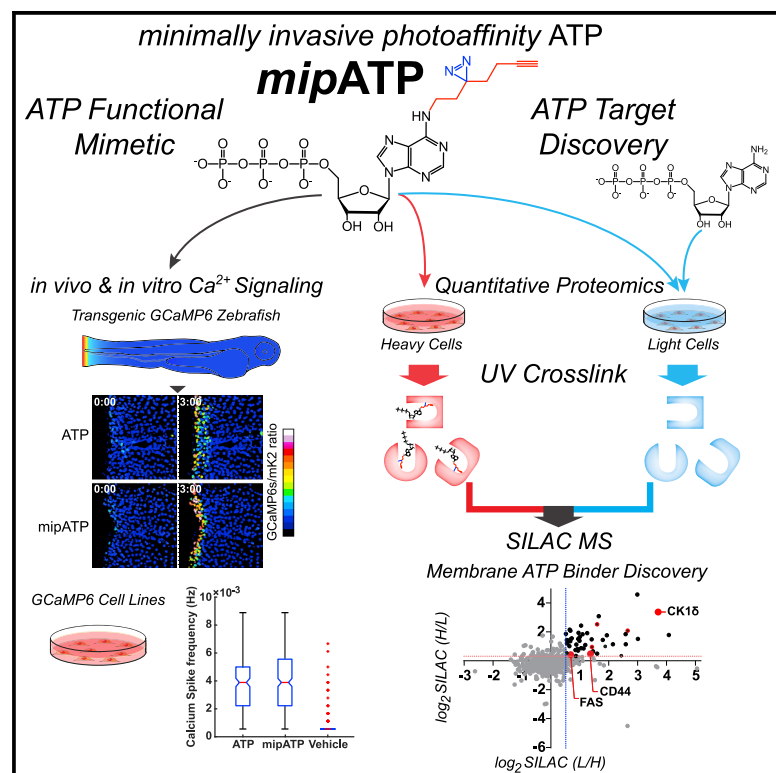


# Cell Chemical Biology

## A Photo-clickable ATP-Mimetic Reveals Nucleotide Interactors in the Membrane Proteome

### Graphical Abstract



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### In Brief

Jelcic et al. developed and verified a minimally invasive photoaffinity ATP (mipATP) probe that retains the signaling functions of native ATP *in vivo* and *in vitro* and provide proof-of-principle that mipATP can be used to map ATP-protein interaction space using proteomic screens.

### Highlights

- Developed a compact minimally invasive photoaffinity ATP (mipATP) probe
- mipATP retains the signaling functions of native ATP *in vivo* and *in vitro*
- Proteomic screens with mipATP map ATP-protein interaction space in membranes



## Resource

# A Photo-clickable ATP-Mimetic Reveals Nucleotide Interactors in the Membrane Proteome

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## SUMMARY

ATP is an important energy metabolite and allosteric signal in health and disease. ATP-interacting proteins, such as P2 receptors, control inflammation, cell death, migration, and wound healing. However, identification of allosteric ATP sites remains challenging, and our current inventory of ATP-controlled pathways is likely incomplete. Here, we develop and verify *mipATP* as a minimally invasive photoaffinity probe for ATP-interacting proteins. Its *N*<sup>6</sup> functionalization allows target enrichment by UV crosslinking and conjugation to reporter tags by “click” chemistry. The additions are compact, allowing *mipATP* to completely retain the calcium signaling responses of native ATP *in vitro* and *in vivo*. *mipATP* specifically enriched for known nucleotide binders in A549 cell lysates and membrane fractions. In addition, it retrieved unannotated ATP interactors, such as the FAS receptor, CD44, and various SLC transporters. Thus, *mipATP* is a promising tool to identify allosteric ATP sites in the proteome.

## INTRODUCTION

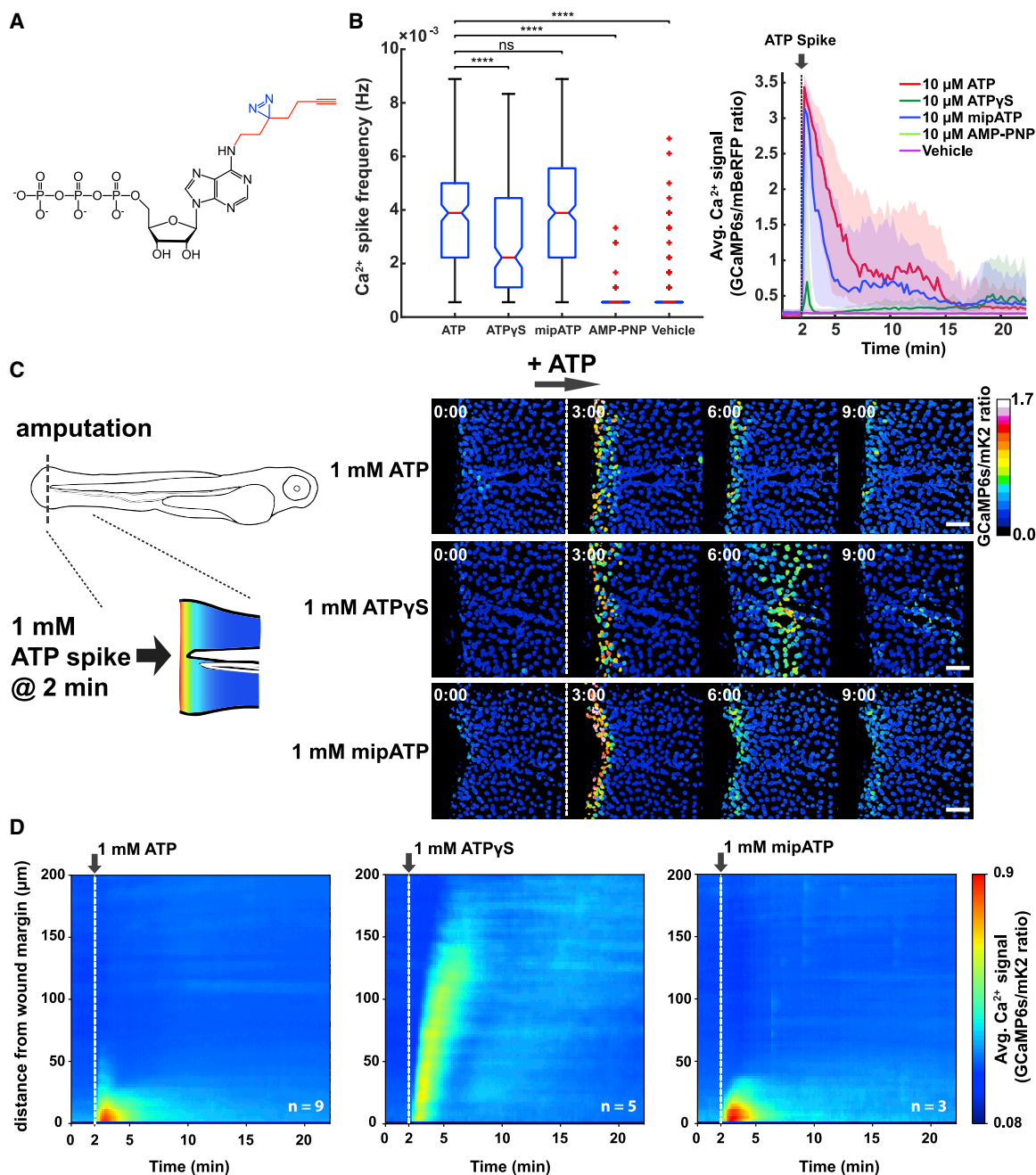
ATP drives cellular processes through the hydrolysis of its high-energy phosphoanhydride bonds. ATP also acts non-catalytically at regulatory sites as an allosteric effector. Catalytic ATP-binding sites can be identified through highly conserved primary sequence and structural motifs, most notably the P-loop (Walker A) NTPase fold (Walker et al., 1982). There are also well-conserved allosteric ATP-binding sites, for instance, the cystathionine beta synthase domain found in AMP-activated protein kinase and inosine 5'-monophosphate dehydrogenase (Bate-man, 1997; Zhang et al., 1999). Yet many non-catalytic ATP-binding sites follow unclear structural rules. For example, the ATP-binding sites of most G protein-coupled purinergic P2 receptors in animals remain unresolved because they lack classic ATP-binding motifs (Zhang et al., 2015, 2014). Such sites are difficult to predict by bioinformatics and must be experimentally identified. One example is the plant ATP receptor DORN1, which shows no sequence homology to known P2 receptors of animals (Choi et al., 2014). DORN1 was experimentally identified in a mutational screen that utilized Ca<sup>2+</sup> responses as a functional readout for purinergic receptor activity.

However, in contrast to plants, about a dozen known P2 receptors can trigger Ca<sup>2+</sup> responses to ATP in animal cells. Identifying novel ATP receptors by gene perturbation and Ca<sup>2+</sup>

measurements amid the large background of known P2 receptors is challenging. In such situations, unbiased biochemical screens with functionalized ATP baits are a powerful tool, because they do not rely on pleiotropic functional readouts, and report on direct, physical interactions.

So far, different nucleotide resins and functionalized ATP probes have been used to identify ATP interactors from complex proteomes (Hanouille et al., 2006; Ito et al., 2006; Mahajan et al., 2015; Patricelli et al., 2007). As previously discussed (Mahajan et al., 2015), site, size, and properties of the functional group(s) attached to ATP biases the interaction profile of the respective probe. Meaningful ATP interaction profiles can only be expected if the probe design does not interfere with ATP's allosteric or enzymatic functions of interest. For example, *N*<sup>6</sup> modifications at the adenine base of ATP hinder probe-binding to protein kinase pockets, whereas modifications to ATP's  $\gamma$ -phosphate are well tolerated by kinases (Patricelli et al., 2007; Shah et al., 1997). Likewise, allosteric ATP binders, such as P2 receptors, prefer certain ATP modifications over others (Burnstock et al., 1994; Fischer et al., 1993). Large functional groups, such as biotin affinity tags (Mahajan et al., 2015), more likely alter ATP binding than small ones; either by not fitting to small pockets, or by enabling non-physiological interactions through their additional hydrophobicity. By contrast, sterically compact additions containing a photo-crosslinking diazirine and a terminal alkyne





**Figure 1. mipATP Reconstitutes Purinergic  $\text{Ca}^{2+}$  Responses *In Vitro* and *In Vivo***

(A) Structure of the minimally invasive photoaffinity ATP probe. Minimalist photo-clickable handle highlighted in blue and red.

(B) Single-cell measurements of extracellular ATP-mediated  $\text{Ca}^{2+}$  signals in HeLa cells expressing a nuclear targeted, ratiometric GCaMP6s transgene (also see Figure S1). Left, boxplot indicating the median frequency (Hz) of  $\text{Ca}^{2+}$  transients per cell upon exposure to 10  $\mu\text{M}$  ATP or ATP analog in HeLa cells expressing a GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS transgene. On each box, the central mark (red) indicates the median, and the bottom and top edges of the box (blue) indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points not considered outliers, and the outliers are plotted with the "+" symbol (red). Right, average  $\text{Ca}^{2+}$  signal of HeLa cells after exposure to 10  $\mu\text{M}$  ATP or ATP analog as determined by the nuclear GCaMP6s/mBeRFP fluorescence ratio. Data represent the mean GCaMP6s/mBeRFP fluorescence ratio and the bottom and top edges of the shaded region indicate the 25th and 75th percentiles, respectively. Data was compiled from (n) number of cells per condition from at least two independent experiments for each nucleotide: ATP, n = 211; ATP $\gamma\text{S}$ , n = 198; mipATP, n = 336; AMP-PNP, n = 104; and vehicle, n = 308. Asterisks, unpaired, two-tailed t tests between the ATP-treated cells versus the indicated ATP analog; \*\*\*\*p < 0.0001; ns, not significant.

(C) Ratiometric imaging of ATP-mediated  $\text{Ca}^{2+}$  transients in zebrafish larvae tail fins (also see Video S1). Left, scheme of experimental setup. Tail fin tips of 2.5–4 days post-fertilization (dpf) transgenic  $\text{Ca}^{2+}$  reporter zebrafish larvae *Tg(krt4:QF2; QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)* are amputated and wounded tail fins are subsequently perfused with a bolus of 1 mM ATP or ATP analog at 2 min into imaging. Right, ratiometric imaging of  $\text{Ca}^{2+}$  transients induced by

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allow for “click” chemistry-mediated conjugation with large affinity tags, such as biotin, after protein-probe complexes have formed. Thereby, the affinity tag cannot inhibit physiological or promote non-physiological ATP interactions. Such probes are increasingly used in biochemical screens for small-molecule targets (Li et al., 2013; Niphakis et al., 2015; Parker et al., 2017a, 2017b), but have not yet been implemented to interrogate the ATP interactome.

This work describes the development and verification of a minimally invasive photoaffinity ATP (mipATP) probe, which mimics physiological signaling functions of extracellular ATP *in vitro* and *in vivo*, and specifically enriches for known and unannotated ATP-interacting proteins from lysates and membrane fractions of cultured A549 lung cancer cells.

## RESULTS

### Design and Verification of a mipATP Probe

A subset of ATP-interacting membrane proteins, including P2Y purinergic receptors and various membrane transporters, are equally activated by, or even prefer *N*<sup>6</sup>-substituted over native ATP (Burnstock et al., 1994; Fischer et al., 1993; Zhou et al., 2005). Their “*N*<sup>6</sup>-tolerant” ATP-binding sites structurally differ from classic protein kinase pockets (Shah et al., 1997), but except for P-loop motifs, they remain little characterized, and are thus hard to predict by bioinformatics. To systematically probe their abundance, we functionalized ATP at its *N*<sup>6</sup> position with a sterically compact combination of a photoreactive diazirine and a terminal alkyne (Figure 1A). The linker stabilizes reversible protein-probe interactions by UV crosslinking. Subsequent ligation with a biotin tag allows for affinity enrichment of ATP-protein complexes and protein identification by mass spectrometry (MS). Similar linkers were previously used to screen for fatty acid-protein interactions and targets of other small molecules (Li et al., 2013; Niphakis et al., 2015; Parker et al., 2017a, 2017b).

An important allosteric function of extracellular ATP is to trigger Ca<sup>2+</sup> transients through G protein-coupled, purinergic receptor signaling (Garritsen et al., 1992; Lustig et al., 1993), which one can measure with genetically encoded biosensors, such as GCaMP6 (Chen et al., 2013). To confirm that our probe functionally reconstitutes allosteric interactions of native ATP, we measured ATP-induced Ca<sup>2+</sup> transients in cells and animals. In HeLa and A549 lung cancer cells expressing a genetically encoded, nuclear-targeted GCaMP6 Ca<sup>2+</sup> sensor, addition of unmodified ATP and the *N*<sup>6</sup>-modified probe to the cell supernatant led to similar Ca<sup>2+</sup> transients (Figures 1B and S1A), whereas the phosphate-modified ATP analogs ATP<sub>γ</sub>S and AMP-PNP, caused little or no Ca<sup>2+</sup> signaling (Figures 1B and S1A). *In vivo* experiments led to the same conclusion; when GCaMP6-expressing zebrafish larvae were perfused with ATP or its analogs through an open wound, the *N*<sup>6</sup>-modified ATP probe evoked the same tissue patterns of Ca<sup>2+</sup> fluxes as native ATP, while phosphate-modified analogs did not (Figures 1C, 1D, and S1B). We

conclude that mipATP minimally interferes with ATP's allosteric ligand functions making it suited to search for the underlying membrane protein interactions.

### mipATP Competes with ATP for Membrane Protein Binding

To test whether mipATP binds to ATP sites on proteins, we incubated total membrane fractions and lysates of A549 cells with varying concentrations of mipATP with or without competing, native ATP. Membrane-bound ATPases rapidly hydrolyze ATP to generate ADP, AMP, and adenosine, which may complicate interpretation. Thus, experiments were performed at 4°C to suppress enzymatic (mip)ATP hydrolysis (Figure S2A).

After UV crosslinking, protein targets were conjugated to a fluorescent tetramethylrhodamine (TAMRA) reporter tag by click chemistry and separated by SDS-PAGE. Protein-linked mipATP complexes were visualized on the gel by fluorescence scanning (Figure S2B). UV crosslinking caused significant target enrichment, suggesting that the probe primarily reports on reversible ATP-protein interactions (Figures 2A, S2C, and S2E). Binding was dose dependent, and densitometry analysis of representative gel bands suggested overall affinities in the low micromolar range, that is, within the physiological response range of known ATP receptors (Figures 2B and S2E) (Garritsen et al., 1992). Equimolar amounts of native ATP or ATP<sub>γ</sub>S quenched mipATP target interactions by half. Thus, mipATP binds target proteins with similar affinity as native ATP (Figures 2C, S2D, and S2E).

### mipATP Maps ATP Ligand Space in the Membrane Proteome

The above results encouraged us to use mipATP to screen for *N*<sup>6</sup>-tolerant ATP interactors on membranes. First, we used a label-free MS strategy to search for protein interactors in total membrane fractions of homogenized A549 cells (Figure S3A; Table S1). Second, we used stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) MS to identify protein mipATP interactors on live A549 cells (Figure S3D; Table S1). Finally, we used SILAC-based MS to profile mipATP targets in cell lysates (Figure S4A; Table S1). In all approaches, native ATP was used as competitive quencher of specific probe interactions.

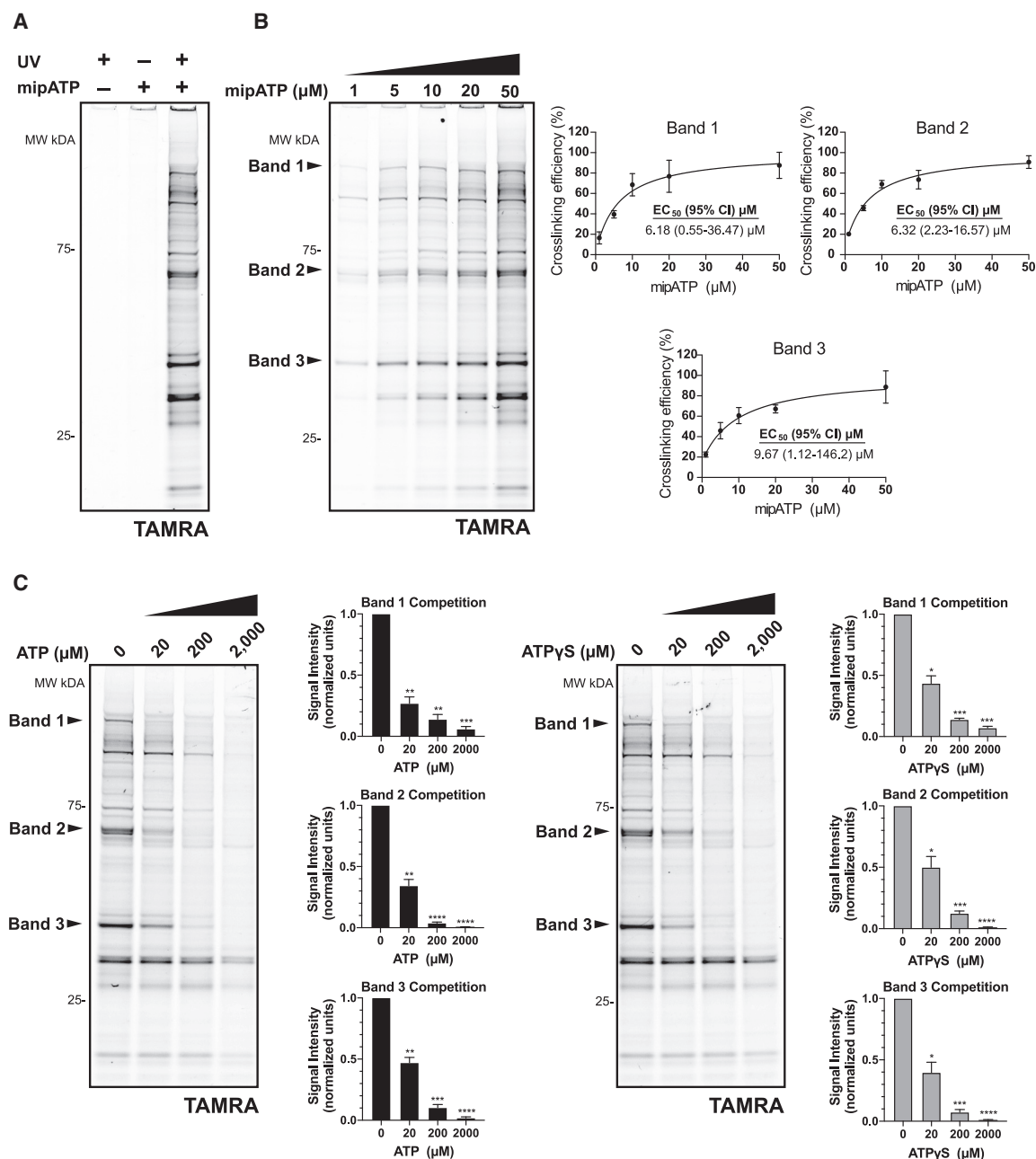
Label-free MS revealed 222 specific ATP interactors in A549 total membrane fractions (Table S1). According to gene ontology (GO) and UniProt database analyses, 123 of these proteins potentially recognize some structural element of the probe, e.g., its sugar, purine, or phosphate moiety (Ashburner et al., 2000; Mi et al., 2019; The Gene Ontology Consortium, 2019). The group contained 59 ATP-, 26 DNA/RNA-, 6 NAD<sup>+</sup>/NADP<sup>+</sup>-, and 6 GTP-binding proteins (Table S1). In addition, mipATP retrieved 99 previously unannotated ATP binders.

To confirm these hits with an orthogonal, quantitative SILAC MS approach, mipATP supplied in extracellular medium was UV-crosslinked onto intact A549 cells in suspension (Figures

perfusion of wounded tail fins with 1 mM ATP or ATP analog. Nuclear GCaMP6s signals are normalized by nuclear mKate2 (mKate2) fluorescence. Scale bars, 50 μm. Time in minutes.

(D) Average spatiotemporal Ca<sup>2+</sup> signal profiles of the indicated (n) number of transgenic Ca<sup>2+</sup> reporter larvae *Tg(krt4:QF2; QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)* after perfusion with 1 mM ATP or ATP analog at 2 min from the start of imaging.

Please also see Figure S1.



**Figure 2. mipATP Reversibly Interacts with A549 Cell Membrane Proteins**

(A) A549 cell membrane fractions were incubated with 50 μM mipATP or vehicle ± UV irradiation (365 nm) before click chemistry with TAMRA-azide. Samples were then separated by SDS-PAGE and gels were scanned for TAMRA fluorescence (25 μg sample per lane). Representative SDS-PAGE in-gel fluorescence scan of three individual experiments.

(B) A549 membrane fractions were incubated with increasing concentrations (1–50 μM) of mipATP then UV-irradiated (365 nm) before click chemistry with TAMRA-azide. Samples were separated by SDS-PAGE and gels were scanned for TAMRA fluorescence (25 μg sample per lane). Left, representative SDS-PAGE in-gel fluorescence scan. Right, analyses of photo-crosslinking efficiencies of mipATP to the selected proteins based on normalized TAMRA fluorescence signal from the isolated bands. Data represent mean ± SEM from three independent experiments.  $EC_{50}$  values (μM) were determined by fitting the data to a dose-response curve. Parentheses, 95% confidence interval (CI). Band 1,  $EC_{50}$  = 6.18 (0.55–36.47) μM; band 2,  $EC_{50}$  = 6.32 (2.23–16.57) μM; band 3,  $EC_{50}$  = 9.67 (1.12–146.2) μM.

(C) A549 membrane fractions were pre-incubated with increasing concentrations of ATP or ATPγS (0, 20, 200, or 2,000 μM) and then probed with 20 μM mipATP. Next, samples were UV-irradiated (365 nm) before click chemistry with TAMRA-azide. Samples were separated by SDS-PAGE and gels were scanned for TAMRA fluorescence (25 μg sample per lane). Left panels, representative SDS-PAGE in-gel fluorescence scans. Right panels, analyses of selected protein TAMRA

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S3B and S3C). In a first, “forward” SILAC experiment, heavy isotope-labeled cells were probed with mipATP, whereas non-labeled cells were probed with mipATP and 100 times excess of competing, native ATP (Figure S3D). In a second, “reverse” SILAC experiment, the isotope labels were swapped between mipATP and mipATP + ATP samples (Li et al., 2012). We affinity-purified the crosslinked proteins from total A549 membrane fractions and combined them into one sample for ratiometric MS analysis. The resulting SILAC ratios are a measure of specific, that is, ATP-quenchable ATP-protein interactions. We ranked these ratios by percentile, and functionally classified hits within the 90<sup>th</sup> percentile by functional clustering (Figures 3A and S3E; Table S1). The two inverted SILAC experiments overlapped on 47 likely ATP binders, 32 of which were previously annotated to likely recognize some structural element of the probe. Those included 21 known ATP-, 2 DNA/RNA-, 4 NAD<sup>+</sup>/NADP<sup>+</sup>-, and 1 GTP-binding protein(s). Fifteen proteins were not previously annotated to bind to nucleotides (Figure 3A; Table S1).

Combining all the data, 73 proteins showed ATP-quenchable mipATP-binding in at least 2 of the 3 MS experiments (label-free MS, forward and reverse SILAC) (Figures 3B and S3F; Table S1). Forty seven of those were predicted ATP/nucleotide binders, leaving 26 previously unannotated, potential ATP interactors. We ranked these hits according to their frequency of appearance in the Contaminant Repository for Affinity Purification (CRAPome), a database for common MS background artifacts (Mellacheruvu et al., 2013). Notably, 32 out of our 73 high-confidence hits had previously come up in SILAC screens using different small-molecule baits (Niphakis et al., 2015; Parker et al., 2017a). This might reflect some propensity of (mip)ATP to undergo pleiotropic target interactions. To focus on likely foreground interactors, we only selected candidates for further consideration that exhibited both a low CRAPome frequency (<5%) and that had not previously been retrieved by other small-molecule baits. Thirty two high-confidence candidates remained after this final filtering, among them 13 previously unannotated, possible ATP interactors (Figure 3C; Table S1). All unannotated mipATP targets are plasma membrane proteins, most of them transporters of the SLC family (SLC35F2, SLC47A1, SLC19A3, SLC22A3, SLC12A9, SLC30A1, SLC12A2). During preparation of this manuscript, SLC19A1 was identified as an immunogenic nucleotide transporter (Luteijn et al., 2019; Ritchie et al., 2019). In addition to SLCs, we found one putative iron-binding, cell surface protein (MELTF), and five signaling/adhesion molecules (FAS, CD44, CESLR1, PTGFRN, ITGA5) as likely ATP interactors.

By contrast, quantitative mipATP-profiling of detergent-solubilized A549 cell lysates primarily retrieved highly abundant, cytosolic ATP binders, such as structural proteins and chaperones (Figures S4B–S4D; Table S1). Nine targets previously enriched by mipATP from membrane fractions also appeared as ATP-quenchable mipATP binders in the cell lysate experiments (Table S1). Eight of them were annotated nucleotide binders, and three of them integral membrane proteins (ABCB6,

ABCC3, SLC12A2). Probe sequestration by highly abundant ATP binders (Table S1) (Thul et al., 2017) possibly contributed to the scarce observation of membrane proteins in the total lysate screens. Removing such overrepresented proteins by membrane (this study) or organelle fractionation appears to be a sensible strategy to enrich for less-represented, lower-affinity binders in proteomics screens using mipATP.

### Select Candidate Validation

For proof-of-principle, we picked three known and three unannotated ATP interactors from our screens for western blot validation.

MRP1 is a multi-specific ATP-binding cassette (ABC) transporter that uses ATP hydrolysis for catalyzing the efflux of organic ions, such as GSH and proinflammatory cysteinyl leukotriene C4, and other lipid mediators (Leier et al., 1994). Western blot analysis confirmed the UV dependency and ATP specificity of mipATP-MRP1 complex formation. Namely, competition with 10-fold excess native ATP and ATP $\gamma$ S blocked the mipATP-MRP1 interaction by ~40% and ~25%, respectively (Figure 4A). These results are in line with previous studies demonstrating that ABC transporters preferentially bind N<sup>6</sup>-substituted ATP analogs over unmodified ATP (Zhou et al., 2005).

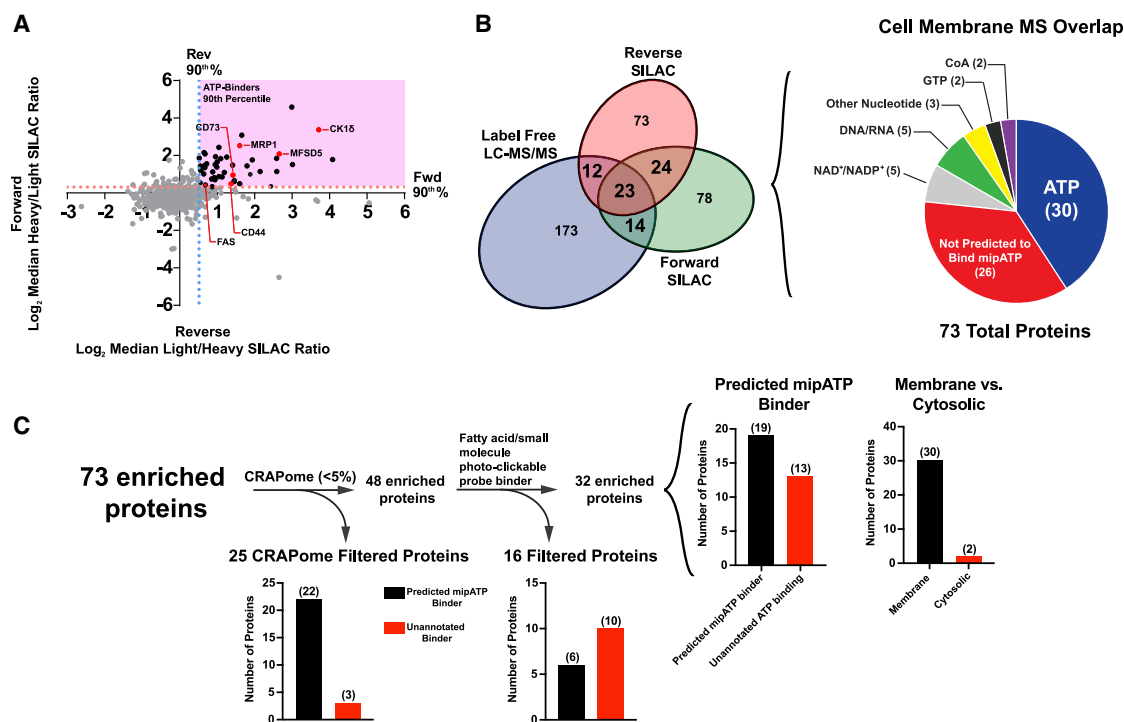
Casein kinase 1 delta (CK1 $\delta$ ) is a serine kinase involved in cell-cycle regulation, DNA damage signaling, and circadian rhythm (Behrend et al., 2000; Etchegaray et al., 2009; Knippschild et al., 1997). CK1 $\delta$  associates with the plasma membrane (Stöter et al., 2005). Interestingly, longdaysin, a small molecule with a N<sup>6</sup>-substituted purine (Hirota et al., 2010) binds and inhibits CK1 $\delta$ . Casein kinase 1 alpha, another longdaysin target, also showed up in our screen. But due to its concomitant appearance in the CRAPome (>5% frequency) and a previous fatty acid interactor screen (Niphakis et al., 2015), we did not classify it as a high-confidence target. Western blot analysis confirmed the UV dependency of CK1 $\delta$ -mipATP complex formation, which was blocked by native ATP and ATP $\gamma$ S (Figure 4A).

Ecto-5'-nucleotidase (CD73) is a glycosylphosphatidylinositol-anchored cell surface enzyme that catalyzes the hydrolysis of extracellular AMP to adenosine. The latter is converted to inosine by adenosine deaminase, imported into cells by nucleoside transporters, or activates P1 G protein-coupled receptors (Antonoli et al., 2013; Knapp et al., 2012). Native ATP and N<sup>6</sup>-substituted nucleotide derivatives (*K*<sub>i</sub>'s in nanomolar and picomolar range) inhibit CD73 activity (Bowman et al., 2019; Grondal and Zimmermann, 1987; Knapp et al., 2012), suggesting that it possesses N<sup>6</sup>-tolerant ATP-binding sites. As expected, our western blot analysis confirmed UV-dependent crosslinking of mipATP to CD73, and an excess of native ATP or ATP $\gamma$ S-blocked mipATP-CD73 interactions (Figure 4A).

Notably, although mipATP reconstituted purinergic Ca<sup>2+</sup> responses (Figures 1 and S1), our screens did not robustly enrich for P2 receptors. Integral membrane proteins are often underrepresented in proteomic studies due to hydrophobic transmembrane domains and low abundance (Speers and Wu, 2007).

fluorescence. The fluorescence intensity value of each band was normalized to the value of the corresponding no competition (0  $\mu$ M) band within the same experiment. Data represent the mean  $\pm$  SEM from three independent experiments. Asterisks, unpaired, two-tailed t tests between the indicated sample and the corresponding no competition (0  $\mu$ M) sample; \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

Please also see Figure S2.



**Figure 3. MS Maps the mipATP Membrane Interactome**

(A) Intact A549 cells were probed with 50  $\mu$ M mipATP alone or in the presence of 100 times excess of unmodified ATP. Labeled proteins were identified by SILAC-based quantitative proteomics. ATP-quenchable mipATP-protein interactions were ranked according to SILAC ratios. Plot of  $\log_2$  values of the SILAC ratios for proteins identified in the “forward” and “reverse” experiments. Dashed lines mark the 90th percentile threshold ratio values. Proteins with SILAC ratios in the 90th percentile of both forward and reverse experiments were considered high-confidence mipATP interactors and are indicated in the top right quadrant highlighted as black dots in a purple background. Data are representative of one forward and one reverse experiment. Please also see [Figures S3D and S3E](#) and [Table S1](#). (B) Left, 73 proteins were identified as high-confidence mipATP interactors in at least two of three MS experiments ([Micallef and Rodgers, 2014](#)). Right, classification of the high-confidence mipATP interactors based on their predicted likelihood of binding mipATP according to GO and UniProt database analyses. Please also see [Figure S3F](#) and [Table S1](#).

(C) High-confidence interactors identified in  $\geq 5\%$  of CRAPome database experiments and/or similar proteomic studies using fatty acid and small-molecule photo-clickable probes ([Niphakis et al., 2015](#); [Parker et al., 2017a](#)) were filtered out resulting in 32 high-confidence mipATP binders. Please also see [Table S1](#).

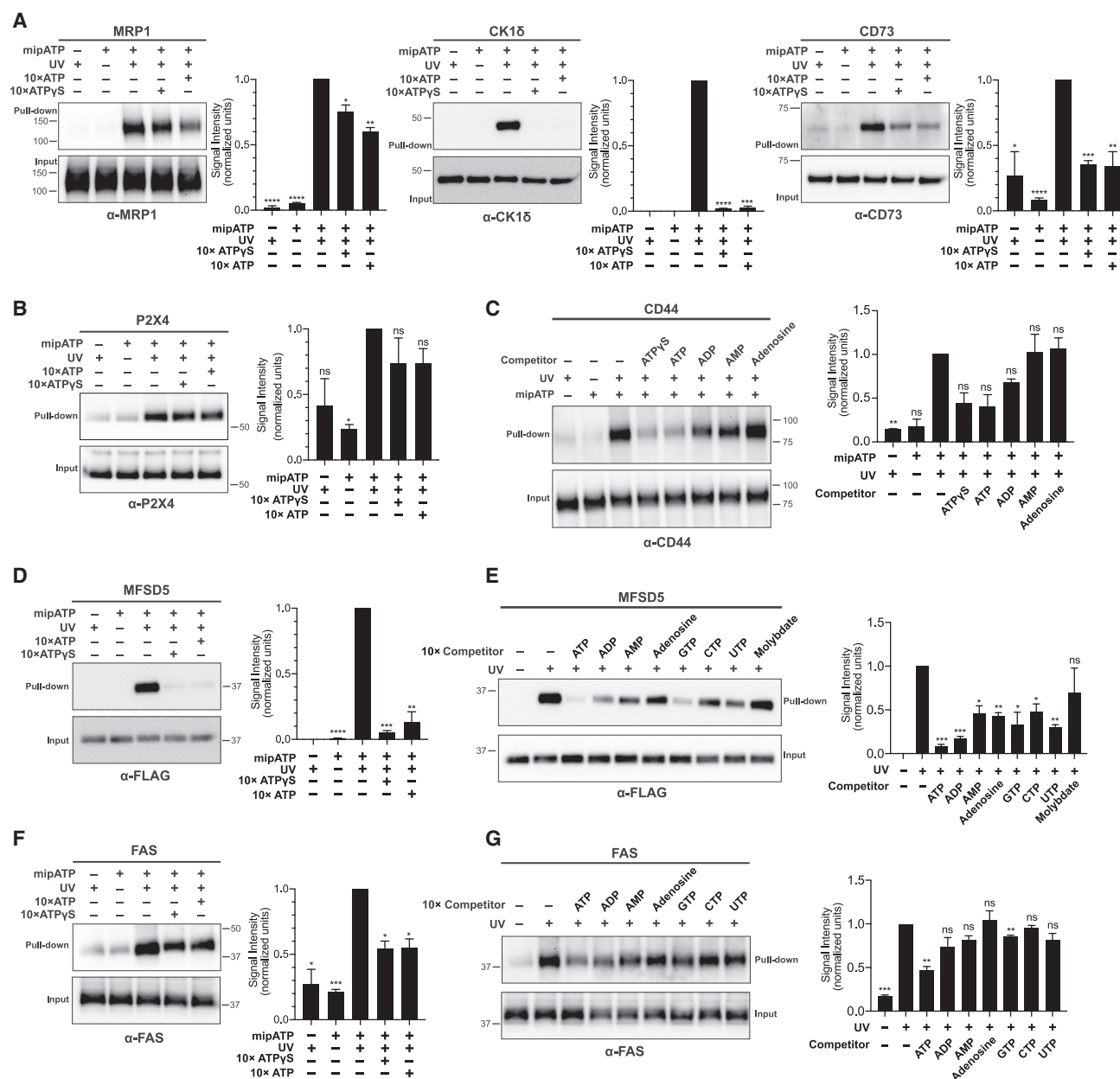
Western blot experiments confirmed the interaction of mipATP with ionotropic P2X4 but not P2Y2 receptors ([Figures 4B and S3G](#)), which are both expressed by A549 cells. This could indicate that mipATP-P2Y2 receptor interactions are weak under the experimental conditions. Lack of P2Y2 receptor identification in our SILAC MS screens may also be the product of agonist-induced desensitization, a process well described for G protein-coupled receptors, such as P2Y2 ([Flores et al., 2005](#)), which could cause receptor internalization and subsequent mipATP inaccessibility, leading to a reduced overall crosslinking efficiency.

Next, we set out to validate three interesting, unannotated ATP targets from our screens. CD44 is a cell surface adhesion receptor involved in a variety of biological processes, including cell migration, lymphocyte activation and homing, and tumor progression and metastasis ([DeGrendele et al., 1997](#); [Günther et al., 1991](#); [Haynes et al., 1989](#); [Liu et al., 2011](#)). CD44 directly binds multiple ligands, including hyaluronic acid, osteopontin, collagens, and matrix metalloproteinases ([Aruffo et al., 1990](#); [Kabayama et al., 2005](#); [Mori et al., 2002](#); [Weber et al., 1996](#)). Western blot analysis confirmed a CD44-mipATP interaction ([Figure 4C](#)). Competition with excess ATP or ATP $\gamma$ S, and to a lesser extent ADP, but not AMP or adenosine blocked CD44

binding to mipATP, highlighting the importance of the phosphate groups for CD44-mipATP binding.

The major facilitator superfamily domain-containing protein 5 (MFSD5) is an atypical solute carrier (SLC) localized to the plasma membrane. It mediates the cellular uptake of molybdate ions ([Tejada-Jiménez et al., 2011](#)). MFSD5 was enriched in all our proteomics experiments, but also appeared in preceding small-molecule target screens ([Niphakis et al., 2015](#); [Parker et al., 2017a](#)). Western blotting confirmed that recombinant MFSD5 directly binds to mipATP. The interaction was UV-dependent and almost completely abolished by excess ATP or ATP $\gamma$ S ([Figure 4D](#)). Various nucleotide triphosphates and ATP hydrolysis products were also capable of blocking the interaction with an apparent rank-order of ATP > ADP > GTP  $\geq$  UTP > AMP  $\sim$  adenosine  $\sim$  CTP, pointing to the importance of the phosphates and adenine base for MFSD5-ATP interactions ([Figure 4E](#)).

Intriguingly, our screen also enriched for the FAS receptor, a cell surface death receptor that triggers apoptosis upon ligation to the FAS ligand ([Itoh et al., 1991](#); [Suda et al., 1993](#)). It is well known that FAS-stimulated apoptosis depends on intracellular ATP, and extracellular ATP triggers apoptosis of various cell types ([Gandelman et al., 2013](#); [Leist et al., 1997](#); [Li et al.,](#)



**Figure 4. Verification of Known and Unannotated ATP Interactors**

(A) Verification of mipATP interactions with high-confidence mipATP targets known to bind ATP. Isolated A549 membrane fractions were probed with 10  $\mu$ M mipATP after preincubation with 100  $\mu$ M ATP $\gamma$ S or ATP competitor or vehicle  $\pm$  UV irradiation (365 nm) before click chemistry with biotin-azide. Biotin-conjugated proteins were purified with streptavidin agarose and separated by SDS-PAGE. MRP1- (left), CK1 $\delta$ - (middle), and CD73-mipATP (right) complexes were detected by western blot with anti-MRP1, anti-CK1 $\delta$ , and anti-CD73 antibodies, respectively. Left panels, representative western blots. Inputs are from 5  $\mu$ g of membrane protein extract before pull-down. Right panels, analyses of protein-mipATP complex signal intensities normalized to the value of the corresponding complex in the absence of competition + UV irradiation within the same experiment. Data represent mean  $\pm$  SEM of at least three independent experiments (four for MRP1 and CD73).

(B) Western blot with anti-P2X4 antibody detects P2X4-mipATP complexes purified from isolated A549 membrane fractions probed with mipATP (10  $\mu$ M) in the presence of the indicated ATP derivative (100  $\mu$ M) or vehicle  $\pm$  UV. Right, analysis of P2X4-mipATP signal intensities. P2X4-mipATP signal values were normalized to the value of the complex in the absence of competition + UV within the same experiment. Data represent mean  $\pm$  SEM of two independent experiments.

(C) Western blot with anti-CD44 antibody detects CD44-mipATP complexes purified from isolated A549 membrane fractions probed with mipATP (10  $\mu$ M) in the presence of the indicated competitor (100  $\mu$ M) or vehicle  $\pm$  UV. Right, analysis of CD44-mipATP signal intensity values normalized to the value of the complex in the absence of competition + UV within the same experiment. Data represent mean  $\pm$  SEM of two independent experiments.

(D) Membrane fractions from A549 cells stably expressing recombinant MFSD5-FLAG were probed with mipATP (10  $\mu$ M) in the presence of the indicated ATP derivative (100  $\mu$ M) or vehicle  $\pm$  UV. Purified MFSD5-mipATP complexes were detected by western blot with an anti-FLAG antibody. Right, analysis of

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1997). Western blotting confirmed the UV-dependent labeling of FAS by mipATP. The interaction was blocked ~40%–50% by excess ATP and ATP $\gamma$ S and to a lesser extent by ADP (Figures 4F and 4G). By contrast, AMP or adenosine, or other nucleotide triphosphates with varying bases (GTP, CTP, and UTP) had no effect. Again, this underlines the importance of ATP's phosphate and nucleic acid moieties for this interaction (Figure 4G).

## DISCUSSION

To identify small-molecule targets, chemical proteomics have become an interesting alternative/complement to functional genomics. Such strategies have identified ATP-interacting proteins from human cell lines, plants, and parasitic organisms, including *Mycobacterium tuberculosis* (Adachi et al., 2014; Ansong et al., 2013; Mahajan et al., 2015; Patricelli et al., 2007; Villamor et al., 2013). To this end, ATP probes are commonly functionalized at the  $\gamma$ -phosphate, favoring the identification of enzymatic over allosteric ATP-binding sites, or contain physiologically invasive, bulky hydrophobic additions. To illuminate underexplored regions of the ATP-binding landscape, we thus developed and characterized mipATP, which contains a very compact, photo-clickable linker at the  $N^6$  position of its adenine base (Li et al., 2013). Importantly, mipATP faithfully mimics allosteric calcium signaling responses of extracellular ATP in cells and tissues, unlike commonly used  $\gamma$ -phosphate-modified ATP derivatives, and is thus physiologically less invasive.

In this study, we focused on ATP-interacting membrane proteins. Besides the known panel of P2 receptors, those likely comprise additional unannotated ATP-regulated signaling proteins. Underlining the robustness of our screen, most of our hits were previously known nucleotide-interacting membrane proteins, such as transporters, ATPases, and others. Demonstrating the explorative value of mipATP, we identified and confirmed various ATP interactors in prominent cellular signaling pathways, such as the FAS receptor and CD44 (Haynes et al., 1989; Itoh et al., 1991).

For all tested targets, our experiments suggest binding affinities in the ~10–100 micromole range. Except for CD73, which has no intracellular domain, the location of the nucleotide-binding sites remains uncertain. If these sites were extracellular, they should be unoccupied under basal conditions, because extracellular steady-state concentrations of nucleotides are extremely low (Schwiebert and Zsembery, 2003). Upon tissue

damage, however, extracellular [ATP] may temporally rise into the micromolar to millimolar range to support allosteric regulation or enzymatic activity. Conversely, if these sites were intracellular, they should always be occupied amid millimolar cytoplasmic [ATP]. However, after severe homeostatic perturbation, for example, during cell death, low intracellular ATP levels will cause dissociation of micromolar affinity interactions. Surveillance of cytoplasmic nucleotide concentrations, for example, by AMPK or the apoptosome, crucially regulates energy metabolism or cell death decisions, respectively (Hardie et al., 2012; Li et al., 1997).

We were intrigued to find the FAS receptor among our high-confidence ATP interactors. Despite being at the top of the apoptotic cascade FAS' direct regulation by nucleotides, to our knowledge, has not been reported. Our result raises the possibility that ATP directly regulates cell death fate decisions at the death receptor level.

A larger  $N^6$ -functionalized ATP photoaffinity probe (AdoR) (Mahajan et al., 2015) consistently enriched highly abundant cytosolic ATP binders from cell extracts, similar to mipATP. Nineteen of the top 50 proteins enriched by AdoR were known ATP binders. In comparison, 34 of the 40 enriched mipATP targets in lysates are known ATP binders. Furthermore, mipATP labeled protein classes not strongly enriched by AdoR, such as transporters and helicases. In conclusion, mipATP improves upon existing ATP probes by highlighting novel ATP interaction space, while retaining ATP's allosteric signaling functions.

## SIGNIFICANCE

**Adenosine 5'-triphosphate (ATP) modulates cell signaling as an allosteric effector and ligand through non-catalytic interactions with proteins at sites that can follow unclear structural rules and are difficult to identify by computational prediction. Functionalized ATP probes are powerful tools to biochemically identify ATP-binding proteins in complex proteomic mixtures; however, the current set of probes rely on structurally conserved ATP-binding sites and/or contain bulky functional groups that can interfere with ATP-protein interactions. We developed and verified a compact ATP photoaffinity probe that retains the signaling functions of native ATP *in vivo* and *in vitro*. Photo-crosslinking and proteomic screening identified ATP-quenchable probe-binding proteins in cell membranes and lysates. In addition to robust enrichment of known**

MFSD5-mipATP signal intensity values normalized to the value of the complex in the absence of competition + UV within the same experiment. Data represent mean  $\pm$  SEM of three independent experiments.

(E) Membrane fractions from A549 cells stably expressing recombinant MFSD5-FLAG were probed with mipATP (10  $\mu$ M) in the presence of the indicated competitor (100  $\mu$ M)  $\pm$  UV. Purified MFSD5-mipATP complexes were detected by western blot with an anti-FLAG antibody. Right, analysis of MFSD5-mipATP signal intensity values normalized to the value of the complex in the absence of competition + UV within the same experiment. Data represent mean  $\pm$  SEM of three independent experiments.

(F) Western blot with anti-FAS antibody detects FAS-mipATP complexes purified from isolated A549 membrane fractions probed with mipATP (10  $\mu$ M) in the presence of the indicated ATP derivative (100  $\mu$ M) or vehicle  $\pm$  UV. Right, analysis of FAS-mipATP signal intensities normalized to the value of the complex in the absence of competition + UV within the same experiment. Data represent mean  $\pm$  SEM of three independent experiments.

(G) Western blot with anti-FAS antibody detects FAS-mipATP complexes purified from isolated A549 membrane fractions probed with mipATP (10  $\mu$ M) in the presence of the indicated nucleotide/nucleoside (100  $\mu$ M)  $\pm$  UV. Right, analysis of FAS-mipATP signal intensities normalized to the value of the complex in the absence of competition + UV within the same experiment. A lane for molybdate competition (right of the UTP competition lane) was removed from the blot image because it is irrelevant to this dataset. Data represent mean  $\pm$  SEM of three independent experiments.

All inputs are from 5  $\mu$ g of solubilized membrane protein extract before pull-down. Please also see Figure S3G. Asterisks, unpaired, two-tailed t tests between the indicated sample and the corresponding no competition + UV irradiation sample; \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001; ns, not significant.

nucleotide binders, profiling of cell membranes consistently enriched for proteins that are not predicted by database annotations to bind nucleotides, some of which we further validated as ATP-quenchable probe binders by western blotting. In summary, we provide proof-of-principle that this compact ATP photoaffinity probe can be used to map ATP-protein interaction space using proteomic screens.

## STAR★METHODS

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## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.chembiol.2020.05.010>.

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## AUTHOR CONTRIBUTIONS

M.J. and P.N. conceived the project. M.J. conceived the ATP probe structure, designed and performed the experiments, analyzed data, and prepared the figures. K.W. and X.-C.C. conceived the synthesis of and synthesized the ATP probe under the supervision of M.L. K.L.H. designed and generated the stable calcium reporter cell lines and transgenic zebrafish, wrote the MATLAB code to analyze the *in vitro* calcium signals, and analyzed the data. B.E. designed and constructed recombinant DNA for transgenesis. M.J. and P.N. wrote the paper.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE                                        | SOURCE                               | IDENTIFIER                       |
|------------------------------------------------------------|--------------------------------------|----------------------------------|
| <b>Antibodies</b>                                          |                                      |                                  |
| Rabbit monoclonal anti-MRP1/ABCC1                          | Cell Signaling                       | Cat#14685S; RRID: AB_2798572     |
| Rabbit polyclonal anti-CK1δ                                | Bethyl Laboratories                  | Cat#A302-136A; RRID: AB_1720340  |
| Rabbit monoclonal anti-CD73/NT5E                           | Cell Signaling                       | Cat#13160S; RRID: AB_2716625     |
| Rabbit polyclonal anti-CD44                                | Cell Signaling                       | Cat#3578S; RRID: AB_2076463      |
| Mouse monoclonal anti-FLAG M2                              | Sigma-Aldrich                        | Cat#F1804; RRID: AB_262044       |
| Rabbit monoclonal anti-FAS                                 | Abcam                                | Cat#ab133619                     |
| Rabbit monoclonal anti-P2X4                                | Cell Signaling                       | Cat#70659; RRID: AB_2799789      |
| Rabbit polyclonal anti-P2Y2                                | Novus Biologicals                    | Cat#NB110-39032; RRID: AB_791447 |
| Anti-rabbit IgG, HRP-linked                                | Cell Signaling                       | Cat#7074; RRID: AB_2099233       |
| Anti-mouse IgG, HRP-linked                                 | Cell Signaling                       | Cat#7076; RRID: AB_330924        |
| <b>Bacterial and Virus Strains</b>                         |                                      |                                  |
| NEB® 5-alpha Competent <i>E. coli</i> (High Efficiency)    | NEB                                  | Cat#C2987H                       |
| One Shot™ TOP10 Chemically Competent <i>E. coli</i>        | Thermo Fisher Scientific             | Cat#C404010                      |
| One Shot™ ccdB Survival™ 2 T1 <sup>R</sup> Competent Cells | Thermo Fisher Scientific             | Cat#A10460                       |
| <b>Chemicals, Peptides, and Recombinant Proteins</b>       |                                      |                                  |
| L-Arginine:HCl (13C6, 99%; 15N4, 99%)                      | Cambridge Isotope Laboratories, Inc. | Cat#CNLM-539-H                   |
| L-Lysine:2HCl (13C6, 99%; 15N2, 99%)                       | Cambridge Isotope Laboratories, Inc. | Cat#CNLM-291-H                   |
| L-Arginine monohydrochloride                               | Sigma-Aldrich                        | Cat#A6969                        |
| L-Lysine monohydrate                                       | Sigma-Aldrich                        | Cat#L9037                        |
| Ethyl 3-aminobenzoate methanesulfonate                     | Sigma-Aldrich                        | Cat#E10521                       |
| 6-Chloropurine riboside-5'-triphosphate                    | BioLog                               | Cat#C 049                        |
| Adenosine 5'-triphosphate disodium salt hydrate            | Sigma-Aldrich                        | Cat#A26209                       |
| Adenosine 5'-(β,γ-imido)triphosphate lithium salt hydrate  | Sigma-Aldrich                        | Cat#A2647                        |
| ATPγS tetralithium salt                                    | Tocris                               | Cat#4080                         |
| mipATP                                                     | This paper                           | N/A                              |
| Adenosine 5'-diphosphate sodium salt                       | Sigma-Aldrich                        | Cat#A2754                        |
| Adenosine 5'-monophosphate sodium salt                     | Sigma-Aldrich                        | Cat#A1752                        |
| Adenosine                                                  | Sigma-Aldrich                        | Cat#A9251                        |
| Guanosine 5'-triphosphate sodium salt hydrate              | Sigma-Aldrich                        | Cat#G8877                        |
| Cytidine 5'-triphosphate disodium salt                     | Sigma-Aldrich                        | Cat#C1506                        |
| Uridine 5'-triphosphate trisodium salt hydrate             | Sigma-Aldrich                        | Cat#U6750                        |
| Sodium molybdate                                           | Sigma-Aldrich                        | Cat#243655                       |
| Low Melt Agarose                                           | Gold Biotechnology                   | Cat#A-204-25                     |
| cComplete™, EDTA-free Protease Inhibitor Cocktail          | Sigma-Aldrich                        | Cat#11873580001                  |
| TAMRA Azide                                                | Click Chemistry Tools                | Cat#AZ109                        |
| Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)         | Sigma Aldrich                        | Cat#C4706                        |
| Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA)   | Cayman Chemical                      | Cat#18816                        |
| Copper(II) sulfate pentahydrate                            | Sigma-Aldrich                        | Cat#203165                       |

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### Continued

| REAGENT or RESOURCE                                                                                                                                             | SOURCE                             | IDENTIFIER                   |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------|------------------------------|
| Biotin Azide                                                                                                                                                    | Click Chemistry Tools              | Cat#1265                     |
| Dde Biotin Azide                                                                                                                                                | Click Chemistry Tools              | Cat#1136                     |
| Diazo Biotin Azide                                                                                                                                              | Click Chemistry Tools              | Cat#1041                     |
| cOmplete™ Protease Inhibitor Cocktail                                                                                                                           | Sigma-Aldrich                      | Cat#11697498001              |
| Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA)                                                                                                        | Sigma-Aldrich                      | Cat#678937                   |
| Sodium Hydrosulfite                                                                                                                                             | Sigma-Aldrich                      | Cat#157953                   |
| Iodoacetamide                                                                                                                                                   | Sigma-Aldrich                      | Cat#11149                    |
| Hydrazine monohydrate                                                                                                                                           | Sigma-Aldrich                      | Cat#207942                   |
| Trichloroacetic acid (TCA)                                                                                                                                      | Sigma-Aldrich                      | Cat#T6399                    |
| Coomassie Brilliant Blue R-250                                                                                                                                  | Bio-Rad                            | Cat#1610436                  |
| Critical Commercial Assays                                                                                                                                      |                                    |                              |
| Luminescent ATP Detection Assay Kit                                                                                                                             | Abcam                              | Cat#ab113849                 |
| Pierce™ BCA Protein Assay Kit                                                                                                                                   | Thermo Fisher Scientific           | Cat#23227                    |
| Deposited Data                                                                                                                                                  |                                    |                              |
| Label-free and SILAC MS data identifying targets of mipATP in A549 cell membranes and lysates                                                                   | This paper                         | <a href="#">Table S1.</a>    |
| Experimental Models: Cell Lines                                                                                                                                 |                                    |                              |
| Human: A549 cells                                                                                                                                               | ATCC                               | Cat#CCL-185; RRID: CVCL_0023 |
| Human: HeLa cells                                                                                                                                               | ATCC                               | Cat#CCL-2; RRID: CVCL_0030   |
| Human: A549 pSBbi-GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS-Pur stable cell line                                                                                           | This paper                         | N/A                          |
| Human: HeLa pSBbi-GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS-Pur stable cell line                                                                                           | This paper                         | N/A                          |
| Human: A549 pSBbi-MFSD5-FLAG-Pur stable cell line                                                                                                               | This paper                         | N/A                          |
| Experimental Models: Organisms/Strains                                                                                                                          |                                    |                              |
| Zebrafish: <i>casper</i>                                                                                                                                        | <a href="#">White et al., 2008</a> | N/A                          |
| Zebrafish: <i>Tg(krt4:QF2): mk111Tg</i>                                                                                                                         | This paper                         | <i>mk111Tg</i>               |
| Zebrafish: <i>Tg(QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS): mk112Tg</i>                                                                                              | This paper                         | <i>mk112Tg</i>               |
| Zebrafish: <i>Tg(krt4:QF2; QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS): mk113Tg</i>                                                                                    | This paper                         | <i>mk113Tg</i>               |
| Oligonucleotides                                                                                                                                                |                                    |                              |
| Forward primer for MFSD5-FLAG cloning into pSB-CMV-Puro from A549 cDNA <u>NheI</u> :<br>5'- agtgcgtagcgccaccATGCTGGTGACTGCCTACCTT-3'                            | This paper                         | N/A                          |
| Reverse primer for MFSD5-FLAG cloning into pSB-CMV-Puro from A549 cDNA <u>NotI</u> :<br>5'- atcgggccgcCTACTTGTCGTCATCGTCTT<br>TGTAGTCGGCTCCCAGCTCAGGGGCATAGG-3' | This paper                         | N/A                          |
| Forward primer for MFSD5-FLAG cloning into pSBbi-Pur from pSB-CMV-Puro-MFSD5-FLAG, <u>SfiI</u> : 5'- agtggcctctgagggccgcccaccATGCTGGTGACT-3'                    | This paper                         | N/A                          |
| Reverse primer for MFSD5-FLAG cloning into pSBbi-Pur from pSB-CMV-Puro-MFSD5-FLAG, <u>SfiI</u> : 5'- agtggcctgacaggccCTACTTGTCGTCATCGTCTTGTAGT-3'               | This paper                         | N/A                          |

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## Continued

| REAGENT or RESOURCE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | SOURCE                                   | IDENTIFIER                                                                                                                                                                                                                                                                              |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| mBeRFP gblock® (Integrated DNA Technologies (IDT)):<br>GTGTCTGAGCTGATTAAGGAGAACATGCACATGAAGC<br>TGTACATGGAGGGCACCGTGAACAACCACTTCAA<br>GTGCACATCCGAGGGCGAAGGCAAGCCCTACGAGGG<br>CAGCCAGACCATGAGAATCAAGGTGGTCGAGGGCGG<br>CCCTCTCCCCTTCGCCTTCGACATCCTGGCTACCAGC<br>TTCATGTACGGCAGCAAAACCTTCATCAACCACACCC<br>AGGGCATCCCCGACTTCTTTAAGCAGTCCTTCCCTGA<br>GGGCTTCACATGGGAGAGAAGCACCATACGAAGA<br>CGGGGGCGTGCTGACCGCTACCCAGGACACAGCC<br>TCCAGGACGGCTGCCTCATCTACAACGTCAAGATCA<br>GAGGGGTGAATTCCCATCCAACGGCCCTGTGATGC<br>AGAAGAAAACACTCGCTGGGAGGCCTCCACCGAGA<br>TGCTGTACCCCGCTGACGGCGGCCTGGAAGGCAGA<br>GATTACATGGCCCTGAAGCTCGTGGGCGGGGCCAC<br>CTGATCTGCAACGCAAGACACATACAGATCCAAGA<br>AACCCGCTAAGAACCTCAAGATGCCCGGCGTCTACTA<br>TGTGGACAGAAGACTGGAAAGAATCAAGGAGGCCGAC<br>AAAGAGACCAGCGTCGAGCAGCAGAGGTGGCTGTG<br>GCCAGATACTGCGACCTCCCTAGCAAACTGGGGCACAAG | This paper                               | N/A                                                                                                                                                                                                                                                                                     |
| Recombinant DNA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                          |                                                                                                                                                                                                                                                                                         |
| pME-QF2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Unpublished, Marnie Halpern Lab; Addgene | Cat#83307; RRID: Addgene_83307                                                                                                                                                                                                                                                          |
| p5E- <i>krt4</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Gong et al., 2002                        | N/A                                                                                                                                                                                                                                                                                     |
| p3E-polyA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Kwan et al., 2007; Tol2kit               | Tol2kit plasmid #302                                                                                                                                                                                                                                                                    |
| pDestTol2CG2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Kwan et al., 2007; Tol2kit               | Tol2kit plasmid #395                                                                                                                                                                                                                                                                    |
| pDONR221-GCaMP6s-3xNLS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Enyedi et al., 2016                      | N/A                                                                                                                                                                                                                                                                                     |
| p5E-QUAS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Unpublished, Marnie Halpern Lab; Addgene | Cat#61374; RRID: Addgene_61374                                                                                                                                                                                                                                                          |
| p3E-P2A-mKate2-3xNLS-polyA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | Enyedi et al., 2016                      | N/A                                                                                                                                                                                                                                                                                     |
| pDestTol2CmKate2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | This paper                               | N/A                                                                                                                                                                                                                                                                                     |
| <i>Tg(krt4:QF2)</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | This paper                               | N/A                                                                                                                                                                                                                                                                                     |
| <i>Tg(QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | This paper                               | N/A                                                                                                                                                                                                                                                                                     |
| pSBbi-Pur                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Kowarz et al., 2015; Addgene             | Cat#60523; RRID: Addgene_60523                                                                                                                                                                                                                                                          |
| pSBbi-GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS-Pur                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | This paper                               | N/A                                                                                                                                                                                                                                                                                     |
| pSBbi-MFSD5-FLAG-Pur                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | This paper                               | N/A                                                                                                                                                                                                                                                                                     |
| Software and Algorithms                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                          |                                                                                                                                                                                                                                                                                         |
| ImageJ/Fiji                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Schindelin et al., 2012                  | <a href="https://fiji.sc/">https://fiji.sc/</a>                                                                                                                                                                                                                                         |
| Anaconda Python 2.7                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | Anaconda Inc., USA                       | <a href="https://www.anaconda.com">https://www.anaconda.com</a>                                                                                                                                                                                                                         |
| MATLAB R2018a                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Mathworks Inc., USA                      | <a href="https://www.mathworks.com/">https://www.mathworks.com/</a>                                                                                                                                                                                                                     |
| GraphPad Prism 8                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | GraphPad Software                        | <a href="https://www.graphpad.com/scientific-software/prism">https://www.graphpad.com/scientific-software/prism</a>                                                                                                                                                                     |
| Image Lab V 5.2 Build 14                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Bio-Rad                                  | <a href="https://www.bio-rad.com/en-us/product/image-lab-software">https://www.bio-rad.com/en-us/product/image-lab-software</a>                                                                                                                                                         |
| ImageQuant TL 8.1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | GE Healthcare                            | <a href="https://www.gelifesciences.com/en-us/shop/protein-analysis/molecular-imaging-for-proteins/imaging-software/imagequant-tl-8-1-p-00110">https://www.gelifesciences.com/en-us/shop/protein-analysis/molecular-imaging-for-proteins/imaging-software/imagequant-tl-8-1-p-00110</a> |
| PANTHER Classification System                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Mi et al., 2019                          | <a href="http://pantherdb.org/">http://pantherdb.org/</a>                                                                                                                                                                                                                               |
| Other                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                          |                                                                                                                                                                                                                                                                                         |
| UVP Blak-Ray Display Lamp 115V; 20 W, 365 nm                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Analytik Jena US                         | Cat#95-0045-04                                                                                                                                                                                                                                                                          |
| 96 Well glass bottom plate #1.5 cover glass                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Cellvis                                  | Cat#P96-1.5H-N                                                                                                                                                                                                                                                                          |
| Zeba™ Spin Desalting Columns, 7K MWCO, 2 mL                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Thermo Fisher Scientific                 | Cat#89889                                                                                                                                                                                                                                                                               |

(Continued on next page)

**Continued**

| REAGENT or RESOURCE                                    | SOURCE                   | IDENTIFIER    |
|--------------------------------------------------------|--------------------------|---------------|
| Zeba™ Spin Desalting Columns, 7K MWCO, 5 mL            | Thermo Fisher Scientific | Cat#89891     |
| Pierce™ High Capacity Streptavidin Agarose             | Thermo Fisher Scientific | Cat#20357     |
| Amersham ECL Western Blotting Detection Reagent        | GE Healthcare            | Cat#RPN2106   |
| Amersham ECL Select Western Blotting Detection Reagent | GE Healthcare            | Cat#RPN2235   |
| Colloidal Blue Staining Kit                            | Thermo Fisher Scientific | Cat#LC6025    |
| Protein LoBind Tubes                                   | Eppendorf                | Cat#022431081 |
| NuPAGE™ 4–12%, Bis-Tris Protein Gels                   | Thermo Fisher Scientific | Cat#NP0322BOX |
| NuPAGE™ 10%, Bis-Tris Protein Gels                     | Thermo Fisher Scientific | Cat#NP0301BOX |
| Ultracel® -3K 0.5 centrifugal filter unit              | Millipore                | Cat#UFC500324 |
| Lipofectamine 3000 reagent                             | Thermo Fisher Scientific | Cat#L300015   |
| DMEM for SILAC                                         | Thermo Fisher Scientific | Cat#88364     |
| GlutaMAX™                                              | Thermo Fisher Scientific | Cat#35050061  |
| NuPAGE™ LDS Sample Buffer (4X)                         | Thermo Fisher Scientific | Cat#NP0007    |
| NuPAGE™ Sample Reducing Agent (10X)                    | Thermo Fisher Scientific | Cat#NP0009    |
| Corning™ Fetal Bovine Serum, Premium (Dialyzed)        | Corning                  | Cat#35071CV   |
| Corning™ Heat Inactivated Regular Fetal Bovine Serum   | Corning                  | Cat#35011CV   |
| Micro Knife – Needle Blade/14.5 cm                     | Fine Science Tools       | Cat#10318-14  |
| LR Clonase™ II Plus enzyme                             | Thermo Fisher Scientific | Cat#12538120  |

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources and reagents should be directed to the Lead Contact, Philipp Niethammer ([niethamp@mskcc.org](mailto:niethamp@mskcc.org)).

### Materials Availability

Materials and reagents are available from the authors upon reasonable request. Note, mipATP is an experimental compound that has been synthesized in limited quantities by M.L.'s laboratory for this study which could be restrictive for further distribution.

### Data and Code Availability

Datasets generated during this study are provided in separate spreadsheets as part of [Table S1](#). Code generated during this study is provided in [Data S2](#).

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### General Zebrafish Procedures

Adult and larval wild-type and transgenic *casper* ([White et al., 2008](#)) zebrafish were maintained as described ([Nüsslein-Volhard and Dahm, 2002](#)) and experiments were carried out according to institutional animal healthcare guidelines and protocols with the approval of the MSKCC Institutional Animal Care and Use Committee (IACUC). Zebrafish larvae were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, and 0.33 mM MgSO<sub>4</sub>) at 28.5°C. For tail fin tip amputation experiments, 2.5–4 days post-fertilization (2.5–4 dpf) *Tg(krt4:QF2; QUAS:GCAMP6s-3xNLS-P2A-mKate2-3xNLS)* (*mk113Tg*) larvae were anaesthetized in E3 medium containing 0.2 mg/mL ethyl 3-aminobenzoate methanesulfonate (Sigma; #E10521). Sex was indeterminate at the stages of development (2.5–4 dpf) that were used in all experiments with zebrafish larvae.

### Cell Culture

A549 (male human origin) and HeLa (female human origin) cells were both maintained in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS, Corning; #35011CV), penicillin (100 U/mL), streptomycin (100 µg/mL), and 2 mM L-alanyl-L-glutamine dipeptide (GlutaMAX™, Thermo Fisher Scientific; #35050061) in a 5% humidified CO<sub>2</sub> incubator at 37°C. For SILAC experiments, A549 cells were passaged at least six times in SILAC DMEM (Thermo Fisher Scientific; #88364) lacking L-lysine and L-arginine and supplemented with 10% dialyzed FBS (Corning; #35071CV), P/S/Q (as above), and either L-arginine-<sup>13</sup>C<sub>6</sub>-<sup>15</sup>N<sub>4</sub> hydrochloride (87.8 µg/mL) (Cambridge Isotope Laboratories; #CNLM-539-H) and L-lysine-<sup>13</sup>C<sub>6</sub>-<sup>15</sup>N<sub>2</sub> dihydrochloride (181.2 µg/mL) (Cambridge Isotope Laboratories; #CNLM-291-H) or L-arginine hydrochloride (84 µg/mL) (Sigma; #A6969) and L-lysine monohydrate (131 µg/mL) (Sigma; #L9037).

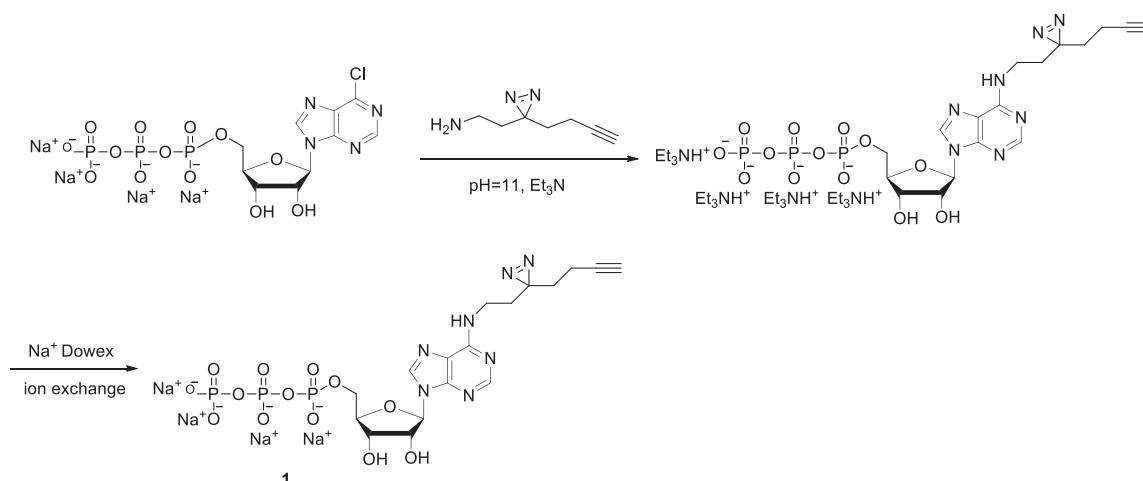
### Bacterial Strains

One Shot™ TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific; # C404010) were used for transformation of LR recombination reactions following the manufacturer's instructions. One Shot™ *ccdB* Survival™ 2 T1<sup>R</sup> Competent Cells (Thermo Fisher Scientific; #A10460) were used for transformation of Destination vectors (which have the *ccdB* gene) following the manufacturer's instructions. All other transformation reactions were conducted with NEB® 5-alpha Competent *E. coli* (High Efficiency) cells (NEB; #C2987H) following the manufacturer's 'High Efficiency Transformation Protocol'. Transformed bacteria were cultured in LB medium with antibiotic selection depending on the plasmid (100 µg/mL ampicillin, 50 µg/mL kanamycin, or a 100 µg/mL ampicillin and 30 µg/mL chloramphenicol combination for Destination vectors). Cultures were incubated for growth at 37°C on a rotary shaker at 200-250 rpm for 12-16 hrs.

### METHOD DETAILS

#### Chemical Synthesis of mipATP

Reagents for chemical reactions were purchased from Sigma-Aldrich without purification unless mentioned otherwise. 6-Chloropurine riboside-5'-triphosphate was purchased from BioLog Inc (Germany). Chemical reactions were carried out under argon atmosphere at the temperatures displayed by thermocouple or at ambient temperature (22°C) unless described otherwise. NMR spectra were recorded on Bruker AVIII 600MHz spectrometers and reported with chemical shifts (ppm), multiplicities (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, and br = broad), integration and coupling constants (*J* in Hz). Chemical shifts were recorded with residual proton peaks of deuterated solvents as references (residual <sup>1</sup>H of D<sub>2</sub>O, 4.80 ppm). <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were collected at 24°C. Mass spectra for compound characterization were collected by Waters Acuity SQD LC-MS with the mode of electron spray ionization (ESI). High resolution mass spectra (HRMS) for compound characterization were collected with a Waters Acuity Premiere XE TOF LC-MS by electrospray ionization (ESI) at the MSKCC Analytical Core Facility.



To a solution of 6-chloropurine riboside-5'-triphosphate (10 mg) in 5 mL buffer (pH = 11, triethylamine in water) was added 2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine (4 mg) and the mixture was stirred at 40°C under Ar. After the reaction was completed as indicated by LC-MS analysis, the resulting material was purified by reverse phase HPLC with aXBridge Prep C18 column (OBD 19 × 150 mm) and a 5–50% gradient of CH<sub>3</sub>CN in 20 mM triethylammonium bicarbonate aqueous solution (volume ratio) in 24 min at a flow rate of 10 mL/min. The desired product was collected at around 9 min. The product was lyophilized, dissolved in 2 mL water and passed through a 0.8 × 4.0 cm, (~ 3 g) column of Dowex AG50W-X8 (100-200 mesh, Na<sup>+</sup> form) cation-exchanged resin preequilibrated with two column volumes of water. The product was eluted with four column volumes of water and lyophilized to afford 4 mg of Compound 1. Compound 1: <sup>1</sup>H-NMR (D<sub>2</sub>O): δ 8.46 (s, 1H), 8.24 (s, 1H), 6.10 (d, 1H, *J* = 6.2), 4.77 (t, 2H, *J* = 7.4), 4.56-4.54 (m), 4.37-4.36 (m), 4.26-4.22 (m), 4.18-4.15 (m), 3.44, 3.21-3.20 (m), 1.98-1.95 (m), 1.87 (t, 2H, *J* = 6.60 Hz), 1.68 (t, 2H, *J* = 7.08 Hz) (Data S1); <sup>13</sup>C NMR, 154.4, 152.7, 139.3, 86.3, 84.1, 74.2, 70.4, 69.6, 65.2, 35.3, 31.4, 12.4 (Data S1). HRMS: calculated for C<sub>17</sub>H<sub>23</sub>N<sub>7</sub>O<sub>13</sub>P<sub>3</sub> ([M-H]<sup>-</sup>) 626.0567, found 626.0588 (Data S1).

#### Plasmid Construction

Plasmids for transgenesis were created using the Tol2kit system (Kwan et al., 2007). To generate the *Tg(krt4:QF2)* plasmid, the pME-QF2 (Addgene; #83307, Halpern lab unpublished) entry clone was recombined with p5E-*krt4* (Gong et al., 2002) and p3E-polyA (Kwan et al., 2007) constructs into the pDestTol2CG2 backbone. To generate the *Tg(QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)* plasmid, the pDONR221-GCaMP6s-3xNLS (Enyedi et al., 2016) entry clone was recombined with the p5E-QUAS (Addgene; #61374, Halpern lab unpublished) and p3E-P2A-mKate2-3xNLS-polyA (Enyedi et al., 2016) constructs into the pDestTol2CmKate2

vector backbone. Plasmid recombination was conducted using LR Clonase™ II plus enzyme (Thermo Fisher Scientific; #12538200) following the manufacturer's instructions. 2–3  $\mu$ L of a completed recombination reaction was then transformed into one 50  $\mu$ L vial of One Shot™ TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific; #C404010) following the manufacturer's instructions and plated onto LB/ampicillin (100  $\mu$ g/mL) agar plates for overnight growth at 37°C.

For mammalian expression, MFSD5-FLAG (Ensembl: ENSG00000182544, ENST00000329548.4; Uniprot: Q6N075-1), was first subcloned from A549 cell cDNA into the pSB/CMV/MCS/Puro transposon plasmid by NheI and NotI digestion (Enyedi et al., 2013) and subsequently cloned into the pSBbi-Pur transposon plasmid (Addgene; #60523) (Kowarz et al., 2015) by SfiI digestion to create the pSBbi-MFSD5-FLAG-Pur plasmid. A pSBbi-GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS-Pur plasmid was created by first subcloning a codon optimized gBlock® (IDT) containing the mBeRFP (Yang et al., 2013) coding sequence into the p3E-P2A-mKate2-3xNLS-polyA construct in order to create a QUAS:GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS-polyA plasmid. Next, the GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS sequence was subcloned into the pSBbi-Pur plasmid by NcoI and XbaI digestion. Plasmid transformation was conducted using NEB® 5-alpha Competent *E. coli* (High Efficiency) cells (NEB; #C2987H) following the manufacturer's 'High Efficiency Transformation Protocol' and plated onto LB agar plates with selection (100  $\mu$ g/mL ampicillin or 50  $\mu$ g/mL kanamycin) for overnight growth at 37°C.

### Generation of Stable Cell Lines

For establishing stable A549 and HeLa cell lines expressing MFSD5-FLAG or GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS, cells were co-transfected with the pSBbi-Pur vector encoding the gene or reporter of interest and a vector encoding the SB100X *Sleeping Beauty* transposase (Izsvák et al., 2009) at a 1:2 ratio using the Lipofectamine 3000 reagent (Thermo Fisher Scientific; #L300015) following the manufacturer's instructions. A549 and HeLa cells were subsequently grown in the presence of 1  $\mu$ g/mL and 2  $\mu$ g/mL of puromycin, respectively, for 1 week.

### Widefield Fluorescence Imaging of *In Vitro* Ca<sup>2+</sup> Signals

A549 and HeLa cells stably expressing a GCaMP6s-3xNLS-P2A-mBeRFP-3xNLS reporter transgene were seeded into the wells of a black 96-well glass bottom plate (Cellvis; #P96-1.5H-N) at a density of  $2 \times 10^4$  cells per well 24 hrs before imaging. Before the experiment, cells were washed 2 times with L-15 medium and equilibrated to 50  $\mu$ L of L-15 medium for 30 min at RT. Images were acquired every 15 sec from two different fields of view for a period of 22 min. During the experiment, 20  $\mu$ M of ATP (Sigma; #A26209), ATP $\gamma$ S (Tocris; #4080), mIPATP, AMP-PNP (Sigma; #A2647) or vehicle was added in 50  $\mu$ L of L-15 medium to the well prior to the 2-min timepoint. (final concentration of 10  $\mu$ M nucleotide).

Images were captured at RT (~26°C) using NIS-Elements (Nikon) on an Eclipse Ti microscope (Nikon) equipped with a 20 $\times$  Plan Apochromat NA 0.75 air objective lens, a Clara CCD camera (Andor), and a motorized stage. GCaMP6s and mBeRFP fluorescence were excited with a LED light source (Lumencor) using a 475/28 bandpass filter and a 470/40 excitation filter in conjunction with a multispectral dichroic (59022 bs, Chroma). GCaMP6s emission was acquired using a 525/50 filter (Chroma) and mBeRFP emission was acquired using a 632/60 emission filter (Chroma).

### Quantification of *In Vitro* Ca<sup>2+</sup> Signals

Image processing and calcium signal analyses were performed with the MATLAB R2018a software. Nuclei (mBeRFP channel) are first identified and separated by watershed segmentation in individual frames and then the same nuclei are connected between consecutive frames, by looking for the nearest neighbor. The sum of GCaMP6s signal within individual nuclei was divided by that of mBeRFP signal to give a ratiometric measure of calcium. For more details, please see the supplemental MATLAB scripts (Data S2).

### Generation of Transgenic Zebrafish Lines

To generate transgenic lines, a solution containing 25–25 pg of *Tg(krt4:QF2)* or *Tg(QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)* plasmid and *in vitro* transcribed Tol2 transposase mRNA was injected into the cytosol of one-cell stage *casper* embryos. Injected larvae with mosaic cardiac EGFP or mKate2 expression were raised to sexual maturity and screened by crossing with wild-type fish to identify founders. F1 embryos were identified by cardiac EGFP or mKate2 expression and raised to sexual maturity. This generated the two independent stable lines *mk111Tg* and *mk112Tg* which are referred to in the text as *Tg(krt4:QF2)* and *Tg(QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)*, respectively. F1 *Tg(krt4:QF2)* and *Tg(QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)* were crossed and F2 *mk113Tg* (referred to in the text as *Tg(krt4:QF2; QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)*) embryos were identified by epithelial expression of nuclear mKate2 and cardiac EGFP and then raised to sexual maturity. Experiments were performed on the progeny of F2 *Tg(krt4:QF2; QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)* incross.

### Spinning Disk Confocal Imaging of Ca<sup>2+</sup> Signals in Zebrafish Larvae

For amputation experiments, anesthetized 2.5–4 dpf *Tg(krt4:QF2; QUAS:GCaMP6s-3xNLS-P2A-mKate2-3xNLS)* individual zebrafish larvae were washed (3  $\times$  1 mL) with isotonic E3 (E3 medium supplemented with 135 mM NaCl) and subjected to tail fin tip amputation using a needle knife (Fine Science Tools; #10318-14) in isotonic E3 on a glass dish. Tail fin amputated larvae were immobilized on a plastic dish by embedding in ~30  $\mu$ L of 1% low-melting agarose (GoldBio; #A-204-25) dissolved in isotonic E3. After solidification, a strip of agarose (~10  $\mu$ L) was removed along the amputated tail fin, and the sample was mounted on the microscope. 50  $\mu$ L of

isotonic E3 supplemented with 1 mM ATP (Sigma; #A26209), ATP $\gamma$ S (Tocris; #4080), mipATP, AMP-PNP (Sigma; #A2647), or vehicle was added to the sample on the imaging dish prior to the 2-minute timepoint.

Experiments were performed at RT (~26°C) on a Nikon Eclipse FN1 microscope equipped with a 25 $\times$  Apochromat LWD NA 1.1 water immersion objective lens, a Yokogawa CSU-X1 Spinning Disk unit, an Andor iXon3 897 EMCCD camera, and 488 nm and 561 nm diode laser lines (Andor Revolution XD). GCaMP6s fluorescence was excited using the 488 nm laser line and emission was collected using a 535/20 bandpass filter (Chroma). mKate2 (mK2) fluorescence was excited using the 561 nm laser line and emission was collected using a 620/30 bandpass filter (Chroma). GCaMP6s and mK2 image acquisition was done simultaneously through triggered excitation and emission collection. Up to 30 Z-stack slices with a resolution of 3  $\mu$ m were acquired per field of view with the NIS-Elements software (Nikon). Images were acquired every 10 sec for a period of 22 min.

### Image Processing and Data Analysis of Ca<sup>2+</sup> Signals in Zebrafish Larvae

Image processing and data analysis was performed as described in (Enyedi et al., 2016). Briefly, image processing tasks were performed with the open-source program Fiji (Schindelin et al., 2012) and the Anaconda distribution of the Python programming language, using custom scripts, based on the pandas, SciPy, NumPy, and scikit-image libraries. To quantify nuclear Ca<sup>2+</sup> signal intensity, background corrected and thresholded maximum intensity projection images of GCaMP6s and mK2 confocal time-lapse Z-stacks were generated and ratio images (GCaMP6s/mK2) were calculated using Fiji. The ratio value of every pixel was then remapped using a custom Python script, to express them as a function of binned distance (2  $\mu$ m) from the moving wound margin. These spatiotemporal maps of the Ca<sup>2+</sup> signals were then averaged over multiple experiments.

### Cell Membrane Isolation

A549 cells were grown to confluence and washed 3 $\times$  with ice-cold PBS. Cells were harvested in PBS with a cell lifter and pelleted by centrifugation at 250  $\times g$  for 5 min at 4°C. Cell pellets were resuspended in 4 volumes of ice-cold homogenization buffer (HB) (250 mM Sucrose, 20 mM HEPES pH 7.4, 1.5 mM MgCl<sub>2</sub>, EDTA-free protease inhibitor cocktail (Sigma; #11873580001)) and pelleted by centrifugation at 1,100  $\times g$  for 10 min at 4°C. Pellets were resuspended in 3 $\times$  ice-cold HB and the resuspended cells were homogenized by 10 passages through a 25G needle at 4°C. The homogenate was centrifuged at 1,000  $\times g$  for 10 min at 4°C and the pellets discarded (nuclei). The supernatant was centrifuged at 33,000  $\times g$  for 30 min at 4°C to obtain the crude membrane fraction. Membrane pellets were resuspended in binding buffer (BB) (150 mM NaCl, 20 mM HEPES pH 7.4, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and EDTA-free protease inhibitor cocktail) and passaged 10 $\times$  through a 25G needle. Membranes were re-pelleted by centrifugation and resuspended in ice-cold BB by passage through a 25G needle. Membrane protein concentrations were determined by Pierce BCA assay (Thermo Fisher Scientific; #23227).

### mipATP Labeling of Proteins in Isolated Membrane Fractions

Isolated membrane proteins were diluted to 1–2 mg/mL in binding buffer (BB) (150 mM NaCl, 20 mM HEPES pH 7.4, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and EDTA-free protease inhibitor cocktail) and then incubated with 1–50  $\mu$ M of mipATP in a 96-well plate for 25–30 min at 4°C in the dark. For competition experiments, isolated membranes were preincubated with the indicated competitor compound (ATP, Sigma #A26209; ATP $\gamma$ S, Tocris #4080; ADP, Sigma #A2754; AMP, Sigma #A1752; Adenosine, Sigma #A9251; GTP, Sigma #G8877; CTP, Sigma #C1506; UTP, Sigma #U6750; molybdate, Sigma #243655) for 10 min at 4°C in the dark prior to the addition of mipATP. Plates containing the samples were then placed on ice and UV-irradiated (365 nm) (Analytik Jena US; #95-0045-04) for 15 min at a distance of 2 cm from the light source. Samples not exposed to UV were placed on a separate 96-well plate that was left in the dark at 4°C for 15 minutes instead of UV-irradiated. Next, membranes were collected and resuspended in 5 volumes of ice-cold BB and centrifuged at 230,000  $\times g$  for 10 min at 4°C. Supernatant was discarded, and the pellets were resuspended in BB and re-pelleted by centrifugation. Membrane pellets were snap frozen with liquid nitrogen or solubilized in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine (TEA) pH 7.4, EDTA-free protease inhibitor cocktail) for downstream click chemistry reactions.

### mipATP Labeling of Proteins in Cell Lysates

A549 cells were grown to confluence and washed 3 $\times$  with ice-cold PBS. Cells were harvested in PBS with a cell lifter and pelleted by centrifugation at 250  $\times g$  for 5 min at 4°C. Cell pellets were resuspended and lysed in 4 volumes of ice-cold lysis buffer (150 mM NaCl, 5 mM MgCl<sub>2</sub>, 20 mM HEPES pH 7.4, 0.1% NP-40, 10% glycerol, EDTA-free protease inhibitor cocktail) and kept on ice for 30 minutes with agitation every 5 minutes. Lysates were cleared by centrifugation at 16,000  $\times g$  for 10 min at 4°C and pellets were discarded. The resulting supernatants were subjected to gel filtration using 7K MWCO Zeba spin desalting columns (Thermo Fisher Scientific; #89889) to remove endogenous nucleotides. Next, filtered lysate protein concentrations were determined by Pierce BCA assay. Lysates were diluted to 1 mg/mL (at 50 or 100  $\mu$ g total) in ice-cold lysis buffer and incubated with 10  $\mu$ M of mipATP in a 96-well plate for 10 min at 4°C in the dark. For competition experiments, filtered lysates were preincubated with the indicated competitor compound (ATP, Sigma #A26209; ATP $\gamma$ S, Tocris #4080) for 10 min at 4°C in the dark prior to the addition of mipATP. Plates containing the samples were then placed on ice and UV-irradiated (365 nm) for 10 min at a distance of 2 cm from the light source. Samples not exposed to UV were placed on a separate 96-well plate that was left in the dark at 4°C for 10 minutes instead of UV-irradiated. Next, proteins were precipitated by the sequential addition of 4 volumes of ice-cold MeOH, 1 volume of ice-cold CHCl<sub>3</sub>, and 3 volumes of ice-cold H<sub>2</sub>O. Samples were centrifuged at 16,000  $\times g$  for 10 min at 4°C and the aqueous phase was discarded. Protein discs were washed (2  $\times$  1 mL) with ice-cold MeOH and air-dried for ~10 min.

### mipATP Labeling of Proteins on Intact Cells

A549 cells were grown to confluence and washed 3× with PBS. Cells were then detached from tissue culture dishes by 10–15 min incubation with 1 mM EDTA in PBS at 37°C. Detached cells were pelleted by centrifugation at 250 ×g for 5 min at 4°C, and sequentially washed 1× with ice-cold PBS and 1× with ice-cold HEPES-buffered extracellular medium (EC) (123 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.9 mM MgCl<sub>2</sub>, 0.9 mM MgSO<sub>4</sub>, 2 mM NaHCO<sub>3</sub>, 5 mM glucose, and 10 mM Na-HEPES pH 7.4; or 113.65 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 5 mM KCl and 25 mM Na-HEPES pH 7.4). Cells were resuspended to ~2–4 × 10<sup>7</sup> cells/mL in ice-cold EC and incubated with mipATP at the indicated concentrations (5–50 μM) in a well of a 24-well plate or a 10 cm plate for 25–30 min at 4°C in the dark. For competition samples, cells were preincubated with the competitor compound (ATP; Sigma #A26209) for 10 min at 4°C in the dark prior to the addition of mipATP. The plates containing the samples were then placed on ice and UV-irradiated (365 nm) for 15 min. Samples not exposed to UV were placed on a separate plate that was left in the dark at 4°C for 15 minutes instead of UV-irradiated. Cells were collected and resuspended in 10 volumes of ice-cold EC + EDTA-free protease inhibitor cocktail and pelleted by centrifugation at 250 ×g for 5 min at 4°C. Cells were then washed in 10 volumes of ice-cold PBS + EDTA-free protease inhibitor cocktail and pelleted by centrifugation at 250 ×g for 5 minutes at 4°C. Next, cells were resuspended in 3 volumes of ice-cold HB (250 mM Sucrose, 20 mM HEPES pH 7.4, 1.5 mM MgCl<sub>2</sub> + EDTA-free protease inhibitor cocktail) and homogenized by 10 passages through a 25G needle at 4°C. Homogenates were centrifuged at 1,000 ×g for 10 min at 4°C and pellets (nuclei) were discarded. The supernatant was centrifuged at 100,000 ×g for 1 hr at 4°C. Supernatants were discarded and pellets were resuspended in ice-cold HB + EDTA-free protease inhibitor cocktail by passage through a 25G needle. Samples were re-pelleted by centrifugation, supernatants were discarded, membranes were solubilized in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail), and then protein concentrations were determined by Pierce BCA assay.

### SDS-PAGE In-gel Fluorescence Analysis

Probe-crosslinked membranes and dried protein pellets from labeled lysates (50–100 μg; determined by Pierce BCA assay) were solubilized in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail) and brought to a final concentration of 1 mg/mL and ~0.5% SDS with SDS-free lysis buffer (150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). Solubilized membranes and lysates were reacted with a freshly premixed click chemistry reaction cocktail containing TAMRA-azide (25 μM, 10 mM stock in DMSO) (Click Chemistry Tools; #AZ109), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared in H<sub>2</sub>O) (Sigma; #C4706), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (100 μM, 1.7 mM in 20% DMSO/80% *tert*-butanol) (Cayman; #18816), and CuSO<sub>4</sub> (1 mM, 50 mM freshly prepared in H<sub>2</sub>O) (Sigma; #203165) for 1 hr at RT in the dark. Reactions were terminated and protein precipitated by the sequential addition of 4 volumes of ice-cold MeOH, 1 volume of ice-cold CHCl<sub>3</sub>, and 3 volumes of ice-cold H<sub>2</sub>O. Samples were centrifuged at 15,000 ×g for 10 min at 4°C and the aqueous phase was discarded. Proteins were washed (2 × 1 mL) with ice-cold MeOH and air-dried in the dark for ~10 min. Dried proteins were solubilized in 1× NuPAGE LDS Sample Buffer (Thermo Fisher Scientific; #NP0007) + Reducing Agent (Thermo Fisher Scientific; #NP0009) at a final of 1 mg/mL and subsequently incubated at 70°C for 10 min in the dark. Next, 25 μg of protein was loaded per gel lane for separation by SDS-PAGE using NuPAGE 4–12% Bis-Tris Protein Gels (Thermo Fisher Scientific; #NP0322BOX). Gels were subsequently fixed in freshly prepared 50% MeOH, 10% acetic acid solution for 1 hr at RT in the dark and then washed with H<sub>2</sub>O (3 × 20 min) in the dark and imaged using a GE Typhoon FLA 9500. Band intensity analyses (Figures 2B and 2C) were conducted using ImageQuant TL 8.1 software (GE Healthcare). For Figure 2B, values were fit to an [Agonist] vs. response (three parameters) curve (fixed Hill Slope = 1.0) using GraphPad Prism 8 software. Band intensity values were normalized to the Best-fit Top value of each band as predicted by the fitted curve and these normalized values are displayed as “Crosslinking efficiency (%)” (Arguello et al., 2017). EC<sub>50</sub> and 95% confidence interval (CI) values were determined using the fitted curve on the GraphPad Prism 8 software. For Figure 2C, band intensity values were normalized to the intensity value of the no competition band within the same experiment and displayed as “Signal Intensity (normalized units)”. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad; #1610436) after fluorescence imaging (Figure S2). Coomassie blue images of gels were generated using the ChemiDoc™ Touch Imaging System and Image Lab V 5.2 software (Bio-Rad).

### Streptavidin Affinity Purification of mipATP-crosslinked Proteins

mipATP-labeled membrane fractions (200–500 μg) were solubilized in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail) and brought to a final concentration of 0.5 mg/mL and ~0.5% SDS with SDS-free lysis buffer (150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). Next, a freshly premixed click chemistry reaction cocktail was added to the sample for a final of 100 μM biotin-azide (Click Chemistry Tools; #1265), 1 mM TCEP (Sigma; #C4706), 100 μM TBTA (Cayman; #18816), and 1 mM CuSO<sub>4</sub> (Sigma; #203165) and allowed to react for 1 hr at RT. Reactions were terminated and protein precipitated by the sequential addition of 4 volumes ice-cold MeOH, 1 volume ice-cold CHCl<sub>3</sub>, and 3 volumes ice-cold H<sub>2</sub>O or by the addition of 5 volumes of ice-cold MeOH and O/N incubation at -80°C. After protein precipitation, samples were centrifuged at 6,000 ×g for 20 minutes at 4°C and the aqueous phase was discarded in order to isolate the precipitated proteins. The precipitated proteins were washed 2× with 5 mL of ice-cold MeOH and then air-dried for ~10 min. Dried proteins were solubilized in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). 5 μg of each solubilized sample was diluted to a final volume of 20 μL in 1× NuPAGE LDS Sample Buffer + Reducing Agent as input in western blot to indicate equal protein concentrations across samples prior to pull-down. Samples were brought to a final concentration of 0.5 mg/mL and

0.4% SDS with SDS-free lysis buffer (150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). Pre-washed (3 × 1 mL) with washing buffer (0.4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) Streptavidin agarose (Pierce; #20357) was brought to a 50% bead slurry and 20  $\mu$ L of bead slurry was added to each sample. Samples were incubated rotating for 2 hr at RT. Next, beads were pelleted by centrifugation at 500  $\times$ g for 2 min at RT and subsequently washed (4 × 1 mL) with washing buffer (0.4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) for 5 minutes rotating per wash. After the final wash, washing buffer was completely removed with an aspirator and the beads were resuspended in 20  $\mu$ L of 1 $\times$  NuPAGE LDS Sample Buffer + Reducing Agent and then incubated at 95°C for 10 min. Samples were vortexed and then centrifuged at 16,000–20,000  $\times$ g for 1 min and subsequently separated by SDS-PAGE with NuPAGE 4–12% Bis-Tris Protein Gels.

### Western Blotting

Proteins were transferred onto a PVDF membrane at 100 V for 90 min at 4°C in Towbin buffer (25 mM Tris, 192 mM glycine, 20% (v/v) MeOH). Blots were then blocked with 5% nonfat dried milk in TBST (0.1% Tween-20 in TBS) for 1 hr at RT. Next, blots were probed with the following primary antibodies: anti-MRP1 (Cell Signaling; #14685S, 1:1,000), anti-CK1 $\delta$  (Bethyl Laboratories; #A302-136A, 1:1,000), anti-CD73 (Cell Signaling; #13160S, 1:500), anti-CD44 (Cell Signaling; #3578S, 1:250 or 1:500), anti-FLAG M2 (Sigma; #F1804 1:2,500), anti-FAS (Abcam; #ab133619, 1:1,000), anti-P2X4 (Cell Signaling; #70659, 1:500), anti-P2Y2 (Novus Biologicals; #NB110-39032, 1:1,000) in 5%-milk TBST O/N at 4°C or for 2 hr at RT. Membranes were washed with TBST (3 × 10 min) and incubated for 1 hr at RT with goat anti-rabbit (Cell Signaling; #7074, 1:5,000) or goat anti-mouse (Cell Signaling; #7076, 1:5,000) HRP-linked secondary antibody in 5%-milk TBST depending on the species of the primary antibody used. Membranes were washed with TBST (3 × 10 min) and visualized with Amersham ECL (GE Healthcare; #RPN2106) or ECL Select Western Blotting Detection (GE Healthcare; #RPN2235). Chemiluminescence was detected using the ChemiDoc™ Touch Imaging System (Bio-Rad). For [Figure 4](#) analysis, band intensity values were normalized to the intensity value of the UV-irradiated, no competition band within the same blot using the Image Lab V 5.2 software (Bio-Rad) and displayed as “Signal Intensity (normalized units)”.

### Sample Preparation for Cell Lysate SILAC Analysis

Two independent SILAC experiments were conducted to profile mipATP targets in A549 cell lysates: one “forward” and one “reverse”. Isotopically labeled A549 cells were grown to confluence (~80–90%) and washed 3 $\times$  with ice-cold PBS. Cells were harvested in PBS with a cell lifter and pelleted by centrifugation at 250  $\times$ g for 5 min at 4°C. Cell pellets were lysed in 4 volumes of ice-cold lysis buffer (150 mM NaCl, 5 mM MgCl<sub>2</sub>, 20 mM HEPES pH 7.4, 0.1% NP-40, 10% glycerol (5% glycerol for “reverse” samples), 1 mM NaF, 1 mM  $\beta$ -glycerophosphate, and EDTA-free protease inhibitor cocktail) and kept on ice for 30 minutes with agitation every 5 minutes. Lysates were cleared by centrifugation at 16,000  $\times$ g for 10 min at 4°C and pellets were discarded. The resulting supernatants were subjected to gel filtration using 7K MWCO Zeba spin desalting columns (Thermo Fisher Scientific; #89891) to remove endogenous nucleotides. Next, filtered lysate protein concentrations were determined by Pierce BCA assay. 2 mg of each lysate was diluted to 1 mg/mL in ice-cold binding buffer (150 mM NaCl, 5 mM MgCl<sub>2</sub>, 20 mM HEPES pH 7.4, 0.1% NP-40, 1 mM NaF, 1 mM  $\beta$ -glycerophosphate, and EDTA-free protease inhibitor cocktail). For the “forward” SILAC experiment, isotopically heavy lysate was incubated with 10  $\mu$ M mipATP (10 min at 4°C in the dark) and light lysate was preincubated with unmodified 100  $\mu$ M ATP (Sigma #A26209) (10 min at 4°C in the dark) prior to the addition of 10  $\mu$ M mipATP (10 min at 4°C in the dark). Cell isotope labels were swapped for the complementary “reverse” SILAC experiment. Next, samples were placed into wells of a pre-chilled 6-well plate on ice and then UV-irradiated (365 nm) for 10 min at distance of 2 cm from the light source. Next, proteins were precipitated by the sequential addition of 4 volumes of ice-cold MeOH, 1 volume of ice-cold CHCl<sub>3</sub>, and 3 volumes of ice-cold H<sub>2</sub>O. Samples were centrifuged at 6,000  $\times$ g for 10 min at 4°C and the aqueous phase was discarded. Protein discs were washed (3 × 10 mL) with ice-cold MeOH and then air-dried for ~10 min. Next, dried protein pellets were solubilized in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail) and protein concentrations were determined for each sample by BCA assay. For the “forward” experiment, 1.2 mg each of heavy and light lysate were combined 1:1. For the “reverse” experiment, 1.4 mg each of heavy and light lysate were combined 1:1. Next, samples were brought to 0.7 mg/mL and 0.5% SDS with SDS-free lysis buffer (150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). Samples were reacted with a freshly premixed click chemistry reaction cocktail at a final of 100  $\mu$ M dde-biotin-azide (Click Chemistry Tools; #1136), 1 mM TCEP (Sigma; #C4706), 100  $\mu$ M TBTA (Cayman; #18816), and 1 mM CuSO<sub>4</sub> (Sigma; #203165) for 1 hr at RT. Reactions were terminated and proteins were precipitated by the sequential addition of 4 volumes of ice-cold MeOH, 1 volume of ice-cold CHCl<sub>3</sub>, and 3 volumes of ice-cold H<sub>2</sub>O. Samples were centrifuged at 6,000  $\times$ g for 10 min at 4°C and the aqueous phase was discarded. Protein discs were washed (3 × 25 mL) with ice-cold MeOH and then air-dried for ~10 min. Dried pellets were solubilized in SDS lysis buffer with EDTA (4% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM TEA pH 7.4, with cOmplete™ protease inhibitor cocktail (Sigma; #11697498001)). Next, samples were diluted to 1 mg/mL and 0.2% SDS with 150 mM NaCl, 1 mM EDTA, 50 mM TEA pH 7.4, and cOmplete™ protease inhibitor cocktail. Streptavidin agarose slurry (pre-washed 3 $\times$  with 150 mM NaCl, 1 mM EDTA, 0.2% SDS, 50 mM TEA pH 7.4) was added to a final bed volume of 50  $\mu$ L and samples were incubated rotating at RT for 1.5 hrs. Next, the beads were pelleted by centrifugation at 1,000  $\times$ g for 2 min at RT and sequentially washed (6 × 5 mL for 5 min per wash) with wash buffer (250 mM NaCl, 1 mM EDTA, 50 mM HEPES pH 7.4, 0.2% SDS, and cOmplete™ protease inhibitor cocktail). After the last wash, the beads were transferred to Protein LoBind tubes (Eppendorf; #022431081) and washed quickly (3 × 1 mL) with hydrazine-free elution buffer (150 mM NaCl, 25 mM HEPES pH 7.4, 0.05% SDS). Next, beads were resuspended in 100  $\mu$ L of hydrazine elution buffer (150 mM NaCl, 25 mM HEPES pH 7.4, 0.05% SDS, 2% hydrazine (Sigma; #207942)) and incubated for 30 min at RT with agitation every 5 minutes. Beads were pelleted by centrifugation

at 1,000  $\times g$  for 2 min at RT, eluates were transferred to fresh Protein LoBind tubes, and beads were resuspended in fresh hydrazine elution buffer and incubated for 30 min at RT. First and second eluates were combined, and the beads were washed one last time with 100  $\mu$ L of hydrazine- and SDS-free elution buffer (150 mM NaCl, 25 mM HEPES) which was subsequently added to the corresponding eluate. Combined eluates were brought to 0.1% sodium deoxycholate (2% stock solution) and incubated at RT for 10 min. Next, eluates were brought to 10% trichloroacetic acid (TCA; 100% stock solution, Sigma; #T6399) and incubated on ice for 30 minutes to precipitate proteins. Samples were centrifuged at 16,000  $\times g$  for 10 min at 4°C to pellet proteins. Protein pellets were washed 2 $\times$  with 500  $\mu$ L of ice-cold acetone and allowed to air-dry for  $\sim$ 10 min. Dried samples were solubilized in 20  $\mu$ L of 1 $\times$  NuPAGE LDS Sample Buffer + Reducing agent and separated on a 10% NuPAGE Bis-Tris Gel (Thermo Fisher Scientific; #NP0301BOX) until the dye front entered  $\sim$ 2 cm into the gel. Samples were stained with Coomassie Brilliant Blue R-250 (Bio-Rad; #1610436) and four gel slices were excised for each sample and sent to the Harvard Medical School Taplin Mass Spectrometry Facility for analysis.

### Sample Preparation for Label-Free MS Analysis

The data generated reports on a single independent experiment. A549 cells were grown to confluence and a crude membrane fraction was isolated as described above (Cell Membrane Isolation) except that homogenization was performed by  $\sim$ 30 strokes in a Teflon Potter-Elvehjem homogenizer at 4°C in homogenization buffer (HB) (250 mM Sucrose, 30 mM Tris-HCl pH 7.4, and EDTA-free protease inhibitor cocktail). The homogenate was centrifuged at 600  $\times g$  for 10 minutes at 4°C to remove nuclei. After isolation, crude membrane protein concentrations were determined by BCA assay and split into two equal 7.5 mg fractions in 2.5 mL (3 mg/mL) in Binding Buffer (BB) (150 mM NaCl, 25 mM HEPES pH 7.4, 1.3 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, EDTA-free protease inhibitor cocktail). ATP (Sigma #A26209) diluted in BB was added to the 'Competition' sample to a final concentration of 2 mM while an equal volume of BB was added to the 'Experimental' sample and both samples were incubated at 4°C for 10 min. Next, mipATP was added to each sample to a final concentration of 20  $\mu$ M and the samples were then incubated at 4°C for 25 minutes in the dark. Next, the samples were placed into separate wells of a pre-chilled 6-well plate on ice and UV-irradiated (365 nm) for 15 min. Then, the samples were washed with 10 volumes of ice-cold BB and pelleted by centrifugation at 100,000  $\times g$  for 30 min at 4°C. Pellets were solubilized with SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail) and subsequently brought to a final concentration of 1 mg/mL and  $\sim$ 1% SDS with SDS-free lysis buffer (150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). A freshly prepared premixed click chemistry reaction cocktail was then added to solubilized membranes to a final of 100  $\mu$ M diazo-biotin-azide (Click Chemistry Tools; #1041), 1 mM TCEP (Sigma; #C4706), 500  $\mu$ M TBTA (Sigma; #678937), and 1 mM CuSO<sub>4</sub> (Sigma; #203165) and incubated rotating for 1.5 hr at RT. Reactions were stopped by the addition of 5 volumes of ice-cold MeOH and placed overnight at -80°C for protein precipitation. The following day, precipitated proteins were pelleted by centrifugation at 6,000  $\times g$  for 30 min at 4°C and subsequently washed with ice-cold MeOH (2  $\times$  25 mL). The final MeOH wash was aspirated and the protein pellets were air-dried for  $\sim$ 10 min and subsequently solubilized in 6 M Urea, 0.4% SDS in PBS pH 7.4. TCEP (10 mM) (Sigma; #C4706) was added and samples were incubated at RT for 30 min. Next, iodoacetamide (20 mM) (Sigma; #11149) was added and samples were incubated in the dark at RT for 30 min. Samples were diluted to 0.5 mg/mL and 0.25% SDS, PBS pH 7.4 + EDTA-free protease inhibitor cocktail. Streptavidin agarose slurry (pre-washed 3 $\times$  with 0.25% SDS, PBS pH 7.4) was added at a final bed volume of 100  $\mu$ L and the samples were incubated rotating at RT for 2 hrs. Next, the beads were pelleted by centrifugation at 1,000  $\times g$  for 5 min at RT and sequentially washed 3 $\times$  with PBS pH 7.4 + 0.25% SDS, 2 $\times$  with PBS pH 7.4, and 3 $\times$  with PBS pH 7.4 + 1% SDS (5 min per wash). Beads were transferred to Protein LoBind tubes (Eppendorf; #022431081), resuspended in elution buffer (50 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> + 1% SDS in PBS, pH 7.4), and incubated for 1 hr at RT with agitation. Beads were then pelleted by centrifugation at 1,000  $\times g$  for 3 min and the eluates were transferred to fresh LoBind tubes. Beads were resuspended in fresh elution buffer and incubated again for 1 hr at RT. First and second eluates from the same sample were combined, concentrated with an Ultracel<sup>®</sup> -3K centrifugal filter (Millipore; #UFC500324), and subsequently dried by speed-vac. Dried samples were solubilized in 25  $\mu$ L of 1 $\times$  NuPAGE LDS Sample Buffer + Reducing agent and separated on a 10% NuPAGE Bis-Tris Gel until the dye front entered  $\sim$ 2 cm into the gel. After Colloidal Coomassie blue staining (Thermo Fisher Scientific; #LC6025), four gel slices were excised for each sample and sent to the Harvard Medical School Taplin Mass Spectrometry Facility for analysis.

### Sample Preparation for Cell Membrane SILAC Analysis

Two independent SILAC experiments were conducted: one "forward" and one "reverse". Isotopically heavy and light A549 cells were grown to confluence and intact cells in suspension were labeled with 50  $\mu$ M mipATP alone or after 10 min preincubation with 5 mM ATP as described above (mipATP Labeling of Proteins on Intact Cells). For the "forward" SILAC experiment, isotopically heavy cells were labeled with mipATP alone (50  $\mu$ M) and light cells were labeled with mipATP (50  $\mu$ M) after preincubation with 100 $\times$  excess ATP (Sigma #A26209) competitor (5 mM). Cell isotope labels were swapped for the complementary "reverse" experiment. After mipATP labeling, membranes were isolated as described above (mipATP Labeling of Proteins on Intact Cells) except that cell homogenization was performed by  $\sim$ 30 strokes of a Teflon Potter-Elvehjem homogenizer at 4°C in homogenization buffer (250 mM Sucrose, 30 mM Tris-HCl pH 7.4, EDTA-free protease inhibitor cocktail). The homogenate was centrifuged at 600  $\times g$  for 10 minutes at 4°C to remove nuclei. After isolation, membranes were solubilized in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail) and protein concentrations were determined by BCA assay. A 1:1 (heavy:-light) combined solubilized 5 mg sample was diluted to 1 mg/mL and  $\sim$ 1% SDS with SDS-free lysis buffer (150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). A freshly prepared premixed click chemistry reaction cocktail was then added to a final of 100  $\mu$ M diazo-biotin-azide (Click Chemistry Tools; #1041), 1 mM TCEP (Sigma; #C4706), 100  $\mu$ M TBTA (Sigma; #678937), and

1 mM CuSO<sub>4</sub> (Sigma; #203165) at a total reaction volume of 5 mL and incubated rotating for 1.5 hr at RT. Reactions were stopped by the addition of 5 volumes of ice-cold MeOH and placed overnight at -80°C for protein precipitation. The following day, precipitated proteins were pelleted by centrifugation at 6,000 ×g for 30 min at 4°C and subsequently washed with ice-cold MeOH (2 × 25 mL). Protein pellets were air-dried for ~10 min then solubilized in EDTA containing SDS lysis buffer (4% SDS, 150 mM NaCl, 10 mM EDTA, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail) and subsequently diluted to 1 mg/mL and 0.4% SDS with SDS-free lysis buffer (150 mM NaCl, 50 mM TEA pH 7.4, EDTA-free protease inhibitor cocktail). Streptavidin agarose slurry (pre-washed 3× with 150 mM NaCl, 0.4% SDS, 50 mM TEA pH 7.4) was added at a final bed volume of 100 µL and the sample was incubated rotating at RT for 2 hrs. The beads were pelleted by centrifugation at 1,000 ×g for 2 min at RT and sequentially washed with 150 mM NaCl, 0.4% SDS, 50 mM TEA pH 7.4 (4× for 5 min) and then with PBS + 1% SDS, pH 7.4 (3× for 5 min). Beads were transferred to a Protein LoBind tube (Eppendorf; #022431081), resuspended in elution buffer (50 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> + 1% SDS in PBS, pH 7.4), and incubated for 1 hour at RT with agitation. Beads were then pelleted by centrifugation at 1,000 ×g for 3 min and the eluate was transferred to a fresh LoBind tube. Beads were resuspended in fresh elution buffer and incubated again for 1 hr at RT. First and second eluates from the sample were combined, concentrated with an Ultracel® -3K centrifugal filter (Millipore; #UFC500324), and subsequently dried by speed-vac. Dried samples were solubilized in 25 µL of 1× NuPAGE LDS Sample Buffer + Reducing agent and separated on a 10% NuPAGE Bis-Tris Gel until the dye front entered ~2-3 cm into the gel. After Colloidal Coomassie blue staining, 5-6 gel slices were excised for each sample and sent to the Harvard Medical School Taplin Mass Spectrometry Facility for analysis.

### Protein Sequence Analysis by LC-MS/MS

Mass spectrometry analysis was performed by the Harvard Medical School Taplin Mass Spectrometry Facility. For the label-free samples, gel pieces were subjected to a modified in-gel trypsin digestion procedure (Shevchenko et al., 1996). Gel pieces were washed and dehydrated with acetonitrile for 10 min followed by removal of acetonitrile then completely dried by speed-vac. Next, gel pieces were rehydrated with 50 mM NH<sub>4</sub>HCO<sub>3</sub> containing 12.5 ng/µL modified sequencing-grade trypsin (Promega) at 4°C. After 45 min, the trypsin solution was removed and replaced with 50 mM NH<sub>4</sub>HCO<sub>3</sub> to just cover the gel pieces. Samples were then placed in a 37°C room O/N. Peptides were later extracted by removing the NH<sub>4</sub>HCO<sub>3</sub> solution, followed by 1 wash with 50% acetonitrile, 1% formic acid solution and then dried by speed-vac. Samples were then stored at 4°C until analysis. For SILAC experiments, gel bands were incubated with 1 mM DTT in 50 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 min at 60°C. Next, the samples were cooled to RT and iodoacetamide was added to 5 mM. Samples were incubated at RT in the dark for 15 min and then DTT (5 mM) was added to quench the reaction. Next, 5 ng/µL of sequence grade trypsin was added and the samples were digested overnight at 37°C. The samples were desalted by an in-house made desalting column the following day.

On the day of analysis, the samples were reconstituted in 5-10 µL of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 2.6 µm C18 spherical silica beads into a fused silica capillary (100 µm inner diameter × ~30 cm length) with a flame-drawn tip (Peng and Gygi, 2001). After equilibrating the column each sample was loaded via a Famos auto sampler (LC Packings) onto the column. A gradient was formed, and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid).

As peptides eluted, they were subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences were determined by matching protein databases with the acquired fragmentation pattern by the software program, Sequest (Thermo Fisher Scientific) (Eng et al., 1994). For SILAC experiments, each dataset was searched with light and heavy parameters. For the heavy search, differential modifications on lysine (+8.0141988132 Da) and arginine (+10.0082685996 Da) were specified. All databases include a reversed version of all the sequences and the data was filtered to between a one and two percent peptide false discovery rate.

Label-free MS analysis was conducted with assistance from the Harvard Medical School Taplin Mass Spectrometry Facility. A single label-free experiment was conducted consisting of one “Experimental” sample (20 µM mipATP) and one “Competition” sample (20 µM mipATP + 2 mM ATP) (Figure S3A). For each sample, carryover and common contaminants (human keratins and trypsin) were removed and peptides with a ΔCn value ≥ 0.08 and minimum XCorr values of 1.8, 2.0, 3.0, and 4.0 for single-, double-, triple-, and quadruple-charged spectra were retained. Next, identified proteins from the “Experimental” (20 µM mipATP) sample were compared to the proteins from the “Competition” (20 µM mipATP + 2 mM ATP) sample. The total intensity sum of the peptides for each identified protein was calculated in both samples and the “Experimental” to “Competition” ratios were determined. Proteins with a peptide intensity sum ratio ≥ 4.5 were retained. Next, proteins with < 1.5× more total peptides in the “Experimental” sample compared to the “Competition” were filtered out. Finally, only proteins in the filtered list with at least 2 total peptides in the “Experimental” sample were retained (Table S1; Tabs 1, 2, and 9).

SILAC sample analysis was conducted by the Harvard Medical School Taplin Mass Spectrometry Facility. Two independent biological experiments were conducted for each type of SILAC experiment (membrane (Figures 3A, S3D, and S3E and Table S1; Tabs 3, 4, 10, 12, 13, 14, and 15) or lysate (Figure S4 and Table S1; Tabs 6, 7, 11, 16, 17, 18, and 19)). Carryover and common contaminants were removed. For each protein, the median intensity values of the identified ‘heavy’ and ‘light’ peptides were determined. Next, for each screen, the sum value of the median ‘heavy’ peptide intensities and the sum value of the median ‘light’ peptide intensities for all identified proteins were calculated. For the “forward” membrane SILAC screen the ratio of the sum of all ‘heavy’ signals to the sum of all ‘light’ signals was calculated to be 1.27 (Table S1; Tab 12). A “corrected median ‘light’ peptide intensity” for each identified protein

was calculated by multiplying the median 'light' peptide intensity of the protein by 1.27. The final "forward" SILAC ratio of each identified protein is the ratio of the median 'heavy' peptide intensity to the "corrected median 'light' peptide intensity" ("forward" SILAC ratio: (Heavy/corrected Light)). For the "reverse" membrane SILAC screen, the ratio of the sum of all 'light' signals to the sum of all 'heavy' signals was calculated to be 1.13 (Table S1; Tab 14). A "corrected median 'light' peptide intensity" for each identified protein was determined by dividing the median 'light' peptide intensity of each identified protein by 1.13. The final "reverse" SILAC ratio of each identified protein is the ratio of the "corrected median 'light' peptide intensity" to the median 'heavy' peptide intensity ("reverse" SILAC ratio: (corrected Light/Heavy)). Next, for both "forward" and "reverse" SILAC experiments, only proteins represented by at least 3 total peptides were retained (Table S1; Tab 10). Proteins identified in the "forward" and "reverse" SILAC screens were then ranked as high confidence ATP binders based on the value of their calculated SILAC ratio, as described above. For each screen, proteins in the 90<sup>th</sup> percentile based on SILAC ratio were identified and listed as high confidence ATP interactors (Table S1; Tab 3). For Figure 3A, only proteins identified in both screens were retained and the log<sub>2</sub> values of the SILAC ratios were plotted.

For Figures 3B and 3C, high confidence targets in each of the three membrane proteomic screens were compared (label-free MS, "forward" membrane SILAC, and "reverse" membrane SILAC). Briefly, the 222 proteins identified as high confidence ATP binders in the label-free MS screen were compared to proteins found in the 90<sup>th</sup> percentile of the "forward" SILAC screen and proteins found in the 90<sup>th</sup> percentile of the "reverse" SILAC screen. Proteins found to be high confidence ATP binders in at least two of the three screens were identified (73 total) (Figure 3B and Table S1; Tab 5). Next, we screened the 73 identified proteins for their frequency in the CRAPome database. Proteins found at a high frequency in the CRAPome ( $\geq 5\%$  of listed experiments) were filtered out. Next, the remaining unfiltered proteins were compared to lists of enriched proteins from similar MS screens (Niphakis et al., 2015; Parker et al., 2017a). All proteins that were found enriched by functionally similar photo-clickable probes in either of these screens were filtered out as low priority hits. The remaining unfiltered 32 proteins were considered high confidence mipATP binders (Figure 3C and Table S1; Tab 5).

For the "forward" cell lysate SILAC experiment (Figure S4A), the median 'light' peptide intensity of each protein was corrected for by multiplying by 1.19 (Table S1; Tab 16). The final "forward" cell lysate SILAC ratio for each identified protein is the ratio of the median 'heavy' peptide intensity to the corrected median 'light' peptide intensity ("forward" SILAC ratio: (Heavy/corrected Light)) (Table S1; Tab 16). For the "reverse" cell lysate SILAC experiment, the median 'light' peptide intensity of each protein was corrected for by multiplying by 0.76 (Table S1; Tab 18). The final "reverse" cell lysate SILAC ratio for each identified protein is the ratio of the median 'heavy' peptide intensity to the corrected median 'light' peptide intensity ("reverse" SILAC ratio: (Heavy/corrected Light)) (Table S1; Tab 18). Next, for both "forward" and "reverse" SILAC experiments, only proteins represented by at least 2 total peptides were retained (Table S1; Tab 11). Then, proteins in the 90<sup>th</sup> percentile based on SILAC ratios were identified and listed as high confidence ATP interactors for each screen (Table S1; Tab 6). For Figure S4B, proteins found in the 90<sup>th</sup> percentile of both screens were identified (40) and classified based on the predicted likelihood to interact with nucleotides/nucleic acids based on Gene Ontology (GO) and Uniprot database analyses (Table S1; Tab 7). In Figure S4C, the 40 overlapping proteins were first filtered by identifying the proteins found at high frequency in the CRAPome database ( $\geq 5\%$  of experiments in the database). Next, remaining proteins found as targets in screens to similarly functionalized photo-clickable small molecule probes (Niphakis et al., 2015; Parker et al., 2017a) were filtered out, leaving 16 high confidence protein targets (Table S1; Tab 7).

### ATPase Activity Detection

A549 cell membranes were isolated as described above (Cell Membrane Isolation). 50  $\mu$ g of isolated membranes at a final of 1 mg/mL in BB (150 mM NaCl, 20 mM HEPES pH 7.4, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub> + EDTA-free protease inhibitor cocktail) was incubated  $\pm$  100  $\mu$ M ATP for 30 min at 0, 4, or 37°C. For membrane protein-less samples, 100  $\mu$ M ATP in BB was incubated for 30 min at the indicated temperature. ATPase activity detection was performed on an opaque 96-well plate using the Luminescent ATP Detection Assay Kit (Abcam; #ab113849) following the manufacturer's instructions. Luminescence counts from 20% of each sample were measured and displayed as "Luminescence arbitrary units [A.U.]". Data is representative of three independent experiments.

### Gene Ontology (GO) Analysis

GO Molecular Function analyses were done using the PANTHER Overrepresentation Test (Released 20200407) (Mi et al., 2019). The GO Ontology database used for analysis was released on 2020-02-21. The reference gene list for each test used was the entire *Homo sapiens* gene list in the GO database. The annotation data set used was the "GO molecular function complete" dataset. Significance was determined using Fisher's exact test with FDR (False Discovery Rate) correction as calculated by the Benjamini-Hochberg procedure.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Unless otherwise stated, p values were derived by unpaired, two-tailed t tests assuming unequal variances (heteroscedastic) using GraphPad Prism version 8.0 for Mac (Welch's t test), Microsoft Excel, or MATLAB. Significance was assumed with \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001; ns = not significant. All data points are shown as the mean  $\pm$  SEM unless otherwise stated in the figure legend. For Figure 2B, EC<sub>50</sub> and 95% confidence interval (CI) values were derived using GraphPad Prism 8. FDR (False Discovery Rate) values used to determine significance of GO terms in GO enrichment analyses were derived by Fisher's exact test with FDR correction as calculated by the Benjamini-Hochberg procedure by PANTHER.