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Essential requirement for JPT2 in NAADP-evoked Ca^{2+} signaling

Gihan S. Gunaratne^{1,2}, Eugen Brailoiu³, Shijun He⁴, Ellen M. Unterwald³, Sandip Patel⁵, James T. Slama^{4,†}, Timothy F. Walseth^{1,†}, Jonathan S. Marchant^{2,†,*}

¹Department of Pharmacology, University of Minnesota Medical School, 312 Church St., Minneapolis, MN 55455

²Department of Cell Biology, Neurobiology & Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

³Center for Substance Abuse Research, Lewis Katz School of Medicine at Temple University, Philadelphia, PA 19140

⁴Department of Medicinal & Biological Chemistry, University of Toledo College of Pharmacy and Pharmaceutical Sciences, 3000 Arlington Avenue, Toledo, OH 43614

⁵Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

Abstract

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a second messenger that releases Ca^{2+} from acidic organelles through the activation of two pore channels (TPCs) to regulate endolysosomal trafficking events. NAADP action is mediated by NAADP-binding protein(s) of unknown identity that confer NAADP-sensitivity to TPCs. Here, we used a 'clickable' NAADP-based photoprobe to isolate human NAADP-binding proteins and identified Jupiter microtubule-associated homolog 2 (JPT2) as a TPC accessory protein required for endogenous NAADP-evoked Ca^{2+} signaling. JPT2 was also required for the translocation of a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pseudovirus through the endolysosomal system. Thus, JPT2 is a component of the NAADP receptor complex that is essential for TPC-dependent Ca^{2+} signaling and control of coronaviral entry.

Introduction

The second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) releases Ca^{2+} from acidic Ca^{2+} stores within the endolysosomal system (1, 2). NAADP-evoked Ca^{2+} release regulates numerous cellular processes (2, 3), including the trafficking of physiological substrates and pathological cargoes (such as viruses (4–7)) through the

*Corresponding Author: JMarchant@mcw.edu.

†Co-Senior Authorship

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endolysosomal system. Dysfunction of this pathway has been implicated in several disease states (8).

Despite the (patho)physiological importance of NAADP-evoked Ca^{2+} signaling, our understanding of the molecular basis for NAADP action remains incomplete. Several ion channel targets for NAADP have been proposed, with the majority of evidence supporting NAADP activation of two-pore channels (TPCs) that reside in endosomes and lysosomes (9, 10). However, no direct binding site for NAADP has been identified on TPCs in over a decade since their first characterization in mammals (11–13). Rather, NAADP is thought to exert its potent Ca^{2+} -mobilizing activity by binding to unidentified NAADP-binding accessory protein(s) within the TPC channel complex (14). In T-lymphocytes, NAADP has alternatively been shown to release Ca^{2+} from the endoplasmic reticulum (ER) through the type 1 ryanodine receptor (RyR1, (15)). No direct NAADP binding site has been identified on RyR1.

Direct experimental support for the NAADP-binding protein model derives from photolabeling studies in which NAADP-derivatized photoprobes identify a low molecular weight NAADP-binding protein (~22/23kDa) distinct from TPCs and RyRs in various mammalian cells (9, 16–20). The binding characteristics of NAADP to the NAADP-binding protein recapitulate key properties of NAADP-evoked Ca^{2+} release, including a characteristic pharmacology, high affinity and selectivity for NAADP over NADP (~11-fold, (16)). Photolabeling of the NAADP-binding protein, but not NAADP-evoked Ca^{2+} release, persists in TPC knockout models (9, 16) and NAADP-binding proteins are co-immunoprecipitated with TPCs in sea urchin egg homogenates (17). Therefore, the ~23kDa NAADP-binding protein is a distinct molecular entity from TPCs, the identity of which is unknown. Here, we utilized a chemical biology and proteomic approach to identify the photolabeled ~23kDa mammalian NAADP-binding protein as Jupiter microtubule-associated homolog 2 (JPT2) and reveal its essential role in both NAADP-evoked Ca^{2+} signaling and cell entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pseudovirus.

Results

Photolabeling of JPT2 by a 'clickable' NAADP-derived affinity probe

A bifunctional photoprobe, [^{32}P]-alkyne-'all-in-one-click' (AIOC)-NAADP, was synthesized and used to profile NAADP-binding proteins in various human cell types. In human erythrocytes, analysis of radiolabeling patterns revealed strong and selective photolabeling of a ~23kDa NAADP-binding protein (Fig. 1, A and B) compared to other human cell lines (16, 18–20). Photolabeling of the ~23kDa NAADP-binding protein was protected by co-incubation with NAADP (Fig. 1, A and B).

Fractionation of erythrocyte whole-cell lysate revealed that the NAADP-binding protein was exclusively found in the soluble ('S20') supernatant (Fig. 1, C and D). Incubation with other nucleotide analogs or other Ca^{2+} -mobilizing messengers failed to shield photolabeling of the NAADP-binding protein (Fig. 1, E and F). However, incubation with NAADP, unlabeled alkyne-AIOC-NAADP, or high concentrations of NADP inhibited photolabeling.

Characterization of selectivity demonstrated ~11-fold selectivity for NAADP ($IC_{50}=80\pm 7\text{nM}$) over NADP ($IC_{50}=871\pm 120\text{nM}$, Fig. 1, G and H) in agreement with previous photolabeling data (16). Together, these data authenticate human erythrocytes as an enriched biological source of the ~23kDa NAADP-binding protein.

Previous efforts to identify this NAADP-binding protein from other mammalian cells has proved challenging, owing to low expression of the endogenous NAADP-binding protein. However, the strong photolabeling of the ~23kDa NAADP-binding protein in erythrocytes provided better opportunity for a purification effort from the gram-quantities of protein available from an easily sourced material (Fig. 2A). Additionally, synthesis of a bifunctional probe ($[^{32}\text{P}]$ -alkyne-AIOC-NAADP, Fig. 2B) with both a photoactivatable azide and a clickable alkynyl moiety permitted coupling of photolabeling of the NAADP-binding protein to a subsequent enrichment strategy (Fig. 2A). Proteins were sequentially fractionated using a multistep protocol (fig. S1, A). A phosphoprotein enrichment resin was then employed to concentrate the NAADP-binding protein using a batch-binding method. When the NAADP-binding protein was not photolabeled it was retained by the phosphoprotein resin, whereas probe-bound NAADP-binding protein passed through (fig. S1, B). This property was exploited to collect unlabeled eluate following an initial fractionation and elution from the phosphoprotein resin. The unlabeled fraction was then photolabeled with a saturating concentration of alkyne-AIOC-NAADP (which was spiked with a traceable amount of $[^{32}\text{P}]$ -alkyne-AIOC-NAADP) to ensure that all NAADP-binding sites were occupied. Probe-bound samples were then incubated with fresh phosphoprotein enrichment resin for a second round of fractionation (Fig. 2, C and D and fig. S1, C). Probe-bound NAADP-binding proteins were biotinylated using a copper-catalyzed alkyne-azide cycloaddition (CuAAC) using ‘click chemistry’ and the biotinylated proteins captured using neutravidin. This protocol resulted in the isolation of a ~23kDa protein detectable by both silver stain and phosphorimaging (Fig. 2E).

Gel bands from both input control samples and biotin pulldowns were excised and processed for mass spectrometry. Inspection of total spectra revealed JPT2 as the most highly enriched protein (Table 1). Few JPT2 spectra were detected in input control samples, whereas >70-fold enrichment of JPT2 spectra was detected in CuAAC biotin-pulldown samples. No JPT2 spectra were detected in non-biotinylated CuAAC control samples. Moreover, nearly complete peptide coverage of JPT2 was detected (186 out of 190 amino acids). As an alternative ranking measure, total spectral counts from protein hits were processed to yield normalized spectral abundance factor (NSAF) values. Hits were ranked after NSAF values for non-biotinylated control samples were subtracted from NSAF values for the CuAAC biotin-azide-Plus pulldown samples, which again ranked JPT2 as the top NAADP-binding protein candidate (Fig. 2F). Immunoblotting of erythrocyte chromatography fractions confirmed a progressive enrichment of JPT2 during the serial chromatography procedure (Fig. 2G).

JPT2 properties and expression profile—JPT2 is a short, highly basic protein containing four repeated consensus (‘PPGGxxSxxF’) sequences, with an additional motif (‘MASNIF’) characteristic of this gene family located between the first two of these repeats (Fig. 3A, (16)). *JPT2* mRNA is broadly expressed in human cell lines but is particularly

abundant in human cell lines (SKBR3 (7), U2OS (15)) commonly used to study endogenous NAADP-evoked Ca^{2+} signals (Fig. 3A). In terms of evolutionary pedigree, both members of this conserved gene family (*JPT1* and *JPT2*) are present in numerous vertebrates (21). However, only a single *JPT* gene is present in basal chordates, echinoderms and most protostomal phyla (Fig. 3B). Identification of JPT homologues in sea squirts, sea urchins, starfish, and sea slugs is consistent with functional NAADP responses in these organisms (22–25). JPTs are notably absent in the round worm (*C. elegans*) as are TPCs (Fig. 3B). However, JPT isoforms appear to be absent in platyhelminths despite the presence of TPCs (Fig. 3B). Conversely, a *JPT* gene is present in fruit flies (*Drosophila*) in which TPCs are absent (Fig. 3B). *Drosophila* Jupiter (which has ~27% sequence identity with human JPT2) is a microtubule-associated protein (26) whereas JPT2 is localized to the cell surface, cytoplasm and nucleus in human cells (21, 27, 28).

Knockdown and pulldown of endogenous JPT2—To validate JPT2 as the photolabeled NAADP-binding protein, we used two independent siRNAs to knockdown endogenous JPT2 in HEK293 and U2OS cells. Lysates from siRNA-treated cells were photolabeled, and the relative intensity of photolabeling patterns assessed. Cells transfected with either of two *JPT2*-specific siRNAs exhibited reduced photolabeling of the endogenous ~23kDa NAADP-binding protein (decrease of ~60% in HEK293, ~50–65% in U2OS, Fig. 4, A to D). In contrast, transfection of two non-targeting siRNAs did not reduce photolabeling of the ~23kDa NAADP-binding protein (Fig. 4, A to D). JPT2 knockdown was verified by Western blotting (fig. S2, A and B). JPT2 immunoprecipitated with either of two independent antibodies from erythrocytes (S20) and U2OS cells (S200 lysates, (20)) were probed with [^{32}P]-alkyne-AIOC-NAADP. Two different JPT2-specific antibodies, but not an isotypic control IgG antibody, pulled down the 23kDa NAADP-binding protein (Fig. 4, E to H). Therefore, both knockdown and immunoprecipitation of endogenous JPT2 validated its identity as the ~23kDa protein labelled by the NAADP-derived photoprobe.

Recombinant JPT2 selectively binds NAADP—To interrogate whether JPT2 acts a NAADP-binding protein using an orthogonal approach, we performed [^{32}P]-NAADP binding experiments to recombinant human JPT2 isolated from *E. coli*. Recombinant JPT2 was incubated with [^{32}P]-NAADP in the presence of increasing NAADP or NADP concentrations and binding determined after filtration (Fig. 5A). Densitometry revealed JPT2 selectively bound NAADP, with displacement of bound [^{32}P]-NAADP being ~17-fold more potent with NAADP ($\text{IC}_{50}=20\pm3.6\text{nM}$) than with NADP ($\text{IC}_{50}=334\pm76\text{nM}$, Fig. 5B).

JPT2 is a TPC1 accessory protein that regulates responsiveness to NAADP—

Does JPT2 interact with TPCs to regulate NAADP-sensitive Ca^{2+} release? First, to assess TPC interactivity, reciprocal immunoprecipitations were performed. In HEK293 cells overexpressing GFP-tagged TPC constructs, the pull-down of endogenous JPT2 resulted in co-immunoprecipitation of TPC1-GFP but not TPC2-GFP (Fig. 6A). Reciprocally, pull-down of GFP-tagged TPC1, but not TPC2, resulted in co-immunoprecipitation of endogenous JPT2. Second, to assess the impact of JPT2 on endogenous NAADP-evoked Ca^{2+} signals, Ca^{2+} imaging experiments were performed in single cells directly microinjected with NAADP. In U2OS cells, microinjection of NAADP, but not buffer alone,

evoked a rapid Ca^{2+} transient, as shown previously (20). This NAADP-evoked Ca^{2+} signal was similar in cells treated with two unique control siRNAs (Fig. 6, B and C). However, in cells treated with either of the dual siRNAs targeting endogenous JPT2, the amplitude of Ca^{2+} signals caused by NAADP microinjection was inhibited (Fig. 6, B and C). The size of NAADP responses in cells treated with siRNA targeting JPT2 was not statistically different from signals evoked by buffer injection (Fig. 6C).

JPT2 regulates coronavirus entry—NAADP-evoked Ca^{2+} release is important for controlling the trafficking of pathogens within the endolysosomal system that are internalized as part of their infective cycle (4–6, 29, 30). For example, we have demonstrated that TPCs regulate endolysosomal translocation of a Middle East Respiratory Syndrome pseudovirus (MERS-CoV, (5, 30)). Is JPT2, as an essential component of NAADP-evoked Ca^{2+} responsiveness (Fig. 6, B and C), also required for CoV infectivity? To investigate this issue, we analyzed the role of JPT2 and the TPC complex in the translocation of a SARS-CoV-2 pseudovirus. Viral translocation trafficking was monitored using a reporter assay (5, 30, 31) in which the pseudovirus generates a luminescence signal after release into the cytoplasm, thereby reporting the efficiency of subcellular translocation events (which includes ACE2 receptor binding, internalization, endolysosomal processing and transit, and cytoplasmic release). First, we tested the effect of the Ca^{2+} chelating agent BAPTA-AM, which resulted in nearly complete inhibition of SARS-CoV-2 pseudovirus infectivity, indicating that intracellular Ca^{2+} signaling plays an essential role for SARS-CoV-2 cellular entry. Next, we analyzed the effects of previously screened inhibitors of NAADP-evoked Ca^{2+} release. Tetrandrine, a TPC blocker (4), effective against replication-competent SARS-CoV-2 (32), caused a marked inhibition of SARS-CoV-2 pseudovirus translocation (Fig. 7A). Four other anti-MERS-CoV compounds that inhibit NAADP-evoked Ca^{2+} release (PF-543, SKF96365, racecadotril and salmeterol (30)) also inhibited cell entry of the SARS-CoV-2 pseudovirus (Fig. 7A). However, drugs targeting IP₃Rs (2-APB, xestospongine) or RyRs (dantrolene, ryanodine) had no effect (Fig. 7B).

Loss of function analyses were then performed using siRNAs targeting either JPT1, JPT2, TPCs, or the endolysosomal ion channel TRPML1 (Fig. 7C). Whereas independent control siRNAs or dual siRNAs targeting JPT1 or TRPML1 did not inhibit SARS-CoV-2 pseudovirus translocation, knockdown of endogenous JPT2 markedly inhibited SARS-CoV-2 infectivity, decreasing luminescence to values comparable seen with siRNAs targeting TPCs (Fig. 7B). Inhibition of SARS-CoV-2 was specific to pseudoviral translocation through the acidic Ca^{2+} stores (mediated by spike protein engagement of ACE2), because overexpression of the membrane-anchored protease TMPRSS2 (to cleave the S protein into a fusogenic state at the cell surface) circumvented the observed inhibition (Fig. 7E).

Collectively, these data show that JPT2 is both a NAADP-binding protein (Fig. 5, A and B) and TPC accessory protein (Fig. 6A). JPT2 function is essential for conferring NAADP sensitivity in human cells, as shown by its role in NAADP-evoked Ca^{2+} signaling (Fig. 6B) and SARS-CoV-2 cell entry, a NAADP-dependent cellular process (Fig. 7, B and D).

Discussion

Two advances facilitated the unmasking of JPT2 as the ~23kDa NAADP-binding protein previously identified in human cells (16, 18). The first was the synthesis of a bifunctional probe (^{32}P -alkyne-'all-in-one clickable'-NAADP) to selectively photolabel and then enrich NAADP-binding proteins. The second was the serendipitous discovery that human erythrocytes exhibited strong and selective photolabeling of the ~23kDa NAADP-binding protein. The latter was a surprising finding given the lack of cytoplasmic organelles and intracellular Ca^{2+} stores in mature red blood cells (33), although NAADP has been shown to be present (34). The erythrocyte NAADP-binding protein exhibited identical characteristics to the previously characterized mammalian ~23kDa NAADP-binding protein including a characteristic pharmacology (Fig. 1E) and selectivity for NAADP over NADP (Fig. 1G (9, 16–20)). Consistent with the required properties of a Ca^{2+} release mechanism that depends on an NAADP-binding protein (14, 35), JPT2 binds to NAADP with nanomolar affinity (Fig. 5, A and B), associates with TPC1 (Fig. 6A) and is required for NAADP-evoked Ca^{2+} signals in U2OS cells (Fig. 6, B and C) and NAADP-dependent endolysosomal trafficking processes (Fig. 7, B and D). These data establish JPT2 as an essential component of an NAADP-binding protein complex that confers endogenous NAADP sensitivity to TPCs.

JPT2 (also known as hematological and neurological expressed 1-like, HN1L) was originally identified in a mouse fertilized egg library (21) and is broadly expressed in human primary tissues and cell lines (Fig. 2G, (11, 19–21, 36)). The JPT gene family (*JPT1* [*HN1*] and *JPT2* [*HN1L*]) is also evolutionarily conserved (Fig. 3B, (21)) although comparatively little is known about the roles of these family members. JPT2 contains many positively charged residues which may be relevant for NAADP binding, given the presence of negatively charged pyrophosphate, phosphate, and carboxylate groups in NAADP at physiological pH. Further work will be needed to define this interaction in detail, and to investigate the resemblance of the repeat motifs in JPT2 to canonical NA(A)DP-binding sequences (37).

Using T cells, Roggenkamp *et al.* also identified HN1L/JPT2 as a photolabeled NAADP-binding protein (38) that is critical for the generation of localized Ca^{2+} microdomains observed in the earliest phases of T cell activation (39). These localized Ca^{2+} microdomains appeared to depend on NAADP-dependent activation of the ryanodine receptor isoform 1 (RyR1) in the ER (40). Knockdown of *HN1L/JPT2* in both human Jurkat cells as well as rat primary T cells impaired local, and consequently global Ca^{2+} signals, evoked by T cell receptor and CD3 stimulation (39). Therefore, studies performed in different cell types both converge to implicate JPT2 as a component of NAADP receptor but diverge in their observations that JPT2 confers NAADP sensitivity to different types of intracellular Ca^{2+} channel: TPCs (this study) or RyRs (38). The hypothesis that NAADP-binding proteins may act to confer NAADP-sensitivity to different families of intracellular Ca^{2+} channel, an attractive idea in this field for over a decade (14, 35, 41), receives experimental support from this pair of studies.

Considering the shared pedigree between TPCs and other members of the voltage-gated ion channel family (Ca_v , Na_v , K_v , (42)), the idea of a multifunctional accessory subunit that

binds to NAADP is not unreasonable. The function of each major subfamily of voltage-operated channels is modulated by accessory subunits (14). Many of these accessory protein subunits are promiscuous with physiological roles dependent on, and independent from, their ion channel partners. Many ion channel accessory proteins have ligand-binding sites, such as FKBP which bind to IP₃Rs and RyRs (43); $\alpha_2\delta$ subunits which bind to Ca_v (44); nucleotides, which bind to K_{ATP} K⁺ channels (45); and sigma receptors which bind to Kv1.4 (46)). A pertinent example is the Na_v channel β subunit, which is both promiscuous and fulfills physiological roles independent of the pore-forming subunit in both excitable and non-excitable cells (47). This provides a precedent for JPT2 acting independently from TPCs (in organisms such as *Drosophila* lacking TPCs, Fig. 3B) or TPCs functioning without NAADP-activation (acting solely as PI(3,5)P₂-gated channels (10)). The ability for JPT2 to associate with both TPC1 and RyR1 is also intriguing in the context of close coupling of these ion channels at tightly apposed membrane contact sites between acidic Ca²⁺ stores and the ER that amplify local NAADP-evoked Ca²⁺ signals into global, whole cell Ca²⁺ signals (48). The preferential association of JPT2 with TPC1 compared with TPC2 also raises the possibility that multiple NAADP-binding proteins may exist to confer NAADP sensitivity and possibly unique functionalities to TPC isoforms. Identification of JPT2 now provides a molecular handle to interrogate these possibilities.

The identification of JPT2 as the photolabeled NAADP-binding protein also implicates a role of NAADP-evoked Ca²⁺ signaling in processes for which a functional requirement for JPT2/HN1L has been already established. JPT2 interacts with multiple signaling pathways (49–51), but a consistent theme is a role in cell growth and tumorigenesis. JPT2 overexpression stimulates cell proliferation, tumor growth and metastasis, whereas JPT2 knockdown suppresses cell growth and migration (49–51). JPT2 expression is elevated in cancer tissues compared with matched controls and this upregulation correlates with poorer patient survival outcomes in several types of cancer (49–52), including non-small cell lung cancer (49, 52), hepatocellular carcinoma (50) and triple negative breast cancer (51). These findings are of special interest given an emerging focus on NAADP-evoked Ca²⁺ signaling in cancer cell growth (8, 53, 54).

A second area of pathophysiological relevance, underscored by our data (Fig. 7, B and C), relates to viral infection. JPT2/HN1L inhibits apoptosis in response to viral infection (27). JPT2/HN1L also localizes to Nipah viral particles (a zoonotic paramyxovirus) within host cells (55). Here, we showed that JPT2 supported the endolysosomal translocation but not direct cell surface entry of a SARS-CoV-2 pseudovirus (Fig. 7B), data that extend our previous discovery of a role of the TPC complex in coronaviral infectivity (5, 30). Pharmacological blockade of NAADP-evoked Ca²⁺ release (5, 32) or knockdown of JPT2 (Fig. 7B) impaired the endolysosomal translocation of two, different coronaviral spike proteins (MERS and SARS-CoV-2). Given the endolysosomal processing of SARS-CoV-2 resembles that of MERS-CoV (31), this is not unexpected and provided good rationale to identify ligands that block JPT2 function and NAADP action as broader anti-CoV therapeutics (7). This additional insight into the role of the JPT2/TPC complex in SARS-CoV-2 translocation holds particular importance given the current COVID-19 pandemic which has infected >100 million people, with more than two million fatalities at the time of writing.

In summary, the identification of JPT2 will further our understanding of the mechanism of NAADP action, aiding analysis of how NAADP sensitivity is conferred to TPCs and possibly other intracellular Ca^{2+} channels (14, 35). Resolution of JPT2 as an endogenous mediator of NAADP signaling also opens new opportunities to understand the roles of this understudied gene family in Ca^{2+} signaling processes in both normal physiology and disease states.

Materials and Methods

Sample preparation.

Human erythrocytes were collected from outdated blood bags supplied by the University of Minnesota Blood Bank Laboratory. Erythrocytes were washed 3 times in 0.9% saline solution. Packed erythrocytes were then lysed in 3 volumes of hypotonic solution of 10mM Tris-HCl, pH 8.5, supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche). Erythrocyte lysate was centrifuged at 20,000xRCF for 30 minutes, and the supernatant (S20) was reserved for further processing. Protein concentration was determined by Bradford assay (Thermo Scientific). Jurkat, U2OS, and HEK293 lysates were prepared as previously described (16, 18, 20). Briefly, cells were washed with PBS and collected by centrifugation and scraping. Cell pellets were suspended in 20mM HEPES, pH 7.4 supplemented with protease inhibitors. Cell suspensions were sonicated to produce whole cell lysates, and subsequently centrifuged at 200,000xRCF. Supernatant (S200) fractions were collected for analysis.

Photoaffinity labeling of NAADP-binding proteins.

When determining a scheme for chromatographic enrichment of NAADP binding proteins, 5 μ g of fractionated protein was incubated with ^{32}P -alkyne-AIOC-NAADP at a final concentration of 1–7nM, and reactions were incubated on ice for 1 hour prior to 2 minutes of ultraviolet irradiation. When photolabeling protein prior to click chemistry-based purification, fractionated protein was separately incubated with either alkyne-AIOC-NAADP at a final concentration of 1 μ M or incubated with ^{32}P -alkyne-AIOC-NAADP at a final concentration of 5nM. All reactions were incubated on ice for 1 hour prior to 2 minutes of ultraviolet irradiation and mixed again prior to click chemistry experiments. Photolabeled protein was incubated (<15min) with SDS sample buffer supplemented with 2-mercaptoethanol (10%). Samples were separated by SDS-PAGE on 12% TGX gels (BioRad). Gels were silver stained using ProteoSilver (Sigma Aldrich) and air-dried. The photolabeling was analyzed by exposing the dried gels to MP storage phosphor screens (Packard Instruments). The screens were developed using a Typhoon storage phosphor system. Densitometric analysis was accomplished using Image J software.

Chromatographic enrichment of NAADP-binding proteins.

Human erythrocyte S20 supernatant was fractionated over various resins as follows. For anion exchange chromatography, a column of Q Sepharose Fast Flow (GE Healthcare) resin was equilibrated with 10mM HEPES, pH 8.5. Protein samples were pumped onto the column at approximately 50mg of protein/ml resin. Retained protein was washed with 3 column volumes of 10mM HEPES + 50mM NaCl, pH 8.5. Protein was eluted with 10mM

HEPES + 150mM NaCl, pH 8.5. For cation exchange chromatography, a column of SP Sepharose Fast Flow (GE Healthcare) resin was equilibrated with 10mM HEPES, pH 7.0. Protein samples were adjusted to pH 7.0 and pumped onto the column at approximately 50mg protein/ml resin. Retained protein was washed with 3 column volumes of 10mM HEPES + 30mM NaCl, pH 7.0. Protein was eluted with 10mM HEPES + 100mM NaCl, pH 7.0. For hydrophobic interaction chromatography (HIC), Phenyl-Sepharose CL-4B (GE Healthcare) resin was equilibrated with 1.5M $(\text{NH}_4)_2\text{SO}_4$, pH 7.0. Protein samples were supplemented with 1.5M $(\text{NH}_4)_2\text{SO}_4$ and pumped onto the column. Retained protein was washed with 3 column volumes of 1.5M $(\text{NH}_4)_2\text{SO}_4$, pH 7.0, and protein was eluted in 600mM $(\text{NH}_4)_2\text{SO}_4$, pH 7.0. For Fe^{3+} -NTA fractionation, Ni^{2+} -NTA resin (Qiagen) was stripped according to vendor protocols and re-charged with 0.2M FeSO_4 . Fe^{3+} -NTA resin was equilibrated with 300mM NaCl + 5mM imidazole, 50mM HEPES, pH 8.0. Protein samples were diluted 1:10 in equilibration buffer and pumped onto the column, which was washed with 5 volumes of equilibration buffer; flowthrough was collected. For “serial” chromatography, approximately 8g of RBC S20 was sequentially fractionated over Q, SP, Phenyl, and NTA resins, in that order. Samples were desalted and concentrated using Amicon Ultra 3kDa MWCO spin filters between each step. Phosphoprotein Enrichment Kit (Pierce) was used according to vendor protocol for the final stage of enrichment. Protein was incubated with phosphoprotein enrichment resin, and flowthrough and eluates were collected. Samples were desalted and concentrated prior to photoaffinity labeling as described above. Photolabeled protein samples were then incubated with fresh phosphoprotein enrichment resin for a second time, and flowthrough and eluate fractions were collected.

Click chemistry and capture of NAADP-binding proteins.

After sequential chromatographic fractionation and enrichment, protein samples with probe-bound NAADP-binding proteins were biotinylated using click chemistry. Approximately 250 μg of protein was diluted into 20mM HEPES, pH 7.4, and supplemented with either 10 μM Dde-biotin-picolyl-azide, 10 μM biotin-azide-Plus or DMSO. A 40x stock of 60mM BTAA and 12mM Cu_2SO_4 was prepared in a separate tube. Click reactions were sequentially supplemented with 5mM aminoguanidine, 1.5mM BTAA/300 μM CuSO_4 , and 5mM ascorbic acid, with brief vortexing after each addition. Reactions were incubated for 2 hours at room temperature in an end-over-end mixer. Click reagents were removed by buffer exchange using 3kDa MWCO ultrafiltration spin columns. Biotinylated protein was captured using 100 μl of Neutravidin agarose beads (Thermo Scientific) at 4°C overnight in an end-over-end mixer. Beads were washed twice with RIPA buffer (50mM Tris base, pH 8.0, 150mM NaCl, 0.1% SDS, 1% triton-X-100, 0.5% deoxycholate), twice with PBS + 1% triton-X-100, once with 1M KCl, twice with 100mM NaCO_3 pH 11.5 (excluded with Dde-biotin-picolyl azide samples and corresponding controls), twice with 2M urea, once with RIPA, and three times with PBS. Proteins from CuAAC reactions containing Dde-biotin-picolyl-azide and corresponding controls were eluted by suspending samples in 3% hydrazine + 0.05% SDS and incubating for 30min at room temperature in an end-over-end mixer. Proteins from CuAAC reactions containing biotin-azide-Plus and corresponding controls were eluted by suspending samples in 10mM D(+)-biotin + 10mM EDTA and incubated in a water bath at 90°C for 10min. Supernatants from both sets of reactions were

collected, and elution was repeated two more times. Eluates were concentrated and buffer exchanged to 20mM HEPES, pH 7.4 using 3kDa MWCO spin columns. Eluates were then separated by SDS-PAGE using a 12% Critereon TGX gel and silver stained. The gels were dried overnight and mounted on a phosphorscreen for detection of radiolabeled NAADP binding proteins. Silver-stained bands that aligned with ^{32}P signals were excised and analyzed by mass spectrometry, along with corresponding bands from non-biotinylated click control samples and input samples.

Mass spectrometry.

Proteomic analysis of gel bands was conducted by MS BioWorks (Ann Arbor, Michigan). In-gel digestion was performed using a ProGest robot (DigiLab) with the following protocol. Samples were washed with 25mM NH_4HCO_3 and subsequently with acetonitrile, reduced with 10mM dithiothreitol at 60°C, and alkylated with 50mM iodoacetamide at room temperature. Samples were digested with trypsin (Promega) at 37°C for 4 hours. Digestions were quenched with formic acid and supernatants collected for analysis. Half of each digested sample was analyzed by nano LC-MS/MS with a Waters M-Class HPLC system interfaced to a ThermoFisher Fusion Lumos mass spectrometer. Peptides were loaded on a trapping column and eluted over a 75µm analytical column at 350nL/min; both columns were packed with Luna C18 resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 60,000 FWHM and 15,000 FWHM for MS and MS/MS respectively. The instrument was run with a 3s cycle for MS and MS/MS. Data were searched using a local copy of Mascot (Matrix Science) with the following parameters: enzyme, trypsin/P; databases, SwissProt Human (concatenated forward and reverse plus common contaminants); fixed modifications, carbamidomethyl (C); variable modifications, acetyl (N-term), deamidation (N,Q), oxidation (M), and Pyro-Glu (N-term Q); mass values, monoisotopic; peptide mass tolerance, 10 ppm; fragment mass tolerance, 0.02 Da; max missed cleavages, 2. Mascot DAT files were parsed into Scaffold (Proteome Software) for validation, filtering and creation of a non-redundant list per sample. Data were filtered using at 1% protein and peptide FDR and at least two unique peptides were required per protein. Relative protein abundance was assessed by calculating normalized spectral abundance factor (NSAF) values (56).

$$(\text{NSAF})_k = \frac{\left(\frac{\text{SpC}}{\text{Mw}}\right)_k}{\sum_{i=1}^n \left(\frac{\text{SpC}}{\text{Mw}}\right)_i}$$

Briefly, total spectral counts (SpC) for an identified protein (k) were normalized to protein size by dividing spectral counts by the predicted mass of the intact protein (M_w), yielding a spectral abundance factor (SAF). Next, SAF values for individual identified proteins were divided by the sum of all SAF values for each identified protein in a given biological sample (n).

Cell culture and transfection.

Jurkat cells were maintained in RPMI medium, U2OS and HEK293 cells were maintained in DMEM. Cell culture medium was supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, and 292 µg/ml L-glutamine, and cells were cultured at 5% CO₂ and 37°C. For plasmid transfection, 3.5×10⁵ U2OS or HEK293 cells were seeded in a 35mm well in the absence of antibiotics. The following day, cells were transfected with siRNA (1–20nM) using RNAiMax (Thermo) according to the vendor's protocol. Media was changed 24 hours post transfection and cells were harvested by scraping 48 hours post transfection.

Ca²⁺ imaging.

U2OS cells were incubated with fura-2 AM (5 µM, Invitrogen) in Hanks' balanced salt solution (HBSS, room temperature, 45 min in the dark), washed, and incubated for another 45 min to allow for de-esterification. Coverslips were mounted in a custom-designed bath on the stage of an inverted microscope (Nikon Eclipse TE 2000-U, 40× NA 1.3 oil immersion objective). Cells were superfused (at a flow rate of ~0.5 ml/min) with HBSS and fluorescence emission (510nm) was captured with a charge-coupled device camera (Roper Scientific) after alternate excitation at 340 and 380 nm. Captured images were analyzed using MetaFluor software. Single cell microinjections were performed as previously described (11) using Femtotips II, an InjectMan NI 2 and a Femtojet systems (Eppendorf). Pipettes were back filled with an intracellular solution composed of 110mM KCl, 10mM NaCl and 20 mM Hepes, pH 7.2, and supplemented with NAADP (10µM). siRNA-transfected cells were selected by identifying cells displaying similar intensities of GFP fluorescence.

Immunoblotting and immunoprecipitation.

To immunoprecipitate photolabeled NAADP- binding proteins, erythrocyte S20 and U2OS S200 lysates were incubated with either 2µg/ml rabbit IgG isotype control antibody (Thermo Scientific), 0.5µg/ml anti-HN1L antibody produced in rabbit (Sigma Aldrich, HPA041908, 'anti-JPT2 #1'), or 2µg/ml anti-HN1L antibody produced in rabbit (Thermo Scientific, PA5–59774, 'anti-JPT2 #2') at 4°C for 1 hour before an overnight incubation with protein-G agarose beads (Roche). For co-immunoprecipitation experiments, HEK293 cells overexpressing TPC1-GFP or TPC2-GFP were solubilized in buffer composed of 110mM KCl, 10µM CaCl₂, 1% Triton X-100, 20mM HEPES, pH7.4 supplemented with Halt phosphatase inhibitor (Pierce) and cOmplete EDTA-free protease inhibitor cocktail (Roche). Lysates were centrifuged at 16,000xRCF for 10min/4°C, and supernatant was collected. 1mg of solubilized lysate was incubated with 2µg/ml of either rabbit IgG isotype control antibody, anti-GFP antibody produced in rabbit (Thermo Scientific, G10362), or anti-HN1L antibody produced in rabbit (Sigma Aldrich, HPA041908 at 4°C for 1 hour before an overnight incubation with protein-G agarose beads (Roche). Beads were collected after brief centrifugation and were washed three times with PBS + 1% Triton X-100. For photolabeling experiments, immunoprecipitated protein was eluted by rinsing beads three times with 0.2M glycine pH 2.6 for 1min and rinses were immediately neutralized with 2M Tris base. Immunoprecipitated protein was concentrated and desalted. For co-immunoprecipitation experiments, immunoprecipitated complexes were eluted by incubating beads with 2x

Laemmli sample buffer at 95°C for 10min. For immunodetection of proteins, 20µg of HEK293 or U2OS whole-cell lysate was separated by SDS-PAGE and transferred to nitrocellulose membranes using standard methods. Nitrocellulose membranes were blocked in 5% milk in tris buffered saline supplemented with 0.1% tween-20 for 1 hour at room temperature. Membranes were then incubated with primary antibodies at a concentration of 0.05µg/ml (anti-HN1L, Sigma Aldrich, HPA041908 and anti-GAPDH, Santa Cruz Biotechnology, sc-47724) overnight at 4°C. The following day membranes were incubated with IRDye secondary antibodies (1:5000 dilution, LI-COR) for 1 hour at room temperature. Signals were detected using a LI-COR Odyssey Imaging system.

Production of recombinant JPT2.

cDNA encoding JPT2 (Uniprot ID# Q9H910–3) fused with an N-terminal 6xHis-GST-TEV protease cleavage site was subcloned into pGS-21a bacterial expression vector and transformed into BL21 Star (DE3) *E. coli* cells. A single colony was inoculated into TB medium and cultured at 37°C. When the OD₆₀₀ reached 1.2, the culture was induced with IPTG at 15°C for 16h. Cells were harvested by centrifugation and cell pellets were resuspended with lysis buffer followed by sonication. Protein was loaded onto a Ni-NTA column and 6xHis-GST-TEV-JPT2 was eluted with 400mM imidazole. Protein was treated with TEV protease to remove N-terminal tags, and untagged JPT2 was collected by loading onto a Ni-NTA column and eluting with 10mM imidazole, samples were concentrated, and buffer was exchanged using 20mM HEPES pH 7.4 and 3kDa MWCO ultrafiltration spin columns.

³²P-NAADP binding assays.

Recombinant JPT2 (20µg) was incubated with [³²P]-NAADP (~0.33nM) in the presence of increasing concentrations of cold NAADP or NADP for 1 hour on ice. During the incubation, multiScreen-IP 96-well PVDF plates (Millipore) were mounted on a vacuum manifold. PVDF membranes were wetted with ethanol, followed by two washes with 20mM HEPES, pH 7.4. Binding reactions were transferred to 96-well PVDF plates and filtered through PVDF membranes. Membranes were washed 3 times with ice-cold 20mM HEPES, pH 7.4. Rubber gaskets were removed from the underside of 96-well PVDF plates, and PVDF wells were dried with paper towels. PVDF plates were then placed on phosphor screens, subsequently processed using a Typhoon phosphor storage system. Bound [³²P]-NAADP was quantified by densitometry using ImageJ.

SARS-Cov-2 pseudovirus translocation assays.

Cell infection assays were carried out as described previously for MERS-CoV (5, 57). Spike-pseudotyped retroviruses expressing a luciferase reporter gene were prepared by co-transfecting HEK293T cells with a plasmid carrying Env-defective, luciferase-expressing HIV-1 genome (pNL4–3.luc.RE) and a plasmid encoding SARS-CoV-2 spike (S) protein, which is necessary and sufficient to facilitate cell entry. SARS-CoV-2 pseudovirus particles were harvested from supernatant 72hrs after transfection. HEK-293 cells (overexpressing ACE2) were used to monitor SARS-CoV-2 pseudovirus translocation. Cells were seeded into 96-well plates (Midwest Scientific) at a concentration of 1×10⁴ cells/well. The following day, cells were pre-incubated with individual drugs (10µM, final concentration)

for 1 hour prior to pseudovirus addition. Cells were incubated (5% CO₂/37°C) for an additional 5 hours in the presence of drug and pseudovirus. After 6hrs, the culture media was replaced with complete DMEM and cells were incubated for a further 60hrs. Cells were then washed 3 times with DPBS (Invitrogen) and assayed for luciferase activity. Cells were lysed in 80µl lysis buffer (Promega) per well, and 40µl of lysate was transferred to solid-white 96-well plates (Corning) and mixed with 40µl of luciferase substrate (Promega). Luminescence (relative luminescence units, RLUs) were measured using a Tecan Infinite M100 microplate reader. Luminescence values are reported relative to values measured in cells treated with virus alone, background corrected by luminescence values in cells unexposed to virus, except where indicated.

Statistical analysis.

A univariate regression model with type as a categorical covariate was used to evaluate differences in intensity between type of samples. Type was coded as a categorical variable with the reference category being the control state or appropriate group against which others were being compared. The coefficients calculated from this regression model were used to calculate the *P* statistic (* *P*<0.05, ** *P*<0.01, *** *P*<0.005).

Chemicals and molecular reagents.

NAADP was synthesized by incubating nicotinamide adenine dinucleotide phosphate (NADP, Sigma-Aldrich) with nicotinic acid in the presence of recombinant *Aplysia* ADP-ribosyl cyclase (58) and purified by high-performance liquid chromatography (HPLC). The bifunctional probe (alkyne-AIOC-NAADP, fig. S3) was synthesized as described in the Materials and Methods in the Supplementary Materials. NADP was freshly purified by HPLC prior to experimentation to remove contaminating NAADP (17). Nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide (NAD), nicotinic acid adenine dinucleotide sodium salt (NAAD), adenosine 5'-triphosphate disodium salt hydrate (ATP), β-nicotinamide ribose monophosphate (β-NMN) and D(+)-biotin were purchased from Sigma Aldrich. 2-[4-((bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl)-1H-1,2,3-triazol-1-yl]acetic acid (BTAA), Dde-biotin-picolyl-azide and biotin-azide-Plus were purchased from Click Chemistry Tools (Scottsdale, AZ). Silencer Select siRNAs targeted against JPT2 and non-targeting negative control siRNAs were purchased from Thermo Scientific. siRNA sequences were: JPT2 siRNA #1, GAACCAAAUCGGAUCUUAtt; JPT2 siRNA #2, CCAAGGAUCAUGUUUCUUtt.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024153 and 10.6019/PXD024153. All other data needed to evaluate the conclusions in this study are present within the paper or the Supplementary Materials.

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Fig. 1. Characterization of NAADP-binding proteins in human erythrocytes.

(A and B), Red blood cell (RBC), Jurkat, and U2OS whole cell lysates (WCL) were photolabeled with $[^{32}\text{P}]$ -alkyne-AIOC-NAADP (3nM) in the absence or presence of competing unlabeled NAADP (10 μM). (C and D), RBC WCL, S20 cytosol fraction, and "ghost" membrane preparations were photolabeled with $[^{32}\text{P}]$ -alkyne-AIOC-NAADP (3nM) in the absence or presence of competing unlabeled NAADP (10 μM). (E and F) RBC S20 was photolabeled with $[^{32}\text{P}]$ -alkyne-AIOC-NAADP (3nM) in the presence of the indicated compounds (10 μM). (G and H) RBC S20 was photolabeled with $[^{32}\text{P}]$ -alkyne-AIOC-NAADP (3nM) in the presence of either NAADP or NADP at the indicated concentration. Data in (B, D, F, H) represent mean $\pm$ SD from densitometry analyses of $n = 3$ independent experiments, where representative gels for each specific assay (A, C, E and G) are shown. 30 μg of protein was used for all labeling reactions. Data are shown as mean $\pm$ SEM. Statistical significance was assessed using a univariate regression model (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$). A.U., arbitrary units.

Fig. 2. Biochemical enrichment and isolation of NAADP-binding proteins.

(A) Schematic representation of NAADP-binding protein enrichment. Red blood cell S20 proteins were sequentially fractionated in a series of chromatography steps: *i.* strong anion exchange (SAX) at pH 8.5, *ii.* strong cation exchange (SCX) at pH 7.0, *iii.* hydrophobic interaction chromatography (HIC), *iv.* immobilized metal affinity chromatography (IMAC). Eluates from a phosphoprotein enrichment resin were photoaffinity labeled, and fall-through was collected after passing protein through phosphoprotein enrichment resin a 2nd time (*v-vii*). (B) Structure of alkyne-AIOC-NAADP. Photolabile aryl azide (*) and 'clickable' alkyne (**) moieties are shown. (C) Silver stain (left) and phosphorimage (right) of 10µg of sample enriched with NAADP-binding protein. (D) Depiction of approach. NAADP-binding protein is crosslinked to the "clickable" alkyne-AIOC-NAADP photoprobe, which is biotinylated by copper-catalyzed azide-alkyne cycloaddition (CuAAC). Biotinylated protein is isolated using neutravidin agarose beads. (E) Probe-bound NAADP-binding protein was used in "click" chemistry reaction in absence or presence of biotin-azide-Plus. Biotinylated protein was captured with neutravidin agarose beads, stringently washed, and eluted. Silver stain (left) and phosphorimage (right) of eluates. Bands of interest are highlighted (blue box). (F) Gel bands of isolated NAADP-binding protein and corresponding bands from input samples and unbiotinylated controls were analyzed by mass spectrometry. Normalized

spectral abundance factor (NSAF) after click and capture minus NSAF of unbiotinylated control samples is shown. **(G)** Western blot detection of JPT2 in erythrocyte S20 supernatant after fractionation by SAX, SCX, HIC, IMAC, or sequential fractionation through each of these steps (serial).

Fig. 3. JPT2 expression profile in cells and organisms.

(A) mRNA expression profiling in 64 human cell lines ordered from high to low abundance for *JPT2* (black), *TPC1* (orange) and *TPC2* (blue). SKBR3 and U2OS cells are highlighted (yellow). Data and Image credit to the Human protein atlas (17): ENSG00000206053-JPT2/cell, available from v19.proteinatlas.org. a.u., arbitrary units. Inset, schematic of JPT2 structure to highlight basic residues (circle), four repeat motifs (blue) and the ‘MASNIF’ motif. (B) Phylogenomic profile of *JPT* genes. Schematic depicting the number of *JPT* and *TPC* homologues in major animal phyla. Organisms, from left to right (accession numbers for JPT): *Homo sapiens* (NP_653171.1 and NP_001002032.1), *Ciona intestinalis* (XP_009861954.1), *Branchiostoma floridae* (XP_002611678.1), *Strongylocentrotus purpuratus* (XP_011684190.1), *Asterias rubens* (XP_033631490.1), *Centruroides sculptratus* (XP_023222178.1), *Drosophila melanogaster* (Q9I7K0), *Caenorhabditis elegans*, *Aplysia californica* (XP_005102887.1), *Capitella teleta* (ELU08900.1), *Schistosoma haematobium*.

Fig. 4. Photolabeling of NAADP-binding proteins following immunoprecipitation or knockdown of JPT2.

(A to D) Knockdown of *JPT2* using two non-targeting control siRNAs and two discrete *JPT2*-specific siRNAs in HEK293 cells (A) and U2OS cells (C). Lysates were photolabeled with [32 P]-alkyne-AIOC-NAADP (7nM). Densitometry analysis (B and D) of samples from $n = 3$ independent knockdown experiments. (E to H) *JPT2* was immunoprecipitated from RBC S20 (E) and U2OS S200 (G) using control IgG or two discrete *JPT2*-specific antibodies. Protein-G beads were used to isolate antibody-protein complexes. Left, 10 μ g of

input samples and cleared supernatants after immunoprecipitation were photolabeled with [^{32}P]-alkyne-AIOC-NAADP (7nM). Right, immunoprecipitated protein was eluted and photolabeled with [^{32}P]-alkyne-AIOC-NAADP (7nM). Densitometry analysis (F and H) of photolabeling of immunoprecipitated protein from three independent experiments. Data in (B, D, F, H) are collected from $n = 3$ independent experiments and shown as mean $\pm$ SEM. Statistical significance was assessed using a univariate regression model (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

Fig. 5. Recombinant JPT2 binds to NAADP.

(A) Recombinant JPT2 was incubated with [³²P]-NAADP in the presence of increasing concentrations of unlabeled NAADP or NADP. Representative phosphorimage of multi-well plates used in binding experiments. (B) Quantification of bound [³²P]-NAADP in the presence of various concentrations of unlabeled NAADP (closed circle) or NADP (open circle). Data represents mean densitometry [³²P] values $\pm$ SD from $n = 3$ independent experiments.

Fig. 6. JPT2 interacts with TPC1 to regulate NAADP-evoked Ca^{2+} release.

(A) Co-immunoprecipitation of JPT2 with TPC1. Left, input controls of lysates from HEK293 cells overexpressing TPC1-GFP or TPC2-GFP. Middle, endogenous JPT2 immunoprecipitates immunoblotted for GFP to detect TPC1 or TPC2 and JPT2. Right, GFP immunoprecipitates immunoblotted for GFP to detect TPC1 or TPC2 and endogenous JPT2. Data show representative gels from $n = 3$ independent transfections. (B) Traces of intracellular Ca^{2+} flux in response to microinjection of buffer (black) or NAADP (blue, 10 μM pipette concentration) in individual U2OS cells transfected with two different non-targeting control siRNAs (ctrl siRNA #1 & #2, green) or JPT2-specific siRNAs (siRNA #1 & #2, red). Individual single cell responses are shown, with the averaged trace bolded. (C) Averaged peak amplitude $\pm$ SD (left) and average area under the curve (AUC, right) is shown from $n = 5$ independent cellular injections. P -values compared to NAADP injections in untransfected control cells. Data are shown as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Fig. 7. JPT2 regulates SARS-CoV-2 translocation through the endolysosomal system.

(A) Schematic to show different routes of SARS-CoV-2 cell entry mediated by ACE2 internalization and translocation through acidic Ca^{2+} stores (left) or fusion at the plasma membrane following spike protein activation by TMPRSS2 (right). (B) Luciferase activity was measured in HEK293 cells transduced with a luciferase-encoding SARS-CoV-2 pseudovirus and expressing ACE2 in the presence of the indicated compound (10 μ M), which inhibit IP_3 Rs (2-APB, xestospongol), RyRs (dantrolene, ryanodine), or NAADP-evoked Ca^{2+} release (tetrandrine, PF-543, SKF96365, racecadotril, and salmeterol). (C) Luciferase

activity was measured in HEK293 cells transduced with a luciferase-encoding SARS-CoV-2 pseudovirus and expressing TMPRSS2. Cells were treated as in (B). (**D** and **E**) Luciferase activity in HEK293 cells transfected with the indicated siRNAs and ACE2 (D) or TMPRSS2 (E), except where indicated (untransfected). Data represent results from $n = 3$ independent assays, with values shown as mean $\pm$ SEM. Statistical significance was assessed using a univariate regression model (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

Table 1.**Mass spectrometry dataset.**

Table depicts the total number of spectral counts (56) associated with each identified protein in the indicated experimental samples. Two independent biological replicates were analyzed for each condition (n1, n2). The first two columns present samples prior to treatment with click chemistry reagents ('input') and without neutravidin-agarose pulldown. The next four columns present samples treated with click reagents either in the absence ('negative control') or presence of hydrazine-cleavable Dde-biotin-picolyl-azide ('Dde-biotin') prior to neutravidin agarose pulldown and elution with hydrazine treatment. The final four columns present samples treated with click reagents either in the absence ('negative control') or presence of biotin-azide-Plus ('Plus-biotin') prior to neutravidin agarose pulldown and elution with excess free biotin. Table is ranked by total spectral count as the top twenty hits.

	Identified Protein		MW (kDa)	Input		Negative Control		Dde-biotin click		Negative Control		Plus-biotin click	
				n1	n2	n1	n2	n1	n2	n1	n2	n1	n2
1	Jupiter microtubule associated homolog 2	JPT2	20	3	7	0	0	231	234	0	0	40	23
2	Hemoglobin subunit alpha	HBA1	15	10	12	11	7	47	61	6	10	30	14
3	Hemoglobin subunit beta	HBB	16	10	16	6	3	35	41	5	15	26	18
4	Hemoglobin subunit delta	HBD	16	6	14	5	0	30	32	4	11	21	14
5	Junction plakoglobin	JUP	82	8	33	17	7	6	2	14	16	32	0
6	Flavin reductase (NADPH)	BLVRB	22	12	5	0	0	30	9	0	7	24	11
7	Peroxiredoxin-2	PRDX2	22	18	7	3	0	16	8	3	6	10	7
8	Ubiquitin-40S ribosomal protein S27a	RPS27a	18	19	5	0	0	18	4	3	7	9	3
9	Desmoglein-1	DSG1	114	2	13	5	4	0	0	8	9	20	4
10	Filaggrin-2	FLG2	248	7	11	2	0	5	0	8	7	25	0
11	L-xylulose reductase	DCXR	26	16	8	0	0	21	8	0	0	7	0
12	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	36	5	5	4	0	17	6	3	2	6	2
13	Actin, cytoplasmic 1	ACTB	42	4	2	0	8	5	0	7	7	12	0
14	Peroxiredoxin-1	PRDX1	22	7	5	0	0	10	0	0	0	11	6
15	Serum albumin precursor Isoform 1	ALB	69	6	0	5	4	0	0	7	8	9	0
16	Carbonic anhydrase 1	CA1	29	3	0	2	0	12	4	0	0	6	3
17	Hemoglobin subunit gamma-2	HBG2	16	4	0	0	0	18	0	0	0	6	0
18	Annexin A2	ANXA2	39	2	5	4	2	0	0	3	3	9	0
19	Carbonic anhydrase 2	CA2	29	0	0	3	0	14	4	0	0	5	2
20	Desmocollin-1	DSC1	100	0	3	6	0	0	0	2	5	8	0