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An affinity-directed phosphatase, AdPhosphatase, system for targeted protein dephosphorylation

Graphical abstract

Highlights
- Affinity-directed phosphatase (AdPhosphatase) can mediate protein dephosphorylation
- AdPhosphatase consists of a target binder conjugated to a phosphatase catalytic subunit
- Target dephosphorylation by AdPhosphatase requires phosphatase catalytic activity
- AdPhosphatase system can be used to study the role of target protein phosphorylation

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In brief
Simpson and Fulcher et al. describe the development of an affinity-directed phosphatase (AdPhosphatase) system to promote targeted dephosphorylation of phosphorylated proteins of interest (POIs). The AdPhosphatase consists of a POI-binding nanobody conjugated to the catalytic subunit of a promiscuous phosphatase (PPP1CA/PPP2CA), which can mediate POI dephosphorylation through proximity in cells.
Article

An affinity-directed phosphatase, AdPhosphatase, system for targeted protein dephosphorylation

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SUMMARY

Reversible protein phosphorylation, catalyzed by protein kinases and phosphatases, is a fundamental process that controls protein function and intracellular signaling. Failure of phospho-control accounts for many human diseases. While a kinase phosphorylates multiple substrates, a substrate is often phosphorylated by multiple kinases. This renders phospho-control at the substrate level challenging, as it requires inhibition of multiple kinases, which would thus affect other kinase substrates. Here, we describe the development and application of the affinity-directed phosphatase (AdPhosphatase) system for targeted dephosphorylation of specific phospho-substrates. By deploying the Protein Phosphatase 1 or 2A catalytic subunits conjugated to an antigen-stabilized anti-GFP nanobody, we can promote the dephosphorylation of two independent phospho-proteins, FAM83D or ULK1, knocked in with GFP-tags using CRISPR-Cas9, with exquisite specificity. By redirecting protein phosphatases to neo-substrates through nanobody-mediated proximity, AdPhosphatase can alter the phospho-status and function of target proteins and thus, offers a new modality for potential drug discovery approaches.

INTRODUCTION

Protein phosphorylation is a reversible post-translation modification (PTM) characterized by the covalent addition of a phosphate group to primarily serine, threonine, or tyrosine residues on the surface of a protein.1,2 Protein phosphorylation is catalyzed by protein kinases, while protein phosphatases mediate the reverse reaction by hydrolyzing the phosphorylated amino acid residue.3 The phosphorylation status of a protein can alter its function through potentially several avenues: modulating its enzymatic activity, folding, stability, subcellular localization, and/or by influencing its protein-protein interactions.4 Almost all aspects of mammalian cell biology are regulated by reversible protein phosphorylation, with abnormal phosphorylation being identified as the cause of a wide range of human pathologies, including many cancers and neurodegenerative diseases.2,5 Therefore, significant research efforts have strived to develop specific protein kinase inhibitors and activators for use in both therapeutics and for studying cell signaling processes, albeit progress with phosphatases has been more limited.5-8 However, even highly selective protein kinase inhibitors suffer from the fact that they block phosphorylation of all the downstream substrates of the inhibited kinase, rendering inhibition of specific substrate phosphorylation events very challenging. Furthermore, both kinase and phosphatase inhibitors are often known to elicit off-target effects.7-17 To study substrate-level phospho-control in cells, mutations of phospho-residues to either block phosphorylation (e.g., Ala or Val substitutions) or mimic phosphorylation (negatively charged Asp or Glu substitutions) are common, but these mutations have the potential to alter protein conformation, especially if there are multiple phospho-residues to be mutated, whereas phospho-mimetics are chemically distinct18 and do not always mimic true phosphorylation state.19 Amber stop-codon engineering to allow for site-specific incorporation of phospho-residues20 can directly overcome problems associated with mimetic mutations, but this technology requires complex genetic manipulation of target cells and does not work for every phospho-residue or protein. In contrast, targeted dephosphorylation of a specific phosphorylated protein of interest (phospho-POI) by redirecting a catalytically active phosphatase to a desired neo-substrate has the potential to achieve exquisite substrate-level phospho-control. This approach would have the added benefit of also allowing dephosphorylation of phospho-substrates that may be phosphorylated by multiple upstream kinases, while not affecting the phospho-status of the other non-related substrates of those individual kinases.
It is estimated that 98% of phosphorylated residues in the human proteome are serine or threonine and a majority of these sites are dephosphorylated by just two ubiquitously expressed enzymes: protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). These enzymes, which are two of the most highly conserved proteins in eukaryotes, function within holoenzyme complexes that contain regulatory subunits that function to direct the catalytic subunits toward specific substrates. Many regulatory subunits exist to mediate substrate specificity, including at least 200 known PP1 regulators and four classes of PP2A regulators (B/B55, B'/B56, B'/PR72 and Striatin), with multiple alternatively spliced isoforms evident for each class. Whereas PP1 regulators bind directly to the catalytic domain via short linear motifs (SLiMs), most commonly via an RVxF consensus sequence, the PP2A regulatory subunits assemble with catalytic and scaffolding subunits into a heterotrimeric holoenzyme complex. In the case of the PP2A-B56 class, the B56 regulatory subunit then binds directly to SLiMs to target this holoenzyme complex to specific substrates.

To develop a system for targeted dephosphorylation, we conceptualized an affinity-directed system whereby we would artificially recruit the catalytic subunits of PP1 (PPP1CA) or PP2A (PPP2CA) to distinct phospho-POIs via POI-specific polypeptide binders. We term this approach the affinity-directed phosphatase (AdPhosphatase). To test this concept, we focused on two distinct phospho-POIs: family with sequence similarity 83 phosphatase (AdPhosphatase). To test this concept, we focused on two distinct phospho-POIs: family with sequence similarity 83 member D (FAM83D) and the unc-51-like kinase 1 (ULK1). FAM83D is required for the recruitment of casein kinase 1 member D (FAM83D) and the unc-51-like kinase 1 (ULK1). On two distinct phospho-POIs: family with sequence similarity 83 member D (FAM83D) and the unc-51-like kinase 1 (ULK1). FAM83D directs CK1α to the mitotic spindle to orchestrate proper spindle positioning and timely cell division. During mitosis, FAM83D is heavily phosphorylated at the mitotic spindle in a CK1α-dependent manner, and is subsequently degraded by the proteasome upon mitotic exit. However, the precise role for CK1α-dependent hyperphosphorylation of FAM83D remains elusive. The mitotic phosphorylation of FAM83D results in an electrophoretic mobility shift of approximately 25 kDa when visualized by immunoblotting, which can be collapsed by subjecting extracts to an in vitro dephosphorylation assay using λ-phosphatase. As such, in the absence of phospho-specific antibodies, FAM83D immunoblots can be reliably used to monitor the phosphorylation status of FAM83D. ULK1 is a serine/threonine protein kinase that functions in a complex with both the autophagy-related protein 13 (ATG13) and the focal adhesion kinase family interacting protein levels were analyzed (Figure 1B). In mitotic U2OS WT cells expressing FLAG-empty control, FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA co-precipitated with FAM83D-GFP and either mediate the dephosphorylation of hyper-phosphorylated FAM83D-GFP in mitosis or prevent FAM83D-GFP phosphorylation during mitosis (Figure 1A).

U2OS WT and FFAM83D-GFP cells transduced with retroviruses encoding FLAG-empty control, FLAG-aGFP6M-PPP1CA, or FLAG-aGFP6M-PPP2CA were synchronized in mitosis using the Eg5 inhibitor S-trityl-L-cysteine (STLC) and FAM83D protein levels were analyzed (Figure 1B). In mitotic U2OS WT cells expressing FLAG-empty control, FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA, FAM83D phosphorylation was observed by the striking ~25 kDa electrophoretic mobility shift compared with asynchronous controls. These data suggest that the low levels of expression of FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA alone in cells do not interfere with the native untagged FAM83D phosphorylation during mitosis. In FFAM83D-GFP cells expressing FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA, but not those expressing FLAG-empty control, the mitotic phosphorylation-FFAM83D-GFP electrophoretic mobility shift collapsed, suggesting that targeted dephosphorylation of mitotic phosphorylated FFAM83D-GFP was potentially achieved by both FLAG-aGFP6M-PPP1CA and FLAG-aGFP6M-PPP2CA AdPhosphatases.

To determine that phospho-FFAM83D-GFP dephosphorylation is mediated through an interaction with FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA, extracts from both asynchronous and mitotic U2OS FFAM83D-GFP cells expressing FLAG-empty, FLAG-aGFP6M-PPP1CA, or FLAG-aGFP6M-PPP2CA were subjected to anti-GFP immunoprecipitation (IP) (Figure 1C). In both asynchronous and mitotic extracts, FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA co-precipitated with FFAM83D-GFP. In addition, both CK1α and the microtubule-associated protein hyaluronan-mediated motility receptor be expected to be maintained. A key advantage of this approach would therefore be to limit the overexpression of the AdPhosphatase and thereby avoid potential off-target dephosphorylation. Encouragingly, following the expression of FLAG-aGFP6M-PPP2CA in FFAM83D-GFP U2OS and ULK1GFP/GFP ARPE-19 cells, the levels of FLAG-aGFP6M-PPP2CA are much lower than endogenous PPP2CA when detected using an anti-PPP2CA antibody (Figures S1A and S1B). Armed with the knowledge that aGFP6M was functioning to limit AdPhosphatase levels as intended, we sought to test the ability of these AdPhosphatases to target the dephosphorylation of phospho-FAM83D-GFP and phospho-ULK1-GFP and investigate the downstream biology.

RESULTS

Nanobody-directed recruitment of PPP1CA or PPP2CA mediates dephosphorylation of mitotic phospho-FAM83D-GFP

FAM83D directs CK1α to the mitotic spindle to ensure proper spindle positioning and timely cell division. During mitosis, FAM83D also undergoes CK1α-dependent hyperphosphorylation, resulting in an ~25 kDa phospho-dependent electrophoretic mobility shift. We postulated that upon expression of the FLAG-aGFP6M-PPP1CA or -PPP2CA AdPhosphatases in FFAM83D-GFP and thereby avoid potential off-target dephosphorylation, which can be collapsed by subjecting extracts to an in vitro dephosphorylation assay using λ-phosphatase. As such, in the absence of phospho-specific antibodies, FAM83D immunoblots can be reliably used to monitor the phosphorylation status of FAM83D. ULK1 is a serine/threonine protein kinase that functions in a complex with both the autophagy-related protein 13 (ATG13) and the focal adhesion kinase family interacting protein levels were analyzed (Figure 1B). In mitotic U2OS WT cells expressing FLAG-empty control, FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA, but not those expressing FLAG-empty control, the mitotic phosphorylation-FFAM83D-GFP electrophoretic mobility shift collapsed, suggesting that targeted dephosphorylation of mitotic phosphorylated FFAM83D-GFP was potentially achieved by both FLAG-aGFP6M-PPP1CA and FLAG-aGFP6M-PPP2CA AdPhosphatases.

To determine that phospho-FFAM83D-GFP dephosphorylation is mediated through an interaction with FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA, extracts from both asynchronous and mitotic U2OS FFAM83D-GFP cells expressing FLAG-empty, FLAG-aGFP6M-PPP1CA, or FLAG-aGFP6M-PPP2CA were subjected to anti-GFP immunoprecipitation (IP) (Figure 1C). In both asynchronous and mitotic extracts, FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA co-precipitated with FFAM83D-GFP. In addition, both CK1α and the microtubule-associated protein hyaluronan-mediated motility receptor
HMMR, also referred to as RHAMM or CD168, which are validated endogenous FAM83D mitotic interactors, co-precipitated with FAM83D-GFP from mitotic extracts from cells expressing FLAG-empty, FLAG-aGFP6M-PPP1CA, or FLAG-aGFP6M-PPP2CA, suggesting that FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA does not interfere with the endogenous FAM83D-CK1α-HMMR mitotic interactions. These data suggest that FLAG-aGFP6M-PPP1CA or FLAG-aGFP6M-PPP2CA interact with FAM83D-GFP to mediate the reduction in mitotic phosphorylation of phospho-FAM83D-GFP, and furthermore, their binding to FAM83D-GFP does not appear to interfere with native FAM83D interacting proteins.

To establish if the reduction in mitotic phosphorylation of phospho-FAM83D-GFP is reliant on the catalytic activity of PPP1CA or PPP2CA, asynchronous and mitotic U2OS FAM83DGFP/GFP cells expressing FLAG-empty, FLAG-aGFP6M, or FLAG-aGFP6M-PPP2CA were synchronized in mitosis using STLC (5 mM) for 16 h. Following incubation, mitotic (M) cells were isolated through shake-off. Asynchronous (AS) cells were included as a control. Cells were washed twice with ice-cold PBS, lysed, and subjected to anti-GFP immunoprecipitation (IP).

Figure 1. Anti-GFP nanobody-directed recruitment of PPP1CA or PPP2CA mediates dephosphorylation of mitotic phospho-FAM83D-GFP

(A) Schematic representation of anti-GFP nanobody (aGFP6M)-directed recruitment of either PPP1CA or PPP2CA to GFP-tagged FAM83D to mediate FAM83D-GFP dephosphorylation.

(B) Wild-type (WT) and FAM83D<sup>wt/wt</sup> U2OS cells expressing FLAG-empty, FLAG-aGFP6M-PPP1CA, or FLAG-aGFP6M-PPP2CA were synchronized in mitosis using the Eg5 inhibitor S-trityl-L-cysteine (STLC) (5 μM) for 16 h. Following incubation, mitotic (M) cells were isolated through shake-off. Asynchronous (AS) cells were included as a control.

(C) U2OS FAM83D<sup>wt/wt</sup> FLAG-empty, FLAG-aGFP6M-PPP1CA, or FLAG-aGFP6M-PPP2CA-expressing cells were synchronized in mitosis using STLC, and M cells were isolated through shake-off. AS cells were included as a control. Cells were washed twice with ice-cold PBS, lysed, and subjected to anti-GFP immunoprecipitation (IP).

(D) U2OS FAM83D<sup>wt/wt</sup> FLAG-aGFP<sub>6M</sub>, FLAG-aGFP<sub>6M</sub>-PPP1CA, FLAG-aGFP<sub>6M</sub>-PPP1CA<sup>H125Q</sup>, FLAG-aGFP<sub>6M</sub>-PPP2CA, or FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup> expressing cells were synchronized in mitosis using STLC, and M cells were isolated through shake-off. AS cells were included as a control. Cells were washed twice with ice-cold PBS, lysed, and subjected to anti-FLAG IP. For (B–D), extracts and/or IPs were resolved by SDS-PAGE and transferred to PVDF membranes, which were subjected to immunoblotting with indicated antibodies. Closed square denotes AS endogenous FAM83D, open square denotes M endogenous FAM83D, closed circle denotes AS FAM83D-GFP, open circle denotes M FAM83D-GFP.
expected to phosphorylate FAM83D in mitosis to cause the upward mobility shift did not do so over the course of Cal A treatment. The data were therefore inconclusive to establish that the dephosphorylation of phospho-FAM83D-GFP in mitosis is reliant on the phosphatase activity of FLAG-aGFP<sub>6M</sub>-PPP1CA or FLAG-aGFP<sub>6M</sub>-PPP2CA. Therefore, to definitively establish that targeted dephosphorylation is facilitated by the phosphatase activity of the AdPhosphatases, FLAG-aGFP<sub>6M</sub> empty and WT or catalytically dead AdPhosphatases (FLAG-aGFP<sub>6M</sub>-PPP1CA/-PPP1CA<sub>H125Q</sub> or FLAG-aGFP<sub>6M</sub>-PPP2CA/-PPP2CA<sub>H118Q</sub> respectively) were expressed in FAM83D<sub>GFP/GFP</sub> cells by retroviral transduction (Figure 1D). Asynchronous or mitotic extracts from these cells were then subjected to anti-FLAG IPs to only isolate pools of FAM83D-GFP exclusively bound to FLAG-aGFP<sub>6M</sub> or FLAG-aGFP<sub>6M</sub>-PPP1CA/-PPP2CA (Figure 1D). A robust and complete collapse of the mitotic phospho-FAM83D-GFP electrophoretic mobility shift was observed in anti-FLAG-IPs from mitotic extracts from FAM83D<sub>GFP/GFP</sub> cells expressing either FLAG-aGFP<sub>6M</sub>-PPP1CA or FLAG-aGFP<sub>6M</sub>-PPP2CA compared with FLAG-aGFP<sub>6M</sub> controls, while no substantial mitotic phospho-FAM83D-GFP electrophoretic mobility shift was evident in anti-FLAG-IPs from mitotic extracts from cells expressing FLAG-aGFP<sub>6M</sub>-PPP1CA/-PPP1CA<sub>H125Q</sub> or FLAG-aGFP<sub>6M</sub>-PPP2CA/-PPP2CA<sub>H118Q</sub> catalytically dead mutants relative to FLAG-aGFP<sub>6M</sub> controls. These data establish that mitotic phospho-FAM83D-GFP dephosphorylation is reliant on the catalytic activity of the aGFP<sub>6M</sub>-directed recruitment of PPP1CA or PPP2CA.

AdPhosphatase-mediated dephosphorylation of mitotic phospho-FAM83D prevents degradation of FAM83D

Previously, it has been shown that following CK1<sub>α</sub> phospho-FAM83D prevents degradation of FAM83D at the mitotic spindle, FAM83D is subsequently degraded by the proteasome after mitotic exit,28 although whether phosphorylation of FAM83D is required for its subsequent degradation is not known. To determine the effects of AdPhosphatase-mediated dephosphorylation of mitotic phospho-FAM83D-GFP on FAM83D-GFP protein stability following STLC release, U2OS FAM83D<sub>GFP/GFP</sub> cells expressing FLAG-empty, FLAG-aGFP<sub>6M</sub>-PPP1CA (Figure 2A), FLAG-aGFP<sub>6M</sub>-PPP1CA<sub>H125Q</sub> (Figure 2A), FLAG-aGFP<sub>6M</sub>-PPP2CA (Figure 2B), or FLAG-aGFP<sub>6M</sub>-PPP2CA<sub>H118Q</sub> (Figure 2B) were synchronized in mitosis using STLC and lysed at various time points following STLC washout. Following STLC release, as expected, a reduction in Cyclin B1,41 HMMR, and phosphorylated ULK1GFP/GFP levels at the mitotic spindle was observed 3–6 h after STLC washout, similar to that seen in FAM-empty control cells, suggesting that the expression of FLAG-aGFP<sub>6M</sub>-PPP1CA or FLAG-aGFP<sub>6M</sub>-PPP2CA does not negatively impact the cell cycle-dependent regulation of Cyclin B1 or HMMR. Interestingly, a reduction in the rate of decrease in FAM83D-GFP protein levels following STLC washout was evident in cells expressing FLAG-aGFP<sub>6M</sub>-PPP1CA and FLAG-aGFP<sub>6M</sub>-PPP2CA compared with the corresponding phosphatase-dead mutants or FLAG-empty control cells (Figures 2A and 2B). This suggests that the rate of degradation of mitotic phospho-FAM83D-GFP caused by the proteasome following STLC release is impaired by the AdPhosphatase-mediated dephosphorylation of phospho-FAM83D-GFP.

Finally, to investigate the localization of FAM83D-GFP and CK1<sub>α</sub> at the mitotic spindle following AdPhosphatase-mediated phospho-FAM83D-GFP dephosphorylation, U2OS FAM83D KO and FAM83D<sub>GFP/GFP</sub> cells expressing FLAG-empty, FLAG-aGFP<sub>6M</sub>-PPP1CA, FLAG-aGFP<sub>6M</sub>-PPP1CA<sub>H125Q</sub>, FLAG-aGFP<sub>6M</sub>-PPP2CA, or FLAG-aGFP<sub>6M</sub>-PPP2CA<sub>H118Q</sub> were synchronized using STLC, fixed, and analyzed by anti-CK1<sub>α</sub> and anti-FLAG immunostaining as well as GFP fluorescence microscopy (Figure 2C), and relative CK1<sub>α</sub> spindle fluorescence intensity was quantified (Figure 2D). As expected, a significant reduction in CK1<sub>α</sub> levels at the mitotic spindle was observed in FAM83D KO cells compared with FAM83D<sub>GFP/GFP</sub> cells (Figures 2C and 2D). Overlapping FAM83D-GFP, CK1<sub>α</sub> and FLAG localization signals were observed at the mitotic spindle in FAM83D<sub>GFP/GFP</sub> cells expressing FLAG-aGFP<sub>6M</sub>-PPP1CA, FLAG-aGFP<sub>6M</sub>-PPP1CA<sub>H125Q</sub>, FLAG-aGFP<sub>6M</sub>-PPP2CA, or FLAG-aGFP<sub>6M</sub>-PPP2CA<sub>H118Q</sub> (Figures 2C and 2D). These observations suggest that the AdPhosphatase-mediated dephosphorylation of phospho-FAM83D-GFP does not interfere with the mitotic localization of either FAM83D-GFP or CK1<sub>α</sub>. Interestingly, a significant increase in CK1<sub>α</sub> levels at the mitotic spindle was observed in cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA compared with FLAG-empty controls and FLAG-aGFP<sub>6M</sub>-PPP2CA<sub>H118Q</sub> (Figures 2C and 2D), potentially due to stabilization of FAM83D-GFP upon targeted dephosphorylation.

PP2CA AdPhosphatase targets phospho-GFP-ULK1 for dephosphorylation

To investigate the versatility and broader applicability of the AdPhosphatase system, we next targeted phospho-ULK1 for targeted dephosphorylation. FLAG-aGFP<sub>6M</sub>-PPP1CA or FLAG-aGFP<sub>6M</sub>-PPP2CA was therefore expressed by retroviral transduction in ULK1<sub>GFP/GFP</sub> ARPE-19 cells33 to direct PPP1CA or PPP2CA to GFP-ULK1 for targeted dephosphorylation of phospho-GFP-ULK1 and to assess the impact on downstream autophagy signaling (Figure 3A). First, to determine the interaction between GFP-ULK1 and FLAG-aGFP<sub>6M</sub>-PPP1CA or FLAG-aGFP<sub>6M</sub>-PPP2CA, extracts from ARPE-19 WT control or ULK1<sub>GFP/GFP</sub> cells expressing FLAG-empty, FLAG-aGFP<sub>6M</sub>-PPP1CA, or FLAG-aGFP<sub>6M</sub>-PPP2CA were subjected to anti-FLAG IP (Figure 3B). GFP-ULK1 co-precipitated with IPs only from ULK1<sub>GFP/GFP</sub> cell extracts expressing FLAG-aGFP<sub>6M</sub>-PPP1CA or FLAG-aGFP<sub>6M</sub>-PPP2CA, but not from WT cells or
ULK1GFP/GFP cells expressing FLAG-empty control, confirming that FLAG-aGFP₆M-PPP1CA or FLAG-aGFP₆M-PPP2CA can interact only with GFP-ULK1, but not with untagged ULK1. In addition, both ATG13 and FIP200 co-precipitated in extracts from ULK1GFP/GFP cells expressing FLAG-aGFP₆M-PPP1CA or FLAG-aGFP₆M-PPP2CA, suggesting that the expression of
Figure 3. PPP2CA AdPhosphatase targets phospho-GFP-ULK1 for dephosphorylation

(A) Schematic representation of anti-GFP nanobody (aGFP6M)-directed recruitment of either PPP1CA or PPP2CA to mediate phospho-GFP-ULK1 dephosphorylation.

(B) ARPE-19 wild-type (WT) and ULK1GFP/GFP knockin cells expressing FLAG-empty, FLAG-aGFP6M-PPP1CA, or FLAG-aGFP6M-PPP2CA were lysed and subjected to immunoprecipitation (IP) with anti-FLAG M2 resin. * = heavy chain of IgG.

(C) ARPE-19 ULK1GFP/GFP cells expressing FLAG-empty, FLAG-aGFP6M-PPP1CA, FLAG-aGFP6M-PPP1CAH125Q, FLAG-aGFP6M-PPP2CA, or FLAG-aGFP6M-PPP2CAH118Q were lysed and subjected to IP using anti-FLAG M2 resin. For (B and C), extracts and IPs were resolved by SDS-PAGE and transferred onto PVDF membranes, which were subjected to immunoblotting with indicated antibodies.

(D) ARPE-19 ULK1GFP/GFP cells expressing FLAG-empty, FLAG-aGFP6M-PPP1CA, FLAG-aGFP6M-PPP2CA, or FLAG-aGFP6M-PPP2CAH118Q were starved of amino acids with EBSS and treated with the lysosomal inhibitor Bafilomycin A1 (Baf-A1, 50 nM) for 2 h and subjected to anti-ULK1 and anti-FLAG immunofluorescence microscopy. DNA is stained with DAPI. Scale bars, 10 μm.
either FLAG-aGFP<sub>6M</sub>-PPP1CA or FLAG-aGFP<sub>6M</sub>-PPP2CA does not interfere with the formation of the GFP-ULK1-ATG13-FIP200 complex. Interestingly, a striking downward electrophoretic mobility shift of GFP-ULK1 and ATG13 was observed in extracts from ULK<sub>1</sub>GFP/GFP cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA, but not FLAG-aGFP<sub>6M</sub>-PPP1CA, suggesting potential dephosphorylation of both phospho-GFP-ULK1 and phospho-ATG13 by FLAG-aGFP<sub>6M</sub>-PPP2CA. To confirm if the observed GFP-ULK1 dephosphorylation was reliant on the catalytic activity of the AdPhosphatase system, constructs containing the catalytically dead PPP1CA (FLAG-aGFP<sub>6M</sub>-PPP1CA<sup>H125Q</sup>) or PPP2CA (FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup>) mutants were expressed by retroviral transduction in ARPE-19 ULK<sub>1</sub>GFP/GFP cells (Figure 3C). Following anti-FLAG IP, GFP-ULK1, ATG13, and FIP200 co-precipitated with IPs from ULK<sub>1</sub>GFP/GFP cells expressing FLAG-aGFP<sub>6M</sub>-PPP1CA, FLAG-aGFP<sub>6M</sub>-PPP1CA<sup>H125Q</sup>, FLAG-aGFP<sub>6M</sub>-PPP2CA, or FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup>, indicating that the catalytically dead AdPhosphatase constructs also interact with GFP-ULK1 and do not interfere with the formation of the ULK1 complex. Under these conditions, neither FLAG-aGFP<sub>6M</sub>-PPP1CA nor FLAG-aGFP<sub>6M</sub>-PPP1CA<sup>H125Q</sup> caused any detectable GFP-ULK1 or ATG13 mobility shift. Furthermore, the downward GFP-ULK1 and ATG13 electrophoretic mobility shift evident in FLAG-aGFP<sub>6M</sub>-PPP2CA expressing cells was not observed in FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup> expressing cells, suggesting that this potential dephosphorylation-induced mobility shift is reliant on PPP2CA catalytic activity of the AdPhosphatase. ARPE-19 ULK<sub>1</sub>GFP/GFP cells expressing FLAG-empty control or FLAG-aGFP<sub>6M</sub>-PPP2CA were also treated with the protein phosphatase inhibitors Cal A or Okadaic acid (OA) to investigate the effects of aGFP<sub>6M</sub>-PPP2CA-directed dephosphorylation of GFP-ULK1 on downstream starvation-induced autophagy. During periods of starvation, autophagy is downregulated. Therefore, the comparison of starvation-induced autophagy signaling in ARPE-19 ULK<sub>1</sub>GFP/GFP cells expressing FLAG-empty, FLAG-aGFP<sub>6M</sub>-PPP2CA, or FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup> were starved of amino acids for 2 h with EBSS (Figures 4A, 4B, and S3). During this period, cells were also treated with Baf-A1 to inhibit lysosomal degradation and monitor autophagic flux. As expected, in EBSS-starved ULK<sub>1</sub>GFP/GFP cells expressing FLAG-empty control, a reduction in GFP-ULK1 phosphorylation at S758 with a concomitant increase in ATG13 phosphorylation at S318 was observed compared with amino acid-rich controls, indicating ULK1 activation. However, in FLAG-aGFP<sub>6M</sub>-PPP2CA expressing cells under amino acid starvation, a complete loss of ULK1 phosphorylation at S758 was observed compared with FLAG-empty control cells, but a concomitant increase in phosphorylation of ATG13 at S318 was not observed. Furthermore, the EBSS-induced phosphorylation of ATG13 at S318 in FLAG-empty control cells was absent in cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA. Moreover, inhibition of both basal and starvation-induced autophagy signaling was observed in cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA, as indicated by the large reduction in LC3-II flux, compared with FLAG-empty control cells. These results suggest that one or more phospho-residues on ULK1, other than S758, that are critical for ULK1 activation are also targeted for dephosphorylation by the AdPhosphatase, resulting in the inhibition of ULK1. Indeed, in FLAG-aGFP<sub>6M</sub>-PPP2CA-expressing cells, regardless of amino acid starvation, a complete loss of ULK1 phosphorylation at S555 and S638 was evident compared with FLAG-empty control or FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup>-expressing cells (Figure S4). In EBSS-stimulated cells expressing the FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup> catalytically dead mutant, robust ATG13 phosphorylation at S318 was observed, comparable to that observed in cells expressing FLAG-empty control (Figures 4A, 4B, and S3). Furthermore, in cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup> both basal and starvation-induced LC3-II flux were comparable to cells expressing FLAG-empty control. These data serve to
demonstrate that the reduction in ATG13 S318 phosphorylation and basal and starvation-induced LC3-II flux observed in cells expressing FLAG-aGFP6M-PPP2CA is dependent on FLAG-aGFP6M-PPP2CA catalytic activity.

Next, we sought to compare the efficacy of autophagy inhibition by AdPhosphatase-directed dephosphorylation of GFP-ULK1 to that of GFP-ULK1 inhibition using the small molecule ULK1 inhibitor MRT68921.45,51 ARPE-19 ULK1GFP/GFP cells expressing FLAG-empty control were pre-treated with or without MRT68921 (2 μM, 2 h) and, along with cells expressing FLAG-aGFP6M-PPP2CA that were not treated with MRT68921, were starved of amino acids with EBSS and treated with or without Baf-A1 (50 nM) for 2 h. Under starvation conditions, a comparable reduction in ULK1 phosphorylation at S758 was observed between MRT68921-treated cells and those expressing FLAG-aGFP6M-PPP2CA. Furthermore, the reduction in ATG13 phosphorylation at S318 was observed between MRT68921-treated cells and those expressing FLAG-aGFP6M-PPP2CA. In addition, under starvation conditions, LC3-II levels were comparable between MRT68921-treated FLAG-empty control cells and DMSO-treated cells expressing FLAG-aGFP6M-PPP2CA. These data suggest that the attenuation of starvation-induced autophagy observed following AdPhosphatase-mediated GFP-ULK1 dephosphorylation reflects that observed when cells were treated with a small molecule inhibitor of ULK1.

**Figure 4.** AdPhosphatase-mediated dephosphorylation of phospho-GFP-ULK1 inhibits starvation-induced autophagy

(A) ARPE-19 ULK1GFP/GFP cells expressing FLAG-empty, FLAG-aGFP6M-PPP2CA, or FLAG-aGFP6M-PPP2CAH118Q were starved of amino acids with EBSS and treated with Baf-A1 (60 nM) for 2 h.

(B) Quantification of p-S758 ULK1 normalized to total ULK1 protein levels from (A) ± SD of n = 3 independent experiments.

(C) ARPE-19 ULK1GFP/GFP cells expressing FLAG-empty or FLAG-aGFP6M-PPP2CA were pre-treated with the ULK1 inhibitor MRT68921 (2 μM, 2 h), where indicated, and amino acid starved with EBSS and treated with Baf-A1 (50 nM) for 2 h.

(D) Quantification of p-S758 ULK1 normalized to total ULK1 protein levels from (C) ± SD of n = 3 independent experiments. For (A) and (C), extracts were resolved by SDS-PAGE and transferred onto PVDF membranes, which were subjected to immunoblotting with indicated antibodies. ±AA = amino acid-rich conditions, n.s. = not significant. Statistical analyses were carried out by one-way ANOVA using Tukey’s post-test.

**FLAG-aGFP6M-PPP2CA expression mediates the recruitment of PP2A regulatory subunits**

PP2A catalytic C subunits typically only exist in a complex with a scaffold/structural A subunit (PP2A A) and regulatory B subunits, of which there are 26.52–54 The nature of the PP2A holoenzyme complex determines substrate specificity, subcellular localization, and catalytic activity.52–55 To determine whether FLAG-aGFP6M-PPP2CA expression mediates the recruitment of PP2A regulatory subunits, STLC-synchronized U2OS FAM83DGFP/GFP cells (Figure S1A) or EBSS-starved ARPE-19 ULK1GFP/GFP cells (Figure S1B) expressing FLAG-aGFP6M-PPP2CA extracts were subjected to anti-FLAG IP. The resultant anti-FLAG IPs were
resolved by SDS-PAGE, subjected to in-gel trypsin digestion, and the resulting peptides were analyzed by LC-MS/MS (Figure S1C). FLAG-aGFP<sub>6M</sub>-PPP2CA interactors identified exclusively from mitotic U2OS FAM83D<sup>GFP/GFP</sup> cells including FAM83D, CK1α, and HMNR (Figure S1C), which were validated by western blot (Figure S1A), whereas those identified exclusively from EBSS-starved ARPE-19 ULK1<sup>GFP/GFP</sup> cells included ULK1, ATG13, and FIP200 (Figure S1C), which were also validated by western blot (Figure S1B). Interestingly, FLAG-aGFP<sub>6M</sub>-PPP2CA interactors identified that were common to both mitotic U2OS FAM83D<sup>GFP/GFP</sup> and EBSS-starved ARPE-19 ULK1<sup>GFP/GFP</sup> cells included PPP2CA (bait), the PP2A 65 kDa regulatory subunit A alpha (PPP2R1A) and beta (PPP2R1B) isoforms, the PP2A 55 kDa regulatory subunit B alpha isoform (PPP2R2A), and the PP2A 56 kDa regulatory subunit delta (PPP2R5D) and epsilon (PPP2R5E) isoforms (Figure S1C). These data suggest that, as reported previously for PPP2CA,<sup>52-55</sup> the FLAG-aGFP<sub>6M</sub>-PPP2CA AdPhosphatase does not appear to exist by itself, but rather recruits additional regulatory subunits to mediate targeted dephosphorylation of phospho-FAM83D-GFP and phospho-GFP-ULK1.

Global phospho-proteomics demonstrates remarkable specificity of the FLAG-aGFP<sub>6M</sub>-PPP2CA AdPhosphatase system

To determine the specificity of the FLAG-aGFP<sub>6M</sub>-PPP2CA AdPhosphatase-mediated dephosphorylation of phospho-FAM83D-GFP in mitosis or phospho-GFP-ULK1 following amino acid starvation, an unbiased global phospho-proteomic approach was used (Figure 5). U2OS FAM83D<sup>GFP/GFP</sup> cells expressing

![Volcano plots](figure5.png)

Figure 5. TMT-labeled quantitative global phospho-proteomic analysis of FLAG-aGFP<sub>6M</sub>-PPP2CA AdPhosphatase-mediated FAM83D-GFP and GFP-ULK1 dephosphorylation

Volcano plot following global (A) phospho- (11,821 unique phospho-peptides detected) and (B) total- (7,201 proteins detected) proteomic analysis of STLC-synchronized U2OS FAM83D<sup>GFP/GFP</sup> cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA compared with those expressing FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup>. Volcano plot following global (C) phospho- (22,574 unique phospho-peptides detected) and (D) total- (8,153 proteins detected) proteomic analysis of EBSS-starved ARPE-19 ULK1<sup>GFP/GFP</sup> cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA compared with those expressing FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup>. Gray horizontal threshold indicates significance level of p = 0.05. Gray vertical threshold indicates 2-fold change. The top left quadrant indicates phospho-peptides/peptides that are hypo-phosphorylated/downregulated in cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA over those expressing FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup>, colored red, whereas the top right quadrant indicates phospho-peptides/peptides that are hyper-phosphorylated/upregulated in cells expressing FLAG-aGFP<sub>6M</sub>-PPP2CA over those expressing FLAG-aGFP<sub>6M</sub>-PPP2CA<sup>H118Q</sup>, colored blue. Phospho-peptide detected corresponding to FAM83D is labeled in (A), and ULK1 is labeled in (C).
FLAG-aGFP<sup>6M</sup>PPP2CA or FLAG-aGFP<sup>6M</sup>PPP2CA<sup>H118Q</sup> were synchronized in mitosis with STLC and subjected to quantitative phospho- and total-proteomic analyses (Figures 5A and 5B). Following total protein analysis of STLC-synchronized U2OS FAM83D<sup>GRPP/GFP</sup> cells expressing FLAG-aGFP<sup>6M</sup>PPP2CA compared with those expressing FLAG-aGFP<sup>6M</sup>PPP2CA<sup>H118Q</sup> (Figure 5B), a total of 7,201 proteins were identified and of those, no proteins were observed to significantly change in abundance more than 2-fold between the two conditions. This suggests that the expression of FLAG-aGFP<sup>6M</sup>PPP2CA, compared with FLAG-aGFP<sup>6M</sup>PPP2CA<sup>H118Q</sup>, in U2OS FAM83D<sup>GRPP/GFP</sup> cells does not cause any substantial changes to total protein levels. Following phospho-peptide enrichment and analysis of STLC-synchronized U2OS FAM83D<sup>GRPP/GFP</sup> cells expressing FLAG-aGFP<sup>6M</sup>PPP2CA compared with those expressing FLAG-aGFP<sup>6M</sup>PPP2CA<sup>H118Q</sup> (Figure 5A, Tables 1 and S1), 11,821 unique phospho-peptides were identified. Of these, levels of phospho-peptides corresponding to 21 proteins were significantly downregulated by more than 2-fold, and six were significantly upregulated by more than 2-fold, in U2OS FAM83D<sup>GRPP/GFP</sup> cells expressing FLAG-aGFP<sup>6M</sup>PPP2CA compared with those expressing FLAG-aGFP<sup>6M</sup>PPP2CA<sup>H118Q</sup>. Of the significantly downregulated phospho-peptides detected in FLAG-aGFP<sup>6M</sup>PPP2CA-expressing cells, two FAM83D phospho-peptides were detected (pS493 and pS462), FAM83D pS369 was also found to be significantly downregulated, but not by more than 2-fold. Further work is needed to determine whether phosphorylation of ULK1 at these residues impacts its role in starvation-induced autophagy. Of the other phospho-peptides identified, those corresponding to autophagy-regulating proteins, including Beclin-1, a previously reported ULK1 substrate,<sup>62,63</sup> AP-3 complex subunit beta-1 (AP3B1),<sup>64</sup> and La-related protein 1 (LARP1),<sup>65</sup> were found to be downregulated. Whether the alteration observed in the phospho-peptides corresponding to these or other proteins is a direct consequence of GFP-ULK1 dephosphorylation and inactivation or rather due to being in proximity of FLAG-aGFP<sup>6M</sup>PPP2CA needs to be further investigated. To test this, we evaluated the in vitro kinase activity of GFP-ULK1 associated with anti-FLAG IPs from ARPE-19 ULK1<sup>GRPP/GFP</sup> cells expressing FLAG-aGFP<sup>6M</sup>, FLAG-aGFP<sup>6M</sup>PPP2CA, or FLAG-aGFP<sup>6M</sup>PPP2CA<sup>H118Q</sup> against recombinant ATG13 and found no substantial differences in the levels of phosphorylation of recombinant ATG13, potentially suggesting that the proximity of the phosphatase to ATG13 rather than the kinase activity of ULK1 might have resulted in the AdPhosphatase-mediated dephosphorylation of ATG13 in cells (Figure S6).

**DISCUSSION**

Through the conjugation of a high-affinity polypeptide binder of GFP to a catalytic subunit of a phosphatase (AdPhosphatase), we demonstrate here that phosphatase catalytic activity can be redirected to endogenously GFP-tagged proteins FAM83D and ULK1 to mediate targeted phospho-POI dephosphorylation with exquisite selectivity. By directing an AdPhosphatase construct consisting of aGFP<sub>6M</sub> conjugated to PPP1CA or PPP2CA to FAM83D-GFP in FAM83D<sup>GRPP/GFP</sup> cells, dephosphorylation of phospho-FAM83D-GFP was promoted in mitosis to delay phosphorylation-triggered FAM83D-GFP degradation. Using the

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**Table 1. Phospho-peptides corresponding to FAM83D identified following global phospho-proteomic analysis of AdPhosphatase-mediated FAM83D-GFP dephosphorylation**

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Values of phospho-peptides corresponding to FAM83D identified from global analysis of STLC-synchronized U2OS FAM83D<sup>GRPP/GFP</sup> cells expressing FLAG-aGFP<sub>6M</sub>PPP2CA compared with those expressing FLAG-aGFP<sub>6M</sub>PPP2CA<sup>H118Q</sup>. The corresponding FAM83D phospho-peptide detected is indicated, as is the fold change and p value.
AdPhosphatase system, the role of the mitotic CK1α-mediated phosphorylation of FAM83D in its proteolysis was established, highlighting the applicability of the AdPhosphatase system in understanding the function of phospho-modifications on POIs. The PPP2CA AdPhosphatase-mediated dephosphorylation of phospho-FAM83D-GFP in mitotic FAM83D<sup>GFP/GFP</sup> cells was remarkably specific, with only a small list of other phospho-peptides shown to be significantly downregulated in an unbiased global phospho-proteomic screen.

Similarly, an AdPhosphatase construct consisting of aGFP<sub>SM</sub> conjugated to PPP2CA mediated the targeted dephosphorylation of phospho-GFP-ULK1 when expressed in ULK1<sup>GFP/GFP</sup> cells. AdPhosphatase-mediated GFP-ULK1 dephosphorylation attenuated starvation-induced autophagy to the same extent as chemical inhibition of ULK1. ULK1 undergoes phosphorylation at multiple residues that orchestrate either an activating or inhibitory role on its activity and subsequently its role in autophagy initiation. The PPP2CA AdPhosphatase system resulted in a profound dephosphorylation of phospho-GFP-ULK1 and resulted in the inhibition of starvation-induced autophagy, suggesting that this system targeted the dephosphorylation of dominant ULK1 phospho-residues that potentiate ULK1 activity. Following global phospho-proteomic analysis, three ULK1 phospho-residues, S539, S544, and S694, were found to be significant and time-intensive development of POI-specific dephosphorylation.71−75 Heterobifunctional small molecules recruiting Halo-PP1 linked to selective inhibitors of Akt and EGFR, respectively, albeit with relatively low efficacy.71 A tag-based PosTAC involving a dTAG-/Halo-recruiting heterobifunctional small molecule was also designed recently for chemical proof-of-concept to recruit overexpressed FKB12<sup>38v</sup>-tagged PP2A regulatory subunit to an overexpressed Halo-tagged POI to mediate POI dephosphorylation.72 To understand whether this dTAG-/Halo-recruiting PosTAC approach affects endogenous protein function, FKB12<sup>38v</sup> and Halo would need to be knocked-in on to the respective phosphatase subunit and POI using, for example, CRISPR-Cas9 genome editing technology, as long as the tags are tolerated by the respective proteins. A heterobifunctional peptide-based recruit to facilitate PP2A-Bζ and Tau protein proximity to elicit targeted dephosphorylation of Tau, was reported to lead to degradation of Tau.72 The constitutive AdPhosphatase system described here can be exploited not only to explore the biological role of specific phospho-GFP-POIs, where GFP-tagging is tolerated, but also to inform the suitability of a chosen phosphatase in mediating the targeted dephosphorylation of the substrate of interest, prior to investing in the resource-intensive development of POI-specific dephosphorylation-inducing heterobifunctional small molecules.

**Limitations of the study**

There are a few limitations of the current AdPhosphatase approach. The AdPhosphatase is introduced in cells via retroviral
transduction and hence the target cell genome is modified randomly. The constitutive expression of the AdPhosphatase means that the interaction between the aGFPexo-AdPhosphatase and the GFP-POI and hence the target protein dephosphorylation are also constitutive. In order to use the aGFPexo-based AdPhosphatases against a desired intracellular POI, there is a requirement to introduce a GFP tag on the POI by genome editing methodologies, which can be time-consuming and need testing to ensure the GFP-tagging is tolerated.

**SIGNIFICANCE**

Protein phosphorylation is a fundamental driver of all cell signaling processes and is therefore tightly regulated. Hyperphosphorylation of proteins is a known hallmark of many diseases, including cancer and neurodegenerative diseases. In this study, we describe a new approach termed the AdPhosphatase system, which efficiently and selectively targeted endogenously GFP-tagged FAM83D and ULK1 for dephosphorylation. This AdPhosphatase system is versatile and adaptable, where, in principle, promiscuous phosphatase activity can be redirected toward any intracellular phosphorylated GFP-tagged POI that tolerates a GFP-fusion. If selective nanobodies against endogenous POIs exist, the AdPhosphatase can be adapted to assess targeted dephosphorylation of unmodified POIs. Nonetheless, whether the target protein is dephosphorylated needs to be evaluated on a case-by-case basis. Achieving targeted dephosphorylation of phospho-proteins by small molecule PhosTACs could hold promise as a therapeutic approach, although currently no allosteric ligands against endogenous phosphatases exist. The Ad-Phosphatase system can inform whether dephosphorylating a target POI by a specific phosphatase alters its function resulting in a desired phenotype so that ligand development for potential PhosTACs can be prioritized.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.chembiol.2023.01.003.

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**AUTHOR CONTRIBUTIONS**

L.M.S. and L.J.F. performed experiments, collected and analyzed data, and contributed to the writing of the manuscript. G.S. assisted with quantitative global total and phospho-proteomics experiments and data analysis. A.B. assisted during the optimization of the AdPhosphatase technology and contributed to the writing of the manuscript. J.Z. performed experiments and collected and analyzed data during the revision of the manuscript. D.R.S. assisted during the optimization of the AdPhosphatase technology. N.W., J.C., and M.W. generated constructs used in this study. J.V., R.G., and R.S. assisted during the optimization of the AdPhosphatase technology and contributed to the writing of the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

**INCLUSION AND DIVERSITY**

The authors support inclusive, diverse, and equitable conduct of research.


25. Casamayor, A., and Anito, J. (2020). Controlling Ser/Thr protein phospha-


### STAR METHODS

#### KEY RESOURCES TABLE

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gopal Sapkota (g.sapkota@dundee.ac.uk).

**Materials availability**
All constructs used in this study are available to request from the MRC PPU Reagents & Services webpage (http://mrcppureagents.dundee.ac.uk) and the unique identifier (DU) numbers provide direct links to the cloning strategies and sequence details. All constructs were sequence-verified by the DNA Sequencing Service, University of Dundee (http://www.dnaseq.co.uk).

**Data and code availability**
- Unprocessed Western blot and immunofluorescence data and proteomics data have been deposited at Mendeley Data and PRIDE, respectively, and are publicly available as of the date of publication. Accession numbers are listed in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](mailto:g.sapkota@dundee.ac.uk) upon request.

**Critical commercial assays**

**Continued**

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**Materials availability**

**Critical commercial assays**

**Continued**

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**Experimental models: Cell lines**

- Human: ARPE-19
  - ATCC
  - Cat# CRL-2302
- Human: ARPE-19 ULK1<sup>GSFP/GFP</sup>
  - Simpson et al.33
  - N/A
- Human: HEK293-FT
  - Invitrogen
  - Cat#R70007
- Human: U2OS
  - ATCC
  - HTB-96
- Human: U2OS FAM83D<sup>GFP/GFP</sup>
  - Fulcher et al.28
  - N/A
- Human: U2OS FAM83D KO
  - Fulcher et al.28
  - N/A

**Recombinant DNA**

- pCMV5-gag-pol
  - Cell Biolabs
  - Cat# RV-111
- pCMV5-VSV-G
  - Cell Biolabs
  - Cat# RV-110
- pBabeD-puromycin FLAG-aGFP<sub>ΔN</sub>
  - MRC PPU Reagents & Services
  - DUS7701
- pBabeD-puromycin FLAG-aGFP<sub>ΔN</sub>-PPP1CA
  - MRC PPU Reagents & Services
  - DU62917
- pBabeD-puromycin FLAG-aGFP<sub>ΔN</sub>-PPP1CA<sup>H125Q</sup>
  - MRC PPU Reagents & Services
  - DU62964
- pBabeD-puromycin FLAG-aGFP<sub>ΔN</sub>-PPP2CA
  - MRC PPU Reagents & Services
  - DU62902
- pBabeD-puromycin FLAG-aGFP<sub>ΔN</sub>-PPP2CA<sup>H118Q</sup>
  - MRC PPU Reagents & Services
  - DU62960

**Software and algorithms**

- ImageJ
  - Schneider et al.76
  - [https://imagej.net](https://imagej.net)
- GraphPad Prism v9.4.0
  - GraphPad
  - [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/)
- OMEROD.4.10
  - Allan et al.77
  - [https://openmicroscopy.org/](https://openmicroscopy.org/)
- SoftWoRx
  - GE Healthcare
  - N/A
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
All procedures were carried out under aseptic conditions meeting biological safety requirements. ARPE-19 cells (ATCC, Cat# CRL-2302) are human retinal pigment epithelial cells derived from a 19-year-old male. HEK293-FT cells (Invitrogen, Cat# R70007) are a clonal isolate of HEK293 cells transformed with the SV40 large T antigen. U2OS cells (ATCC, Cat# HTB-98) are human epithelial bone osteosarcoma cells derived from a 15-year-old Caucasian female. For growth, HEK293-FT and U2OS cells were maintained in DMEM (Life Technologies) containing 10% (v/v) foetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM L-glutamine, 100 U/ml penicillin (Lonza), and 0.1 mg/mL streptomycin (Lonza). ARPE-19 cells were maintained in a 1:1 mix of DMEM and Ham’s F-12 nutrient mix (Life Technologies) containing 15% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/mL streptomycin. Cells were grown at 37°C with 5% CO2 in a water-saturated incubator. For passaging, cells were incubated with trypsin/EDTA at 37°C to detach cells.

METHOD DETAILS

Plasmids
For production of retroviral vectors, the following were cloned into pBABED-puromycin plasmids: FLAG-aGFP6M (DU57701), FLAG-aGFP6M-PPP1CA (DU62917), FLAG-aGFP6M-PPP1CAH125Q (DU62964), FLAG-aGFP6M-PPP2CA (DU62902), FLAG-aGFP6M-PPP2CAH118Q (DU62960). All constructs were sequence-verified by the DNA Sequencing Service, University of Dundee (http://www.dnaseq.co.uk). These constructs are available to request from the MRC PPU Reagents and Services webpage (http://mrcppureagents.dundee.ac.uk) and the unique identifier (DU) numbers provide direct links to the cloning strategies and sequence details.

Generation of cell lines using CRISPR/Cas9
The CRISPR/Cas9 genome editing system78 was used to generate U2OS FAM83D homozygous C-terminal GFP knockin (KI) (FAM83DGFP/GFP) cells28, FAM83D knock-out (FAM83D KO) cells28, and ARPE-19 ULK1 homozygous N-terminal GFP KI (ULK1GFP/GFP) cells.33 For FAM83D KO cells, U2OS cells were transfected with vectors encoding a pair of guide RNAs (pBABED-Puro-sgRNA1 and pX335-CAS9-D10A-sgRNA2) targeting around the first exon of FAM83D (1 μg each) along with 1 mg/mL PEI. For FAM83DGFP/GFP cells, U2OS cells were transfected with vectors encoding a pair of guide RNAs (pBABED-Puro-sgRNA1 and pX335-CAS9-D10A-sgRNA2) targeting around the stop codon of FAM83D, along with the respective donor plasmid carrying the GFP KI insert and flanking homology arms (~500 bases) (3 μg each) and PEI. For ULK1GFP/GFP cells, ARPE-19 cells were transfected with vectors encoding a pair of guide RNAs (pBABED-puromycin-sgRNA1 and pX335-CAS9-D10A-sgRNA2) targeting ULK1 exon 1 (1 mg each), along with the respective donor plasmids carrying the GFP KI insert (3 mg) and PEI. 16 hr post-transfection, selection with 2 μg/mL puromycin (Sigma-Aldrich) was carried out and continued for a further 48 hr. The transfection process was repeated one more time. After selection, cells were sorted by flow cytometry and single GFP-positive cell clones were plated on individual wells of two 96-well plates. Viable clones were expanded, and integration of GFP at the target locus was verified by Western blotting and genomic sequencing of the targeted locus.

Retroviral generation of stable cell lines
Retroviral pBABED-puromycin vectors encoding the desired construct (6 μg) were co-transfected with pCMV5-gag-pol (3.2 μg) and pCMV5-VSV-G (2.8 μg) (Cell Biolabs) into a 10 cm diameter dish of ~70% confluent HEK293-F7 cells. Briefly, plasmids were added to 1 mL Opti-MEM medium to which 24 μL of 1 mg/mL PEI was added. Following a gentle mix and incubation at room temperature for 20 min, the transfection mix was added dropwise to HEK293-FT cells. 16 hr post-transfection, fresh medium was added to the cells. 24 hr later, the retroviral medium was collected and passed through 0.45 μm sterile syringe filters. Target cells (~60% confluent) were transduced with the optimised titre of the retroviral medium diluted in fresh medium (typically 1:1-1:10) containing 8 μg/mL polybrene (Sigma-Aldrich) for 24 hr. The retroviral medium was then replaced with fresh medium, and 24 hr later, the medium was again replaced with fresh medium containing 2 μg/mL puromycin for selection of cells which had integrated the constructs. A pool of transduced cells were utilised for subsequent experiments following complete death of non-transduced cells placed under selection in parallel.

Treatment of cells with compounds
The following chemicals were added to cell media at indicated concentrations and times: MG132 (Abcam), Calyculin A (CST), Okadaic acid (CST), Bafilomycin-A1 (Enzo Life Sciences), MRT68921 (MRC PPU Reagents and Services). Cells were synchronised in mitosis using the Eg5 inhibitor S-trityl-L-cysteine (STLC, Sigma-Aldrich, 5 μM, 16 hr).28,33 Following incubation, mitotic cells were lysed after isolation through shake-off or after release into fresh media containing stated compounds for indicated times. For amino acid starvation, cells were washed twice in Earle’s balanced salt solution (EBSS, Gibco) and incubated in EBSS for 2 hr.

Cell lysis and immunoprecipitation
Cells were harvested by washing twice with phosphate-buffered saline (PBS) and scraping into ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 0.27 M sucrose, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate,
50 mM sodium fluoride, 5 mM sodium pyrophosphate and 1% NP-40) supplemented with 1x cComplete protease inhibitor cocktail (Roche). After incubation for 10 min on ice, lysates were clarified by centrifugation at 20,000 xg for 20 min at 4 °C. Protein concentration was determined according to the Bradford assay to enable normalisation between samples.

Following determination of protein concentration by Bradford assay, immunoprecipitation (IP) was utilised to isolate a particular protein of interest. For anti-FLAG IPs, anti-FLAG M2 resin (Sigma-Aldrich) was used; for anti-GFP IPs, GFP-TRAP beads (ChromoTek) were used. Before an IP was performed, an input from each lysate was retained to compare and determine IP efficiency. Samples were incubated for 4 hr at 4 °C on a rotating wheel. Beads were collected by centrifugation at 1000 xg for 1 min at 4 °C and a sample of the supernatant was retained (flow-through). IPs were subsequently washed three times with lysis buffer. Input, IP and flow-through samples were reduced in LDS sample buffer (Invitrogen).

**SDS-PAGE and Western blotting**

Cell lysates containing equal amounts of protein (10-20 μg) were resolved by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked in 5% (w/v) non-fat milk (Marvel) in TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20) and incubated overnight at 4 °C in 5% (w/v) BSA/TBS-T or 5% (w/v) milk/TBS-T with the appropriate primary antibodies. Primary antibodies used at indicated dilutions include: anti-Akt (9272S, CST, 1:1,000), anti-Akt p-S473 (12694, CST, 1:1,000), anti-ATG13 (SAB4200100, Sigma-Aldrich, 1:1,000), anti-ATG13 p-S318 (NPB2-19127, Novus, 1:1,000), anti-CK1ε (A301-991A, Bethyl, 1:1,000; SA527, MRC PPU Reagents & Services, 1:1,000), anti-Cyclin B1 (4138, CST, 1:1,000), anti-FAM83D (SA102, MRC PPU Reagents & Services, 1:1,000), anti-FIP200 (17250-1-AP, Proteintech, 1:1,000), anti-FLAG (A8592, Sigma-Aldrich, 1:2,500), anti-GAPDH (2118, CST, 1:5,000), anti-GFP (S268B, MRC PPU Reagents & Services, 1:2,000), anti-HMMR (ab124729, Abcam), anti-LC3 (S400D, MRC PPU Reagents & Services, 1:2,000), anti-mono- and poly-ubiquitylated conjugates (BML-PW8810, Enzo Life Sciences, 1:2,000), anti-PP2CA (S274B, MRC PPU Reagents & Services, 1:1,000), anti-α-tubulin (MA1-80189, Thermo Fisher Scientific, 1:5,000), anti-ULK1 (8054, CST, 1:1,000), anti-ULK1 p-S555 (5689, CST, 1:1,000), anti-ULK1 p-S638 (14205, CST, 1:1,000), anti-ULK1 p-S757 (6888, CST, 1:1,000).

Membranes were subsequently washed with TBS-T and incubated with HRP-conjugated secondary antibody for 1 hr at room temperature. HRP-coupled secondary antibodies used at indicated dilutions include: goat anti-rabbit-IgG (7074, CST, 1:2,500), rabbit anti-sheep-IgG (31480, Thermo Fisher Scientific, 1:5,000), goat anti-rat IgG (62-9520, Thermo Fisher Scientific, 1:5,000), goat anti-mouse-IgG (31430, Thermo Fisher Scientific, 1:5,000). After further washing, signal detection was performed using ECL (Merck) and ChemiDoc MP System (Bio-Rad). ImageJ v1.49 (National Institutes of Health) was used to analyse protein bands by densitometry.76

**In vitro ULK1 kinase assay**

ARPE-19 ULK1GFP/GFP cells expressing FLAG-aGFP6M, FLAG-aGFP6M-PPP2CA or FLAG-aGFP6M-PPP2CAH118Q were lysed and subjected to IP with anti-FLAG M2 resin to co-IP GFP-ULK1. Following IP, resins were washed twice with lysis buffer and twice with kinase assay buffer (1 mM DTT, 50 mM NaCl, 50mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM MgCl₂). Resins were then incubated within kinase assay buffer containing 100 mM [γ-32P]ATP (~500 cpm/pmol) and 2 μg of GST-ATG13 (DU30086, MRC PPU Reagents and Services). Reactions were incubated at 30 °C for 30 min with shaking, and terminated by adding 2x SDS sample buffer containing 5% (v/v) β-mercaptoethanol and subsequent heating at 95 °C for 5 min.

**Immunofluorescence microscopy**

Cells were seeded onto sterile glass coverslips in 6-well dishes. Coverslips were washed twice with PBS, fixed with 4% (w/v) paraformaldehyde (Thermo Fisher Scientific) for 10 min, washed twice with and incubated for 10 min in DMEM/10 mM HEPS pH 7.4. After one wash in PBS, cell permeabilisation was carried out using 0.2% NP-40 in PBS for 4 min. Samples were blocked by washing twice and incubation for 15 min in blocking buffer (1% (w/v) BSA/PBS). Coverslips were incubated for 1 hr at 37 °C with primary antibodies in blocking buffer and washed three times in blocking buffer. Mouse anti-FLAG monoclonal (F8014, Sigma-Aldrich), rabbit anti-DYKDDDDK Tag (FLAG) monoclonal (14793, CST), sheep anti-CK1ε polyclonal (SA527, MRC PPU Reagents & Services) and rabbit anti-ULK1 (8054, CST) primary antibodies were used at a 1:500 dilution. Coverslips were then incubated for 30 min at room temperature with Alexafluor coupled secondary antibodies in blocking buffer and washed an additional three times in blocking buffer. Donkey anti-rabbit IgG Alexa-Fluor 488 (A21206, Thermo Fisher Scientific), goat anti-mouse IgG Alexa-Fluor 594 (A11005, Thermo Fisher Scientific), donkey anti-rabbit IgG Alexa-Fluor 594 (A11012, Thermo Fisher Scientific) and donkey anti-sheep IgG Alexa-Fluor 647 (A21448, Thermo Fisher Scientific) secondary antibodies were used at a 1:500 dilution. After submerging in ddH₂O, cells were mounted onto glass slides using ProLong gold antifade mountant with DAPI (Life Technologies) and visualised using a DeltaVision system (Applied Precision) and deconvolved using SoftWoRx (Applied Precision). Images were processed using ImageJ and OMERO 5.4.10 software.77 ImageJ macro quantification of CK1ε spindle localisation was performed as previously described.28,33

**Mass spectrometry**

**Interactome analysis**

For interactome analysis, cells were first lysed in NP-40 lysis buffer. Clarified lysates were incubated with Protein A agarose beads for 1 hr on a rotating wheel at 4 °C to pre-clear non-specific binding proteins and filtered through Spin-X columns by centrifugation for 5 min at 13,000 xg. Filtered extracts (5-10 mg protein) were incubated with 10-20 μl of appropriate beads for specified IP for 4 hr at...
4°C on a rotating wheel. Following incubation, beads were washed 3x with standard lysis buffer. Bead-bound proteins were denatured and eluted in 2x LDS for 5 min at 95°C. Samples were then filtered through Spin-X columns to remove the beads from the eluate. The filtered eluate was loaded onto a 4-12% Bis-Tris gradient gel and proteins were separated by SDS-PAGE. Gels were stained with InstantBlue and subsequently de-stained in deionised water. A small portion of the eluate was retained for analysis and validation by Western blotting.

To minimise potential protein contaminants, all steps from this point were performed under a laminar flow hood. Disposable scalps were used to cut protein bands of interest from the InstantBlue stained gels into 1-2 cm cubes, which were subsequently transferred into LoBind 1.5 ml Eppendorf tubes. Gel pieces were washed once in HPLC grade water, and then shrunk in anhydrous acetonitrile (ACN) for 5 min with gentle shaking. The gel pieces were then re-swollen with 50 mM Tris-HCl pH 8.0 for 5 min with shaking. The shrinking-swelling process was repeated once more, and the proteins within the gel pieces were reduced in nitrile (ACN) for 5 min with gentle shaking. The ACN was aspirated, and gel pieces were re-swollen with 50 mM triethylammonium bicarbonate (TEAB) pH 8.0 containing 5 µg/ml trypsin for 16 hr at 30°C for digestion. An equivalent amount of ACN was added to the digest for 15 min with shaking and the supernatant was collected into a fresh LoBind 1.5 ml Eppendorf tube. Gel pieces were then re-swollen with 0.1% (v/v) trifluoroacetic acid (TFA) for 5 min with shaking, and peptides were extracted twice with ACN for 5 min each with shaking. After each extraction, the supernatant was removed and combined with the previous supernatant. The supernatants were then dried by vacuum centrifugation using a SpeedVac.

Digested peptides were reconstituted in HPLC-grade 5% (v/v) ACN containing 0.1% (v/v) formic acid (FA) and injected into a U3000 RSLC (rapid separation liquid chromatography) HPLC chromatography system (Thermo Fisher Scientific) coupled to a linear ion trap-orbitrap hybrid mass spectrometer (Orbitrap Velos Pro, Thermo Fisher Scientific). Peptides were trapped on a nanoViper Trap column (2 cm x 100 µM, 18.5 µM, 100 Å, Thermo Fisher Scientific) and subsequently separated on a 15 cm EasySpray column (Thermo Fisher Scientific) equilibrated with a flow rate of 300 nL/min. Data was acquired in the data-dependent mode, automatically switching between MS1 and MS2 acquisition. Full scan spectra (m/z 400-1,600) were acquired in the orbitrap with resolution set to 60,000 at m/z 400. The 20 most intense ions, above a specific minimum signal threshold of 2,000, were fragmented by collision induced dissociation and recorded in the linear ion trap (full automatic gain control (AGC) target; 30,000, Msn AGC target; 5,000).

Raw files were subsequently converted into a list of identified peptides, along with the precursor intensity of the identified peptides, and submitted to the in-house Mascot server (MRC PPU, University of Dundee). Data was searched against the SwissProt human database with variable modifications allowing for oxidation of Met, phosphorylation of Ser/Thr or Tyr residues, along with oxidation or dioxidation modifications. Carbamidomethylation of Cys was set as a fixed modification. Error tolerances were set to ±0.1 ppm (parts per million) for MS1 and 0.6 Da for MS2. Data analysis was performed using Scaffold v 4.4.6 (Proteome Software).

**Global proteome and phospho-proteome analysis**

Cells were lysed in urea lysis buffer (8 M urea, 20 mM HEPES pH 8.0, supplemented with 1 tablet of cOmplete protease inhibitors per 25 mL lysis buffer and 1 tablet of PhosSTOP phosphatase inhibitors per 10 mL lysis buffer) by Bioruptor® sonication for 15 cycles at 30 sec intervals in LoBind Eppendorf tubes. Lysates were clarified by centrifugation at 13,000 x g for 20 min at 4°C and were then transferred to fresh LoBind Eppendorf tubes. Protein concentration was estimated using the Pierce™ BCA method. Equal protein from each condition were reduced with 5 mM DTT at room temperature for 30 min and alkylated with 20 mM iodoacetic acid (IAA) for 20 min at room temperature. Gel pieces were then re-swollen in 50 mM triethylammonium bicarbonate (TEAB) pH 8.0 containing 5 µg/ml trypsin for 16 hr at 30°C for digestion. An equivalent amount of ACN was added to the digest for 15 min with shaking and the supernatant was collected into a fresh LoBind 1.5 ml Eppendorf tube. Gel pieces were then re-swollen with 0.1% (v/v) trifluoroacetic acid (TFA) for 5 min with shaking, and peptides were extracted twice with ACN for 5 min each with shaking. After each extraction, the supernatant was removed and combined with the previous supernatant. The supernatants were then dried by vacuum centrifugation using a SpeedVac.

Peptides were resuspended in 50 mM TEAB and labelled using tandem mass tag (TMT) labels as per the manufacturer’s instructions. TMT labels were resuspended in ACN, added to assigned samples and incubated for 1 hr at room temperature. Following label check by LC-MS/MS, the labelling reaction was quenched with 5% hydroxyamine for 15 min at room temperature. Labelled peptides from each condition were pooled together and dried. Pooled peptides were separated by basic reversed phase (bRP) chromatography fractionation on a C18, 250 x 4.6 mm column, 5 µm, XBridge (Waters, Milford, MA) with Ultimate 3000 HPLC system (Dionex) operating at 500 µL/min with two buffers: buffer A (10 mM ammonium formate, pH 10) and buffer B (80% ACN, 10 mM ammonium formate, pH 10). Peptides were resuspended in 100 µL of buffer A (10 mM ammonium formate, pH10) and separated on a C18 reverse phase column. A total of 96 fractions were collected. 10% of each fraction were concentrated to 24 fractions for proteome analysis, whilst the remaining 90% were concentrated into 12 fractions for IMAC-based phospho-peptide enrichment. Each concentrated fraction was then dried by SpeedVac.

IMAC beads were prepared from Ni-NTA superflow agarose beads that were stripped with Nickel with 100 mM EDTA and incubated in an aqueous solution of 10 mM iron (III) chloride (FeCl3). Dried peptide fractions were reconstituted to a concentration of 0.5 µg/µL in 80% ACN/0.1% TFA. Peptide mixtures were enriched for phosphorylated peptides with 10 µL IMAC beads for 30 min. Enriched IMAC beads were loaded on Empore C18 silica packed stage tips. Stage tips were equilibrated with methanol followed by 50% ACN/0.1% FA then 1% FA. The beads with enriched peptide were loaded onto C18 stage tips and washed with 80% ACN/0.1% TFA. Phosphorylated peptides were eluted from IMAC beads with 500 mM dibasic sodium phosphate, pH 7.0.

Enriched phospho-peptides and peptides were analysed on an Orbitrap Fusion Trubrid mass spectrometer interfaced with Dionex Ultimate 3000 nanoflow liquid chromatography system. Peptides were separated on an analytical column (75 µm x 50 cm, RSLC C18) at a flow rate of 300 nL/min using a step gradient of 5-7% solvent B (90% ACN/0.1% FA) for the first 10 min, followed by 7-35% up to 150 min. The total run time was set to 180 min. The mass spectrometer was operated in a data-dependent acquisition mode. A survey
full scan MS (from m/z 375-1500) was acquired in the Orbitrap at a resolution of 120,000 at 200 m/z. The AGC target for MS1 was set as standard and ion filling time set at 50 ms. The most intense ions with charge state $\geq 2$ were isolated and fragmented using higher collision dissociation (HCD) fragmentation with 38% normalised collision energy and detected at a mass resolution of 50,000 at 200 m/z. The AGC target for MS2 was set as standard and ion filling time set at 86 ms, while dynamic exclusion was set for 80 s.

The mass spectrometry raw data were searched using Sequest HT search engines with Proteome Discoverer 2.1 (Thermo Fisher Scientific). Phosphopeptide-enriched fractions from each replicate were searched against the Uniprot protein database. The search parameter used were carbamidomethylation of cysteine residues and TMT of lysine and N terminal as a fixed modification. Oxidation of methionine, the phosphorylations of serine, threonine and tyrosine, were selected as dynamic modifications. Trypsin was set as the protease and a maximum of two missed cleavages were allowed. Precursor mass tolerance was set to 10 ppm, and a fragment mass tolerance of 0.02 Da was allowed. All peptide-spectrum matches (PSM) were identified at a 1% false-discovery rate (FDR). The probability of phosphorylation for each site was calculated by the phosphoRS node in Proteome Discoverer.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was determined using unpaired Student’s t-test for single comparisons and for multiple treatments analysis of variance was performed followed by the post-hoc tests described in figure legends using Prism® Version 8.0.