DNA release and activation of the cGAS-STING pathway. *Experimental & Molecular Medicine* 55, 510-519. 10.1038/s12276-023-00965-7.
59. Carvalho, G., Repolês, B.M., Mendes, I., and Wanrooij, P.H. (2022). Mitochondrial DNA Instability in Mammalian Cells. *Antioxidants & Redox Signaling* 36, 885-905. 10.1089/ars.2021.0091.
60. Heo, J.M., Ordureau, A., Paulo, J.A., Rinehart, J., and Harper, J.W. (2015). The PINK1-PARKIN Mitochondrial Ubiquitylation Pathway Drives a Program of OPTN/NDP52 Recruitment and TBK1 Activation to Promote Mitophagy. *Mol Cell* 60, 7-20. 10.1016/j.molcel.2015.08.016.
61. Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M.K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., et al. (2004). Hereditary Early-Onset Parkinson's Disease Caused by Mutations in *PINK1*. *Science* 304, 1158-1160. 10.1126/science.1096284.
62. Narendra, D., Walker, J.E., and Youle, R. (2012). Mitochondrial Quality Control Mediated by PINK1 and Parkin: Links to Parkinsonism. *Cold Spring Harbor Perspectives in Biology* 4, a011338-a011338. 10.1101/cshperspect.a011338.
63. Lazarou, M., Seok, Lesley, and Richard (2012). Role of PINK1 Binding to the TOM Complex and Alternate Intracellular Membranes in Recruitment and Activation of the E3 Ligase Parkin. *Developmental Cell* 22, 320-333. 10.1016/j.devcel.2011.12.014.
64. Yamano, K., Wang, C., Sarraf, S.A., Münch, C., Kikuchi, R., Noda, N.N., Hizukuri, Y., Kanemaki, M.T., Harper, W., Tanaka, K., et al. (2018). Endosomal Rab cycles regulate Parkin-mediated mitophagy. *eLife* 7. 10.7554/eLife.31326.
65. Hegedűs, K., Takáts, S., Boda, A., Jipa, A., Nagy, P., Varga, K., Kovács, A.L., and Juhász, G. (2016). The Ccz1-Mon1-Rab7 module and Rab5 control distinct steps of autophagy. *Molecular Biology of the Cell* 27, 3132-3142. 10.1091/mbc.e16-03-0205.
66. Yamano, K., Sawada, M., Kikuchi, R., Nagataki, K., Kojima, W., Endo, R., Kinefuchi, H., Sugihara, A., Fujino, T., Watanabe, A., et al. (2024). Optineurin provides a mitophagy contact site for TBK1 activation. *The EMBO Journal* 43, 754-779. 10.1038/s44318-024-00036-1.
67. Li, F., Xie, X., Wang, Y., Liu, J., Cheng, X., Guo, Y., Gong, Y., Hu, S., and Pan, L. (2016). Structural insights into the interaction and disease mechanism of neurodegenerative disease-associated optineurin and TBK1 proteins. *Nature Communications* 7, 12708. 10.1038/ncomms12708.
68. Heo, J.M., Ordureau, A., Swarup, S., Paulo, J.A., Shen, K., Sabatini, D.M., and Harper, J.W. (2018). RAB7A phosphorylation by TBK1 promotes mitophagy via the PINK-PARKIN pathway. *Science Advances* 4, eaav0443. 10.1126/sciadv.aav0443.
69. Matsunaga, K., Saitoh, T., Tabata, K., Omori, H., Satoh, T., Kurotori, N., Maejima, I., Shirahama-Noda, K., Ichimura, T., Isobe, T., et al. (2009). Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nature Cell Biology* 11, 385-396. 10.1038/ncb1846.
70. Bhargava, H.K., Tabata, K., Byck, J.M., Hamasaki, M., Farrell, D.P., Anishchenko, I., Dimaio, F., Im, Y.J., Yoshimori, T., and Hurley, J.H. (2020). Structural basis for autophagy inhibition by the human Rubicon–Rab7 complex. *Proceedings of the National Academy of Sciences* 117, 17003-17010. 10.1073/pnas.2008030117.
71. Chang, C., Young, L.N., Morris, K.L., Von Bülow, S., Schöneberg, J., Yamamoto-Imoto, H., Oe, Y., Yamamoto, K., Nakamura, S., Stjepanovic, G., et al. (2019). Bidirectional Control of

- Autophagy by BECN1 BARA Domain Dynamics. *Molecular Cell* 73, 339-353.e336. 10.1016/j.molcel.2018.10.035.
72. Liang, C., Lee, J.-S., Inn, K.-S., Gack, M.U., Li, Q., Roberts, E.A., Vergne, I., Deretic, V., Feng, P., Akazawa, C., and Jung, J.U. (2008). Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nature Cell Biology* 10, 776-787. 10.1038/ncb1740.
 73. Zhong, Y., Wang, Q.J., Li, X., Yan, Y., Backer, J.M., Chait, B.T., Heintz, N., and Yue, Z. (2009). Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1–phosphatidylinositol-3-kinase complex. *Nature Cell Biology* 11, 468-476. 10.1038/ncb1854.
 74. Beltran, S., Nassif, M., Vicencio, E., Arcos, J., Labrador, L., Cortes, B.I., Cortez, C., Bergmann, C.A., Espinoza, S., Hernandez, M.F., et al. (2019). Network approach identifies Pacer as an autophagy protein involved in ALS pathogenesis. *Mol Neurodegener* 14, 14. 10.1186/s13024-019-0313-9.
 75. Pfeffer, S.R. (2023). LRRK2 phosphorylation of Rab GTPases in Parkinson's disease. *Febs Lett* 597, 811-818. 10.1002/1873-3468.14492.
 76. Nirujogi, R.S., Tonelli, F., Taylor, M., Lis, P., Zimprich, A., Sammler, E., and Alessi, D.R. (2021). Development of a multiplexed targeted mass spectrometry assay for LRRK2-phosphorylated Rabs and Ser910/Ser935 biomarker sites. *Biochem J* 478, 299-326. 10.1042/bcj20200930.
 77. Fujita, K., Kedashiro, S., Yagi, T., Hisamoto, N., Matsumoto, K., and Hanafusa, H. (2022). The ULK complex–LRRK1 axis regulates Parkin-mediated mitophagy via Rab7 Ser-72 phosphorylation. *Journal of Cell Science* 135. 10.1242/jcs.260395.
 78. Richter, B., Sliter, D.A., Herhaus, L., Stolz, A., Wang, C., Beli, P., Zaffagnini, G., Wild, P., Martens, S., Wagner, S.A., et al. (2016). Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. *Proc Natl Acad Sci U S A* 113, 4039-4044. 10.1073/pnas.1523926113.
 79. Moore, A.S., and Holzbaur, E.L. (2016). Dynamic recruitment and activation of ALS-associated TBK1 with its target optineurin are required for efficient mitophagy. *Proc Natl Acad Sci U S A* 113, E3349-3358. 10.1073/pnas.1523810113.
 80. Vargas, J.N.S., Wang, C., Bunker, E., Hao, L., Maric, D., Schiavo, G., Randow, F., and Youle, R.J. (2019). Spatiotemporal Control of ULK1 Activation by NDP52 and TBK1 during Selective Autophagy. *Mol Cell* 74, 347-362 e346. 10.1016/j.molcel.2019.02.010.
 81. Nguyen, T.N., Sawa-Makarska, J., Khuu, G., Lam, W.K., Adriaenssens, E., Fracchiolla, D., Shoebridge, S., Bernklau, D., Padman, B.S., Skulsuppaisarn, M., et al. (2023). Unconventional initiation of PINK1/Parkin mitophagy by Optineurin. *Molecular Cell* 83, 1693-1709. 10.1016/j.molcel.2023.04.021.
 82. Tabata, K., Matsunaga, K., Sakane, A., Sasaki, T., Noda, T., and Yoshimori, T. (2010). Rubicon and PLEKHM1 Negatively Regulate the Endocytic/Autophagic Pathway via a Novel Rab7-binding Domain. *Molecular Biology of the Cell* 21, 4162-4172. 10.1091/mbc.e10-06-0495.
 83. Yim, W.W., Yamamoto, H., and Mizushima, N. (2022). A pulse-chasable reporter processing assay for mammalian autophagic flux with HaloTag. *eLife* 11. 10.7554/eLife.78923.
 84. Nakamura, S., Oba, M., Suzuki, M., Takahashi, A., Yamamuro, T., Fujiwara, M., Ikenaka, K., Minami, S., Tabata, N., Yamamoto, K., et al. (2019). Suppression of autophagic activity by Rubicon is a signature of aging. *Nature Communications* 10. 10.1038/s41467-019-08729-6.
 85. Allen, G.F.G., Toth, R., James, J., and Ganley, I.G. (2013). Loss of iron triggers PINK1/Parkin-independent mitophagy. *EMBO reports* 14, 1127-1135. 10.1038/embor.2013.168.
 86. Hara, Y., Yanatori, I., Tanaka, A., Kishi, F., Lemasters, J.J., Nishina, S., Sasaki, K., and Hino, K. (2020). Iron loss triggers mitophagy through induction of mitochondrial ferritin. *EMBO reports* 21. 10.15252/embr.202050202.
 87. Zhao, J.F., Rodger, C.E., Allen, G.F.G., Weidlich, S., and Ganley, I.G. (2020). HIF1alpha-dependent mitophagy facilitates cardiomyoblast differentiation. *Cell Stress* 4, 99-113. 10.15698/cst2020.05.220.

88. Waschbüsch, D., and Khan, A.R. (2020). Phosphorylation of Rab<sc>GTPases</sc> in the regulation of membrane trafficking. *Traffic* 21, 712-719. 10.1111/tra.12765.
89. Boecker, C.A., Goldsmith, J., Dou, D., Cajka, C.G., and Holzbaur, E.L.F. (2021). Increased LRRK2 kinase activity alters neuronal autophagy by disrupting the axonal transport of autophagosomes. *Current Biology* 31, 2140-2154.
90. Cui, Z.C., Napolitano, G., de Araujo, M.E.G., Esposito, A., Monfregola, J., Huber, L.A., Ballabio, A., and Hurley, J.H. (2023). Structure of the lysosomal mTORC1-TFEB-Rag-Ragulator megacomplex. *Nature* 614, 572-579. 10.1038/s41586-022-05652-7.
91. Napolitano, G., Di Malta, C., Esposito, A., de Araujo, M.E.G., Pece, S., Bertalot, G., Matarese, M., Benedetti, V., Zampelli, A., Stasyk, T., et al. (2020). A substrate-specific mTORC1 pathway underlies Birt-Hogg-Dubé syndrome. *Nature* 585, 597-602. 10.1038/s41586-020-2444-0.
92. Tsun, Z.Y., Bar-Peled, L., Chantranupong, L., Zoncu, R., Wang, T., Kim, C., Spooner, E., and Sabatini, D.M. (2013). The Folliculin Tumor Suppressor Is a GAP for the RagC/D GTPases That Signal Amino Acid Levels to mTORC1. *Molecular Cell* 52, 495-505. 10.1016/j.molcel.2013.09.016.
93. Manford, A.G., Mena, E.L., Shih, K.Y., Gee, C.L., McMinimy, R., Martinez-Gonzalez, B., Sherrieff, R., Lew, B., Zoltek, M., Rodriguez-Perez, F., et al. (2021). Structural basis and regulation of the reductive stress response. *Cell* 184, 5375-5390. 10.1016/j.cell.2021.09.002.
94. Malik, N., Ferreira, B.I., Hollstein, P.E., Curtis, S.D., Trefts, E., Novak, S.W., Yu, J.T., Gilson, R., Hellberg, K., Fang, L.J., et al. (2023). Induction of lysosomal and mitochondrial biogenesis by AMPK phosphorylation of FNIP1. *Science* 380, eabj5559.
95. Stolz, A., Ernst, A., and Dikic, I. (2014). Cargo recognition and trafficking in selective autophagy. *Nature Cell Biology* 16, 495-501. 10.1038/ncb2979.
96. Malik, A.U., Karapetsas, A., Nirujogi, R.S., Mathea, S., Chatterjee, D., Pal, P., Lis, P., Taylor, M., Purlyte, E., Gourlay, R., et al. (2021). Deciphering the LRRK code: LRRK1 and LRRK2 phosphorylate distinct Rab proteins and are regulated by diverse mechanisms. *Biochemical Journal* 478, 553-578. 10.1042/bcj20200937.
97. Lee, D.J.W., Hodzic Kuerec, A., and Maier, A.B. (2024). Targeting ageing with rapamycin and its derivatives in humans: a systematic review. *The Lancet Healthy Longevity* 5, e152-e162. 10.1016/s2666-7568(23)00258-1.
98. Juricic, P., Lu, Y.-X., Leech, T., Drews, L.F., Paulitz, J., Lu, J., Nespital, T., Azami, S., Regan, J.C., Funk, E., et al. (2022). Long-lasting geroprotection from brief rapamycin treatment in early adulthood by persistently increased intestinal autophagy. *Nature Aging* 2, 824-836. 10.1038/s43587-022-00278-w.
99. Bitto, A., Ito, T.K., Pineda, V.V., Letexier, N.J., Huang, H.Z., Sutlief, E., Tung, H., Vizzini, N., Chen, B., Smith, K., et al. (2016). Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice. *eLife* 5. 10.7554/elife.16351.
100. Harrison, D.E., Strong, R., Sharp, Z.D., Nelson, J.F., Astle, C.M., Flurkey, K., Nadon, N.L., Wilkinson, J.E., Frenkel, K., Carter, C.S., et al. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* 460, 392-395. 10.1038/nature08221.
101. Baroja-Mazo, A., Revilla-Nuin, B., Ramírez, P., and Pons, J.A. (2016). Immunosuppressive potency of mechanistic target of rapamycin inhibitors in solid-organ transplantation. *World Journal of Transplantation* 6, 183. 10.5500/wjt.v6.i1.183.
102. Cloughesy, T.F., Yoshimoto, K., Nghiemphu, P., Brown, K., Dang, J., Zhu, S., Hsueh, T., Chen, Y., Wang, W., Youngkin, D., et al. (2008). Antitumor Activity of Rapamycin in a Phase I Trial for Patients with Recurrent PTEN-Deficient Glioblastoma. *PLoS Medicine* 5, e8. 10.1371/journal.pmed.0050008.
103. Yamamuro, T., Kawabata, T., Fukuhara, A., Saita, S., Nakamura, S., Takeshita, H., Fujiwara, M., Enokidani, Y., Yoshida, G., Tabata, K., et al. (2020). Age-dependent loss of adipose Rubicon promotes metabolic disorders via excess autophagy. *Nature Communications* 11. 10.1038/s41467-020-17985-w.

104. Tanaka, S., Hikita, H., Tatsumi, T., Sakamori, R., Nozaki, Y., Sakane, S., Shiode, Y., Nakabori, T., Saito, Y., Hiramatsu, N., et al. (2016). Rubicon inhibits autophagy and accelerates hepatocyte apoptosis and lipid accumulation in nonalcoholic fatty liver disease in mice. *Hepatology* *64*, 1994-2014. 10.1002/hep.28820.
105. Peng, Z., Gong, Y., Wang, X., He, W., Wu, L., Zhang, L., Xiong, L., Huang, Y., Su, L., Shi, P., et al. (2022). METTL3-m6A-Rubicon axis inhibits autophagy in nonalcoholic fatty liver disease. *Molecular Therapy* *30*, 932-946. 10.1016/j.ymthe.2021.09.016.
106. Basak, B., and Holzbaur, E.L.F. (2024). Mitochondrial damage triggers concerted degradation of negative regulators of neuronal autophagy. Cold Spring Harbor Laboratory.
107. Nah, J., Zablocki, D., and Sadoshima, J. (2021). The roles of the inhibitory autophagy regulator Rubicon in the heart: A new therapeutic target to prevent cardiac cell death. *Experimental & Molecular Medicine* *53*, 528-536. 10.1038/s12276-021-00600-3.
108. Nah, J., Zhai, P., Huang, C.-Y., Fernández, Á.F., Mareedu, S., Levine, B., and Sadoshima, J. (2020). Upregulation of Rubicon promotes autosis during myocardial ischemia/reperfusion injury. *Journal of Clinical Investigation* *130*, 2978-2991. 10.1172/jci132366.
109. Ruby, M., Gifford, C.C., Pandey, R., Raj, V.S., Sabbiseti, V.S., and Ajay, A.K. (2023). Autophagy as a Therapeutic Target for Chronic Kidney Disease and the Roles of TGF- β 1 in Autophagy and Kidney Fibrosis. *Cells* *12*, 412. 10.3390/cells12030412.
110. Békés, M., Langley, D.R., and Crews, C.M. (2022). PROTAC targeted protein degraders: the past is prologue. *Nature Reviews Drug Discovery* *21*, 181-200. 10.1038/s41573-021-00371-6.
111. Verma, R., Mohl, D., and Deshaies, R.J. (2020). Harnessing the Power of Proteolysis for Targeted Protein Inactivation. *Molecular Cell* *77*, 446-460. 10.1016/j.molcel.2020.01.010.
112. Planelles, V., and Barker, E. (2010). Roles of Vpr and Vpx in modulating the virus-host cell relationship. *Molecular Aspects of Medicine* *31*, 398-406. 10.1016/j.mam.2010.05.002.
113. Yan, J., Shun, M.-C., Hao, C., Zhang, Y., Qian, J., Hrecka, K., Delucia, M., Monnie, C., Ahn, J., and Skowronski, J. (2018). HIV-1 Vpr Reprograms CLR4 ^{DCAF1} E3 Ubiquitin Ligase to Antagonize Exonuclease 1-Mediated Restriction of HIV-1 Infection. *mBio* *9*. 10.1128/mbio.01732-18.
114. Gomes, G.L.B., and Scortecci, K.C. (2021). Auxin and its role in plant development: structure, signalling, regulation and response mechanisms. *Plant Biology* *23*, 894-904. 10.1111/plb.13303.
115. Dong, G., Ding, Y., He, S., and Sheng, C. (2021). Molecular Glues for Targeted Protein Degradation: From Serendipity to Rational Discovery. *Journal of Medicinal Chemistry* *64*, 10606-10620. 10.1021/acs.jmedchem.1c00895.
116. Sakamoto, K.M., Kim, K.B., Kumagai, A., Mercurio, F., Crews, C.M., and Deshaies, R.J. (2001). Protacs: Chimeric molecules that target proteins to the Skp1–Cullin–F box complex for ubiquitination and degradation. *Proceedings of the National Academy of Sciences* *98*, 8554-8559. 10.1073/pnas.141230798.
117. Hon, W.-C., Wilson, M.I., Harlos, K., Claridge, T.D.W., Schofield, C.J., Pugh, C.W., Maxwell, P.H., Ratcliffe, P.J., Stuart, D.I., and Jones, E.Y. (2002). Structural basis for the recognition of hydroxyproline in HIF-1 α by pVHL. *Nature* *417*, 975-978. 10.1038/nature00767.
118. Min, J.-H., Yang, H., Ivan, M., Gertler, F., Kaelin, W.G., and Pavletich, N.P. (2002). Structure of an HIF-1 α -pVHL Complex: Hydroxyproline Recognition in Signaling. *Science* *296*, 1886-1889. 10.1126/science.1073440.
119. Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., Yamaguchi, Y., and Handa, H. (2010). Identification of a Primary Target of Thalidomide Teratogenicity. *Science* *327*, 1345-1350. 10.1126/science.1177319.
120. Chamberlain, P.P., Lopez-Girona, A., Miller, K., Carmel, G., Pagarigan, B., Chie-Leon, B., Rychak, E., Corral, L.G., Ren, Y.J., Wang, M., et al. (2014). Structure of the human Cereblon–DDB1–lenalidomide complex reveals basis for responsiveness to thalidomide analogs. *Nature Structural & Molecular Biology* *21*, 803-809. 10.1038/nsmb.2874.

121. Fischer, E.S., Böhm, K., Lydeard, J.R., Yang, H., Stadler, M.B., Cavadini, S., Nagel, J., Serluca, F., Acker, V., Lingaraju, G.M., et al. (2014). Structure of the DDB1–CRBN E3 ubiquitin ligase in complex with thalidomide. *Nature* *512*, 49-53. 10.1038/nature13527.
122. Bjorklund, C.C., Lu, L., Kang, J., Hagner, P.R., Havens, C.G., Amatangelo, M., Wang, M., Ren, Y., Couto, S., Breider, M., et al. (2015). Rate of CRL4CRBN substrate Ikaros and Aiolos degradation underlies differential activity of lenalidomide and pomalidomide in multiple myeloma cells by regulation of c-Myc and IRF4. *Blood Cancer Journal* *5*, e354-e354. 10.1038/bcj.2015.66.
123. Gandhi, A.K., Kang, J., Havens, C.G., Conklin, T., Ning, Y., Wu, L., Ito, T., Ando, H., Waldman, M.F., Thakurta, A., et al. (2014). Immunomodulatory agents lenalidomide and pomalidomide co-stimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRL4. *British Journal of Haematology* *164*, 811-821. 10.1111/bjh.12708.
124. Liu, Y., Yang, J., Wang, T., Luo, M., Chen, Y., Chen, C., Ronai, Z.E., Zhou, Y., Rupp, E., and Han, L. (2023). Expanding PROTACable genome universe of E3 ligases. *Nature Communications* *14*. 10.1038/s41467-023-42233-2.
125. Atilaw, Y., Poongavanam, V., Svensson Nilsson, C., Nguyen, D., Giese, A., Meibom, D., Erdelyi, M., and Kihlberg, J. (2021). Solution Conformations Shed Light on PROTAC Cell Permeability. *ACS Medicinal Chemistry Letters* *12*, 107-114. 10.1021/acsmchemlett.0c00556.
126. Price, E., Weinheimer, M., Rivkin, A., Jenkins, G., Nijssen, M., Cox, P.B., and DeGoey, D. (2024). Beyond Rule of Five and PROTACs in Modern Drug Discovery: Polarity Reducers, Chameleonicity, and the Evolving Physicochemical Landscape. *Journal of Medicinal Chemistry* *67*, 5683-5698. 10.1021/acs.jmedchem.3c02332.
127. Gironde-Martínez, A., Donckele, E.J., Samain, F., and Neri, D. (2021). DNA-Encoded Chemical Libraries: A Comprehensive Review with Successful Stories and Future Challenges. *ACS Pharmacology & Translational Science* *4*, 1265-1279. 10.1021/acspsci.1c00118.
128. Brenner, S., and Lerner, R.A. (1992). Encoded combinatorial chemistry. *Proceedings of the National Academy of Sciences* *89*, 5381-5383. 10.1073/pnas.89.12.5381.
129. Nielsen, J., Brenner, S., and Janda, K.D. (1993). Synthetic methods for the implementation of encoded combinatorial chemistry. *Journal of the American Chemical Society* *115*, 9812-9813. 10.1021/ja00074a063.
130. Needels, M.C., Jones, D.G., Tate, E.H., Heinkel, G.L., Kochersperger, L.M., Dower, W.J., Barrett, R.W., and Gallop, M.A. (1993). Generation and screening of an oligonucleotide-encoded synthetic peptide library. *Proceedings of the National Academy of Sciences* *90*, 10700-10704. 10.1073/pnas.90.22.10700.
131. Chen, Q., Hall, J., Foley, T.L., Wan, J., Li, Y., and Israel, D.I. (2020). A method for estimating binding affinity from primary DEL selection data. *Biochemical and Biophysical Research Communications* *533*, 249-255. 10.1016/j.bbrc.2020.04.029.
132. Satz, A.L. (2018). What Do You Get from DNA-Encoded Libraries? *ACS Medicinal Chemistry Letters* *9*, 408-410. 10.1021/acsmchemlett.8b00128.
133. Jafari, R., Almqvist, H., Axelsson, H., Ignatushchenko, M., Lundbäck, T., Nordlund, P., and Molina, D.M. (2014). The cellular thermal shift assay for evaluating drug target interactions in cells. *Nature Protocols* *9*, 2100-2122. 10.1038/nprot.2014.138.
134. Troup, R.I., Fallan, C., and Baud, M.G.J. (2020). Current strategies for the design of PROTAC linkers: a critical review. *Exploration of Targeted Anti-tumor Therapy* *1*. 10.37349/etat.2020.00018.
135. Ermondi, G., Garcia-Jimenez, D., and Caron, G. (2021). PROTACs and Building Blocks: The 2D Chemical Space in Very Early Drug Discovery. *Molecules* *26*, 672. 10.3390/molecules26030672.
136. Riching, K.M., Caine, E.A., Urh, M., and Daniels, D.L. (2022). The importance of cellular degradation kinetics for understanding mechanisms in targeted protein degradation. *Chemical Society Reviews* *51*, 6210-6221. 10.1039/d2cs00339b.

137. Machleidt, T., Woodroffe, C.C., Schwinn, M.K., Méndez, J., Robers, M.B., Zimmerman, K., Otto, P., Daniels, D.L., Kirkland, T.A., and Wood, K.V. (2015). NanoBRET—A Novel BRET Platform for the Analysis of Protein–Protein Interactions. *ACS Chemical Biology* *10*, 1797-1804. 10.1021/acscchembio.5b00143.
138. Wurz, R.P., Rui, H., Dellamaggiore, K., Ghimire-Rijal, S., Choi, K., Smither, K., Amegadzie, A., Chen, N., Li, X., Banerjee, A., et al. (2023). Affinity and cooperativity modulate ternary complex formation to drive targeted protein degradation. *Nature Communications* *14*. 10.1038/s41467-023-39904-5.
139. Hollville, E., Joers, V., Nakamura, A., Swahari, V., Tansey, M.G., Moy, S.S., and Deshmukh, M. (2020). Characterization of a Cul9–Parkin double knockout mouse model for Parkinson’s disease. *Scientific Reports* *10*. 10.1038/s41598-020-73854-y.
140. Stefanis, L. (2012). -Synuclein in Parkinson's Disease. *Cold Spring Harbor Perspectives in Medicine* *2*, a009399-a009399. 10.1101/cshperspect.a009399.
141. Gao, Y.-L., Wang, N., Sun, F.-R., Cao, X.-P., Zhang, W., and Yu, J.-T. (2018). Tau in neurodegenerative disease. *Annals of Translational Medicine* *6*, 175-175. 10.21037/atm.2018.04.23.
142. Fellner, L., Gabassi, E., Haybaeck, J., and Edenhofer, F. (2021). Autophagy in α -Synucleinopathies—An Overstrained System. *Cells* *10*, 3143. 10.3390/cells10113143.
143. Tang, Q., Gao, P., Arzberger, T., Höllerhage, M., Herms, J., Höglinger, G., and Koeglsperger, T. (2021). Alpha-Synuclein defects autophagy by impairing SNAP29-mediated autophagosome-lysosome fusion. *Cell Death & Disease* *12*. 10.1038/s41419-021-04138-0.
144. Rose, K., Jepson, T., Shukla, S., Maya-Romero, A., Kampmann, M., Xu, K., and Hurley, J.H. (2023). Tau fibrils induce nanoscale membrane damage and nucleate cytosolic tau at lysosomes. Cold Spring Harbor Laboratory.
145. Flach, K., Hilbrich, I., Schiffmann, A., Gärtner, U., Krüger, M., Leonhardt, M., Waschipky, H., Wick, L., Arendt, T., and Holzer, M. (2012). Tau Oligomers Impair Artificial Membrane Integrity and Cellular Viability. *Journal of Biological Chemistry* *287*, 43223-43233. 10.1074/jbc.m112.396176.
146. Luk, K.C., Kehm, V.M., Zhang, B., O’Brien, P., Trojanowski, J.Q., and Lee, V.M.Y. (2012). Intracerebral inoculation of pathological α -synuclein initiates a rapidly progressive neurodegenerative α -synucleinopathy in mice. *Journal of Experimental Medicine* *209*, 975-986. 10.1084/jem.20112457.
147. Skamris, T., Vestergaard, B., Madsen, K.L., Langkilde, A.E., and Foderà, V. (2023). Identifying Biological and Biophysical Features of Different Maturation States of α -Synuclein Amyloid Fibrils. In (Springer US), pp. 321-344. 10.1007/978-1-0716-2597-2_22.