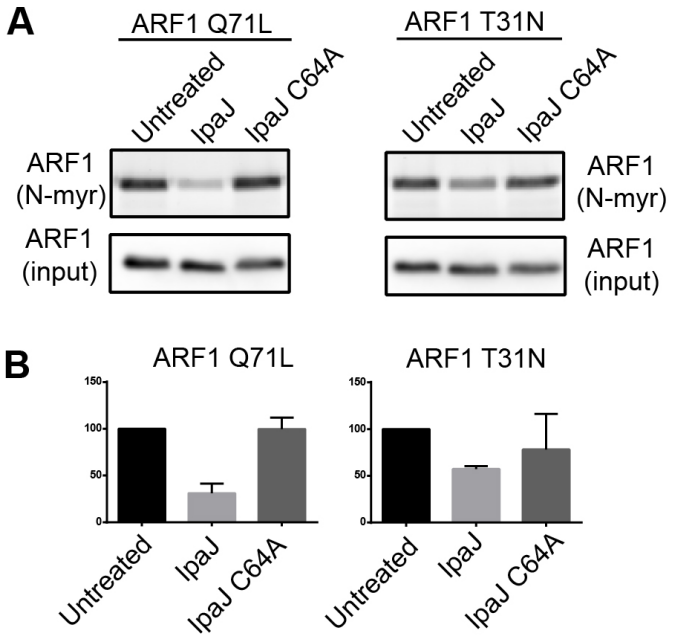


**Figure S1. IpaJ preferentially cleaves ARF1 in the active conformation. Related to Figure 5.**

A. In-gel fluorescence analysis shows myristoylated ARF1 T31N mutant (a GDP-locked mutant) or ARF1 Q71L (a GTP-locked mutant) expressed in HeLa cells and incubated with recombinant IpaJ or catalytic mutant. Equal loading of ARF1 (middle panel) was determined by western blot analysis.

B. Quantification of fluorescence in Figure S1A. Values are presented in arbitrary units (au). Signal of 'untreated' condition is normalized to 100 for both mutants. Fluorescent signal was quantified in three experiment repetitions using ImageJ.

.

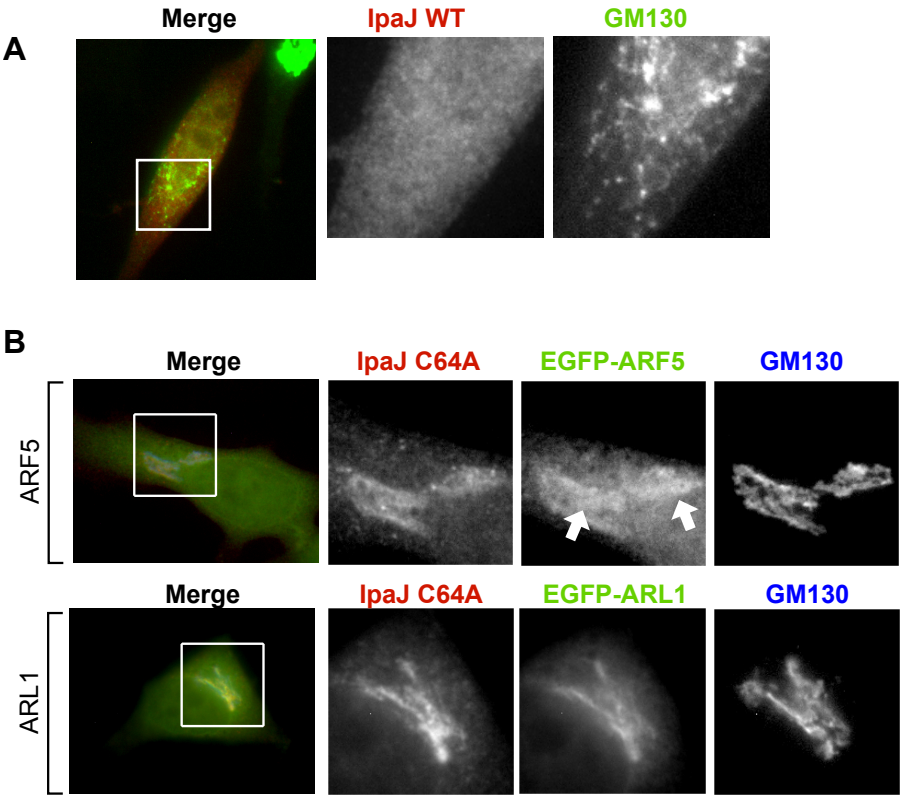


**Figure S2. IpaJ co-localizes with and cleaves ARF1, ARF5 and ARL1. Related to Figure 6**

(A) WT IpaJ exhibits a cytosolic localization pattern. Fluorescent microscopy of HeLa cells transfected with mCherry-tagged IpaJ (red) and fragmented Golgi membranes were labeled with GM130 antibodies and visualized by immunofluorescence (green) (merged image). We found that IpaJ does not localize to Golgi fragments (magnified box) and is cytoplasmic.

(B) IpaJ C64A co-localizes with ARF5 and ARL1. mCherry-tagged IpaJ C64A (red) was co-expressed with ARF5- or ARL1-EGFP (green) in HeLa cells and visualized by fluorescent microscopy (merged image). We found that IpaJ mutant partially co-localized with both GTPases on Golgi (magnified box). *Cis*-Golgi was visualized with GM130 antibodies.

**Table S1. Related to Figure 1.** Abundance of the proteins identified by mass-spectrometry after IpaJ cleavage *in vitro*. 'Control' and "IpaJ-treated" values represent relative abundance of a given protein in each condition based on abundance of its peptides. 'Ratio' is equal to 'IpaJ-treated'/'control' when protein abundance is higher after cleavage. Otherwise, 'ratio' is calculated as 'control'/'IpaJ-treated' and marked by negative sign (e.g -3.697). Ratios higher than +10 or lower than -10 were assigned values of +10 or -10 respectively for ease of visualization. For the proteins with N-myristoylated glycine we indicated whether *N*-myristoylation was already confirmed for them.

**Table S2. Related to Figure 4.** Abundance of the proteins identified by mass-spectrometry after infection with indicated *Shigella flexneri* strains. 'Ratio' is calculated for the samples infected with wild type of Δ*ipaJ Shigella*. Ratios higher than +10 or lower than -10 were assigned values of +10 or -10 respectively for ease of visualization

**Table S3. Related to Figure 4.** Abundance of the proteins identified by mass-spectrometry after infection with indicated *Shigella flexneri* strains. Replicated results under conditions identical to those reported for Table S2.

**TableS4: ARF1 mutations**

| Residue | Mutation | ARF1 region | Interaction | Reference |
|---|---|---|---|---|
| **Ile46** | Asp, Lys, Ala | switch 1 | AP-1, ASAP1 | (Luo et al., 2005; Ren et al., 2013) |
| **Ile49** | Thr | switch 1 | MKLP1, GGA1, AP-1, ASAP1, AGAP1, ARF GAP1, Sec7 domain, golgin-245 | (Goldberg, 1998; Kuai et al., 2000; Luo et al., 2005; Ren et al., 2013; Shiba et al., 2003; Wu et al., 2004) |
| **Phe51** | Tyr | switch 1 | MKLP1, GGA1, AP-1, ASAP1, AGAP1, ARF GAP1, Sec7 domain, golgin-245 | (Goldberg, 1998; Kuai et al., 2000; Luo et al., 2005; Ren et al., 2013; Shiba et al., 2003; Wu et al., 2004) |
| **Trp66** | His | interswitch | AP-1, POR-1, LTA1, GGA1, Sec7 domain, golgin-245 | (Goldberg, 1998; Kuai and Kahn, 2000; Ren et al., 2013; Wu et al., 2004) |
| **Aps72** | Ala | switch 2 | ARF GAP1 | (Luo et al., 2005) |
| **Lys73** | Ala | switch 2 | AP-1, ARF GAP1, Sec7 domain, GGA1, golgin-245 | (Goldberg, 1998; Luo et al., 2005; Ren et al., 2013; Shiba et al., 2003; Wu et al., 2004) |
| **Ile74** | Thr | switch 2 | POR-1, MKLP1, AP-1, Sec7 domain, golgin-245 | (Goldberg, 1998; Kuai et al., 2000; Ren et al., 2013; Wu et al., 2004) |
| **Trp78** | Cys | switch 2 | Sec7 domain, | (Goldberg, 1998) |
| **Arg79** | Gly | switch 2 | POR1 | (Kuai et al., 2000) |
| **Tyr81** | Thr | switch 2 | POR1, MKLP1, LTA1, AP-1, ARF GAP1, Sec7 domain, golgin-245 | (Goldberg, 1998; Kuai et al., 2000; Luo et al., 2005; Ren et al., 2013; Shiba et al., 2003; Wu et al., 2004) |
| **Thr85** | Ala | switch 2 | POR1 | (Kuai et al., 2000) |
| **Glu105** | Ala | helix 3 | ARF GAP1 | (Goldberg, 1999) |
| **Glu106** | Ala | helix 3 | ARF GAP1 | (Goldberg, 1999; Luo et al., 2005) |
| **Arg109** | Ala | helix 3 | ARFGAP | (Goldberg, 1999) |

**Table S4, Related to Figure 5.** ARF1 mutations. We introduced point mutations at ARF1 residues that were previously shown to interact with natural GTPase effectors (see 'Interaction' and 'Reference' columns). The structural localization of mutated residues within the protein is indicated ('ARF1 region').

**Extended Materials and Methods:**

**Plasmids and cloning:**

IpaJ gene (AAK18440) was PCR amplified from *Shigella flexneri* M90T and cloned into pEntr/D, generating a GatewayTM compatible entry clone (Invitrogen) according to manufacturer recommendations. For mammalian expression the effector was then recombined into modified pcDNA3.1 vector carrying NH2-terminal eGFP or mCherry in frame with the Gateway cassette (GatewayTM vector conversion system, Invitrogen). For bacterial expression 50 amino acid NH2-terminal deletion of IpaJ (IpaJΔN50; residues 51-259) was cloned in frame into pET28b-MBP vector, yielding intact catalytic domain of IpaJ COOH-terminally fused to MBP-6His-tag. NH2-terminal deletion of ARF1 (ARF1ΔN17; residues 18-181) was PCR subcloned into pGEX-4T1 vector. Human GGA1 (GAT domain, residues 76-215) was PCR amplified and cloned in frame into pET28b-MBP vector. For co-localization studies full-length ARF1 and ARF5 (Missouri S&T cDNA Resource Center; #ARF0100000, #ARF0500000) were cloned into modified pcDNA3.1 vector carrying COOH-terminal eGFP. For plasma membrane targeting 10 NH2-terminal amino acids of neuromodulin were cloned in front of eGFP in the pcDNA3.1-based vector. ARF1 and ARF5 were then subcloned COOH-terminally to eGFP. Site-directed mutation (IpaJ Cys64Ala) was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

**In vitro cleavage of proteins and in-gel fluorescence:**

To assay *in vitro* substrates HeLa cells were plated on 10cm dishes. 20 uM Alk-12 was added into the media for metabolic labeling. For *in vitro* cleavage of ARF1, cells were transfected with respective STREPtag-containing constructs using Lipofectamine® according to manufacturer recommendations 18 hrs before adding alkyne-myristic acid. Next day cells were washed with PBS and fresh media was added containing alkyne-myristic acid. After 24 hrs of metabolic labeling cells were lysed (with 20 mM Tris HCl pH 7.5, 1.5 mM MgCl2, 350 mM NaCl, 0.5% Igepal CA630, 5% glycerol) and protein content was measured by BCA assay. Lysate was incubated with 5 ug of MBP-IpaJΔ50 (wild type or C64A non-catalytic mutant) at 37$^{o}$C for 30 minutes. 100 ug of protein solutions were used for click-labeling with rhodamine-azide as described previously (Charron et al., 2009). The remaining portions of lysates were used for coupling to biotin and mass-spectrometric analysis (see below). Fluorescently labeled proteins were separated on SDS-PAGE and visualized on Typhoon Trio imaging system. ARF1-strep fusion proteins were then transferred on nitrocellulose paper for western blot with STREPtactin-HRP.

**Bacterial infection of cultured cells:**

For *Shigella* infections, HeLa cells were seeded onto 10 cm dishes the day before infection. For metabolic labeling 20 μM of alk-12 (Charron et al., 2009) were added 18 hours before infection. Cells were washed with PBS and fresh FBS- and antibiotic-free DMEM was added 2 hrs before infection. *Shigella* strains were inoculated from frozen stocks and grown overnight at 30$^{o}$C in Brain-Heart Infusion media (BHI) (DifcoTM, BD Biosciences) with ampicillin. Bacteria were then back diluted with 1:50 and incubated at 37$^{o}$C until reaching OD600~ 0.5-0.6. Bacteria were then washed in 1X phosphate buffer saline (PBS) and incubated at 37$^{o}$C for 15 minutes in 0.003% Congo Red. 130 μl of bacterial suspension was then added to each 10 cm dish. The plates and dishes were centrifuged for 10 minutes at room temperature (1000x g) to facilitate bacterial adherence. The plates were then incubated for 90 minutes at 37$^{o}$C, 5%CO2. The media was removed and the wells were washed three times with PBS containing gentamicin (150 μg/ml) followed by a wash with sterile PBS. Fresh antibiotic-free DMEM was added to each well after that and cells were incubated an additional 4 hours (37$^{o}$C, 5% CO2). Cells were washed again with PBS containing gentamicin and were lysed in 50 mM TEA (triethanolamine), 4% SDS, 150 mM NaCl, pH 7.4. Lysates then were prepared for in-gel visualization or mass-spectrometry as previously described (Wilson et al., 2011).

**Proteomic Identification of alk-12 Labeled Proteins:**

2 mg of protein was clicked with azido-biotin (Click Chemistry tools) for 4 h (1 mM $CuSO_4$, 1 mM TCEP, 200 μM TBTA, 200 μM azido-biotin) at 1 mg/ml in 4% SDS. Protein was precipitated using methanol (8 mL), chloroform (3 mL), and water (6 mL) overnight at –20 °C and washed with methanol (5 mL) twice. Protein pellets were dried and resuspended in 200 μL of 4% SDS. Equal protein amounts were diluted to 2 mg/mL with 50 mM TEA buffer containing 150 mM NaCl at pH 7.4. 60 μL of streptavidin beads (Thermo) were added to each sample and the mixtures were incubated for 1 h at room temperature on a nutating mixer. The beads were then washed three times with 1% SDS (50 mM TEA, 150 mM NaCl, pH 7.4), 5M urea (50 mM TEA, 150 mM NaCl, pH 7.4), and PBS. Centrifugation of the beads between washing steps was carried out (2000 × g, 1 min) to collect the beads. The beads were then resuspended in 200 μL 25 mM ammonium bicarbonate (ABC), reduced with 10 mM DTT for 30 min, and alkylated with 20 mM iodoacetamide in the dark for 30 min. After that, the beads were washed three times with 200 μL 25 mM ABC and suspended in 50 μL of 25 mM ABC. 0.5 μg of trypsin was added to each sample and the mixtures were digested at 37 °C overnight. The supernatant was collected, dried on a Speedvac and resuspended in water containing 5% acetonitrile and 1% formic acid for mass spectrometry.

LC-MS analysis was performed with a Dionex 3000 nano-HPLC coupled to an LTQ-Orbitrap ion trap mass spectrometer (ThermoFisher). Tryptic peptide samples were desalted on a trap column following separation on a 12cm/75μm reversed phase C18 column (NikkyoTechnos Co., Ltd. Japan). A 180 minutes gradient increasing from 10% B to% 45% B in 133 minutes (A: 0.1% Formic Acid, B: Acetonitrile/0.1% Formic Acid) were delivered at 200 nL/min. The liquid chromatography setup (Dionex, Boston, MA, USA) was connected to an Orbitrap XL (Thermo, San Jose, CA, USA) operated in top-8-CID-mode with MS spectra measured at a resolution of 60,000@m/z 400. One full MS scan (300–2000 MW) was followed by three data-dependent scans of the nth most intense ions with dynamic exclusion enabled.

Acquired tandem MS spectra were extracted using ProteomeDiscoverer v. 1.4.0.288 (Thermo, Bremen, Germany) and queried against Uniprots complete human database concatenated with common known contaminants (Bunkenborg et al., 2010) using MASCOT v. 2.3.02 (Matrixscience, London, UK). Peptides fulfilling a Percolator calculated 1% false discovery rate threshold (Kall et al., 2008a, b) were reported. For a matched protein, its abundance was calculated based on the average area of the three most abundant peptides (Silva et al., 2006). All LC-MS/MS analysis was carried out at the Proteomics Resource Center at The Rockefeller University, New York, NY, USA.

**Analysis of protein abundance changes:**

Protein amount ratios were determined by dividing the calculated protein abundances. Larger values were divided by smaller ones and the sign (+ or -) was assigned for overrepresented or underrepresented proteins respectively. If smaller value was equal to 0, the ratio was put equal to 10. All the ratios higher than 10 or lower than -10 was put to +10 or -10 respectively for convenience. All values equaled to 10 (+ or -) were added a small random value (from -0.25 to +0.25) for convenience of visualization.

**Synthesis of the acylated peptides:**

Peptides were synthesized manually on a scale of 50 micromole using Rink-amide copoly-(styrene-divinylbenzene) resin (Novabiochem, CA) with a substitution value of 0.41 mmol/g, Fmoc protected amino acids and HATU activation in NMP. Single coupling cycles were used throughout with a 10-fold excess of activated amino acid. Removal of the orthogonally protected methyltrityl group was achieved using 1% TFA in dichloromethane (DCM) prior to the addition of Fmoc PEG(n=3)-OH and Fluorescein succinimidyl ester. Final deprotection and cleavage of the peptides from the resins was carried out using 5% triisopropylsilane/95% trifluoroacetic acid (TFA) for 90 mins at room temperature. The crude cleavage mixtures were filtered and peptides precipitated from cold diethyl ether and allowed to air dry. Purification was achieved on either a Vydac C4 or C18 (250x10mm) column using a 120 min gradient of

0% buffer A (water/0.1% TFA) to 100% B (acetonitrile/0.1% TFA) and 220 nm wavelength detection. Purified fractions are combined and lyophilized.

**Cleavage of the acylated peptides:**

For enzymatic cleavage myristoylated peptides were incubated with IpaJΔN50 in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl2, 10mM DTT, 0.5% Igepal CA630 at 37$^{o}$C for indicated amounts of time. Reaction was stopped by addition of methanol with 0.5M HCl. After incubation the solution was centrifuged at 40000g for 30 minutes. The supernatant was used for HPLC gel filtration with TOSOH TSK-GEL® SuperSW3000 column. Area under the peaks was used to quantify the abundance of each peptide form.

**Recombinant protein purification and in vitro binding:**

Recombinant proteins (IpaJΔN50, IpaJΔN50 C64A, ARF1ΔN17, GGA1$_{76-215}$) were expressed in BL21-DE3 *E.coli* and were then stored at −80 °C. For *in vitro* nucleotide exchange 60 uM of ARF-GST fusion protein was incubated in TBS buffer with 500 uM GTP or GDP nucleotide, 10 mM of EDTA, 2 mM TCEP, 10% glycerol at 37 °C for 1 hr. After incubation solution was cooled down on ice for 1 min, mixed with 40 mM of MgCl2 and was spun down (15000g, 2 minutes). Supernatant containing non-precipitated protein was used for *in vitro* binding assay. ARFs was mixed with MBP-IpaJΔN50 and GST-agarose beads and incubated in the presence of 0.5% Triton X-100 for 1 hr at 4°C. Beads were washed three times with TBS containing 0.5% Triton X-100 and ARF -GST was eluted with 10 mM glutathione. Proteins were boiled with SDS, separated on SDS-PAGE and visualized with Coomassie staining.

***In vitro* affinity measurements**

ARF1ΔN17 and mutants were incubated in nucleotide loading buffer (40 mM Hepes pH 7.6, 150 mM NaCl, 3 mM EDTA, 10% glycerol, supplemented with 5 mM GTP) for 30 min at 37°C. $MgCl_2$ was added to 10 mM and the reaction was transferred to ice after 15 min at room temperature (25°C). ARF1ΔN17 proteins were assayed for concentration and adjusted to 200 μM. Homologous competition experiments were performed to determine parameters necessary to calculate the dissociation constant ($K_d$) of IpaJ with the various ARF1ΔN17 mutants. In Eppendorf tubes, we sequentially added non-radioactive competitor (as noted in Figure 5H x-axis), a constant 55 nM $^{35}$S-labeled IpaJ (or GGA for a control measurement), and an aliquot of GST-beads saturated in either wt or mutant versions of ARF1ΔN17. After 30 min incubation at room temperature with gentle agitation, we spun down the beads and washed 4x with TBS buffer. We eluted with 50 ml GST elution buffer (3.5 mM reduced glutathione in TBS buffer) and scintillation counted 30 ml of the supernatant. Data was analyzed with GraphPad Prism 6 and $K_d$ was determined using the one site homologous competition model with 95% confidence intervals.

**In vitro cleavage of recombinant ARF1:**

*E. coli* BL-21 cells expressing ARF1-His with or without yeast NMTp were grown overnight at 37$^{o}$C in LB media. Cultures were diluted 1:50 and incubated at 37$^{o}$C till reaching $OD_{600}$~ 0.5-0.6. Myristic acid and azide myristic acid were added at the concentrations 50µM and 5µM respectively and cells were additionally incubated for 30 minutes. Protein expression was then induced with 0.4 mM IPTG and cells were further incubated at 37$^{o}$C for 3 hrs. MBP-IpaJΔN50 was expressed by the same method without adding myristic acid. ARF1 and IpaJΔN50 expressing cells were lysed by 1-minute sonication separately (lysis buffer: 20 mM Tris HCl pH 7.5, 1.5 mM $MgCl_2$, 350 mM NaCl, 0.5% NP-40, 5% glycerol) and the lysates were mixed together and incubated for 30 minutes at 37 $^{o}$C. ARF1 was then purified using nickel agarose (Qiagen). *N*-myristoylated proteins were labeled with Alex Fluor® 647 Alkyne using Click-It® reaction buffer kit (Invitrogen) according to instructions with modifications. Specifically, Click-labeling was performed on the proteins still bound to the column. Column was then washed and proteins were eluted with 500 mM Imidazole/100mM HEPES containing 1% SDS. Myristoylation status was analyzed by in-gel fluorescence and the equal protein load was confirmed by Coommassie stain.

To test the cleavage of non-myristoylated ARF1 a similar experimental setup was used with the following modifications: ARF1-His was co-expressed with MBP-IpaJΔN50 in *E. coli* BL-21 cells (in the absence of NMTp or exogenous myristic acid). ARF1 was then purified with Ni-NTA agarose, eluted with 500 mM Imidazole/100 mM HEPES, and processed for mass spectrometry. Protein samples were cleaned using a C4 ZipTip (Millipore), following which the intact proteins were directly injected into an Agilent 6540 UHD Accurate-Mass Q-ToFmass spectrometer. The acquired mass spectra were deconvoluted using the deconvolution algorithm in the Agilent MassHunter software in order to obtain the molecular weight.

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