Supplementary Information for
Phosphorylation of Arl4A/D promotes their binding by the HYPK chaperone for their stable recruitment to the plasma membrane

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Antibodies
The anti-HYPK antibody was raised by immunizing New Zealand White rabbits with recombinant His-tagged human HYPK, a.a. 1-129 (NM_016400.3). The anti-Arl4C antibody was raised with the synthetic peptide (CIEKQLALHELPATTY) corresponding to a.a. 140-155 of human Arl4C (NM_057377). Anti-phosphorylated (p)Arl4A/D (S143/S144) were raised against a.a. 136-150 of human Arl4A (QDLRNSLPSEIEKL) and a.a. 137-151 of human Arl4D (QDDPGALpSAAEVEKR), respectively. For western blotting, the following antibodies were applied at the noted dilutions: anti-HYPK (1:2000), anti-Arl4A (1:1000) (1), anti-Arl4C (1:1000), anti-Arl4D (1:2500) (2), anti-pArl4A/D (1:1000), anti-HA (1:2000), Covance Inc.), anti-His (1:5000, Clontech), anti-α-tubulin (1:1000, Sigma), anti-EGFP (1:5000, generated in our laboratory), anti-Naa10 (1:2000, GTX125971, GeneTex), anti-Naa15 (1:2000, GTX115958, GeneTex), anti-Arl1 (1:1000, Dr. Chia Jung Yu’s laboratory, Chang Gung University, Taiwan), anti-Arl13b (1:1000, GTX122703, GeneTex), anti-Myc (rabbit) (1:1000, #2278, Cell Signaling), anti-Myc (mouse) (1:5000, #2276S, Cell Signaling), anti-Pak1 (1:1000, #2602S, Cell Signaling), anti-E-cadherin (ab1416, Abcam), anti-pPak (T423) (1:1000, #2601S, Cell Signaling), and the secondary antibodies were goat horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse IgG (1:5000, NA934V/NA931V, GE Healthcare). For IF, dilutions of anti-Arl4C (1:200), anti-Arl4C (1:300), anti-Arl4D (1:300), and anti-HA (1:2000) primary antibodies and Alexa Fluor 488/594 goat anti-rabbit and mouse IgG secondary antibodies (1:1000, A-11034/A-11012 and A-11001/A-11003, Invitrogen) were used.

Plasmids
In the mammalian expression system, untagged Arl4A, Arl4C, Arl4D and their GTP-bound or GTP-deficient mimic mutants, G2A mutants, and phospho-mutants were cloned into pSG5 vector (Stratagene) as described previously (3). Arl4s were also subcloned into pB7m116 (Harvard Medical School) to obtain N-terminal LexA-DBD-tagged proteins for expression in the yeast, and into pETDuet-1 (Novagen) at the first multiple cloning site (MCS1) with HYPK subcloned into the second multiple cloning site (MCS2) for induction of N-terminal His-tagged protein from the E. coli system. Pak1 related constructs were described (3).

HYPK (NM_016400.3) obtained from the human fetal brain cDNA library and its related mutants were cloned into the pACT2 vector (Clontech), which contains an N-terminal Gal4AD-HA tag, and used in the yeast two-hybrid assay. For bacterial expression, HYPK and related mutants were subcloned into pGEX-4T-1 (GE Healthcare) and pET15b (Novagen) to obtain N-terminal GST-tagged and His-tagged fusion proteins (for raising anti-HYPK antibody), respectively. For expression in mammalian cells, untagged, N-terminal EGFP-, and HATagged HYPK were acquired by cloning into pSG5, pEGFP-C2 (Clontech), and pcDNA3.0-HA-NF1 vectors, respectively. The HYPK shRNA-resistant constructs in pSG5 and pcDNA3.0-HA-NF1 were generated by site-directed mutagenesis with the following primers:

\[
\begin{align*}
\text{HYPK WT}^{\text{Res}} & \quad (F, 5’-\text{GGCGGAACACATGGGAAATGTAGCTGCTGCTCTAATTGCCCTAACCAACTGAAGATCTTA} & \quad \text{and } R, 5’-\text{AAAGATCTTTCAATGGTTAGGGAATAGGTAGTGAGCTCTAAATGCCCTCTACCAACACTGAAGATCTTA-3’}) \\
\text{HYPK A1}^{\text{Res}} & \quad (F, 5’-\text{GGCGGAACACATGGGAAATGTAGCTGCTGCTCTCTGAGGCGATCTTA-3’}) \\
\text{HYPK A2}^{\text{Res}} & \quad (F, 5’-\text{GGCGGAACACATGGGAAATGTAGCTGCTGCTCTCTGAGGCGATCTTA-3’}) \\
\end{align*}
\]

All constructs were verified by DNA sequencing.

Cell culture
HeLa (CCL-2) and C33A (HTB-31) human cervical carcinoma cell lines and A549 (CCL-185) human lung carcinoma cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa and C33A cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) with sodium bicarbonate. A549 cells were cultured in Kgaighn's modified Ham's F-12 (F-12K) medium. For normal passage processes, cells were in the complete medium supplementing with 10% fetal bovine serum (FBS) (Biological Industry), pH 7.4, and maintained in a humidified incubator containing 5% CO₂ at 37°C.

Transient transfection and knockdown in mammalian cells
Cells were transfected with desired plasmid DNA or siPak1 (3) by Lipofectamine 2000 or RNAiMax (Thermo Fisher Scientific), respectively, in Opti-MEM (Gibco) according to the manufacturer’s protocols. HYPK knockdown was performed by lentiviral shRNA silencing. pKLO.1-shHYPK#1 and shHYPK#2 (clone ID of #1 is TRCN0000061953; clone ID of #2 is TRCN0000061954, RNAi Core, Academia, Sinica, Taiwan) were used as packaging plasmids to produce shHYPK lentiviruses, which were used to infect cells at a multiplicity of infection (MOI) of 5 with 10 μg/mL polybrene (TR-1003, Sigma). 24 hours after infection, cells were selected with 4 μg/mL puromycin for 2 days.

Transwell migration assay
The bottom of Transwell device (3464, Corning) was precoated with FN (10 μg/mL, 356008, Corning) in PBS for at least one-half hour. After knockdown or/and transient transfection for 24 hours, 1×10⁵ HeLa cells were resuspended in 100-200 μL of serum-free culture medium and seeded in a Transwell device. The rest of the cells were harvested by RIPa (Tris-HCl 50 mM, pH7.5; NaCl 150 mM; SDS 0.1%; sodium deoxycholate 0.5%; NP40 1%; EDTA 1mM) for western blotting, or GENEzol reagent (GZX200, Geneaid) for RT-PCR. The seeded cells were allowed to migrate through the Transwell membrane for 12 hours and analyzed as previously described (3).
Co-immunoprecipitation (co-IP)
For general co-IP, cells expressing the proteins of interest were lysed on ice in 1 mL of lysis buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.1% NP-40; 1 mM EDTA; and protease inhibitor (PI) cocktail) per each 10-cm dish. The lysates were then rotated at 4°C for 30 minutes and centrifuged at 15000 g for 10 minutes to remove insoluble debris. GFP-Trap magnetic beads (gtma-20, ChromoTek) were prewashed with lysis buffer three times and incubated with precleared lysates on an end-on-end rotator at 4°C for 1 hour. The beads were washed with lysis buffer three times on a Magna GrIIP Rack (Millipore) and resuspended in 30 µL of sample buffer. Eluted protein complexes were boiled at 95°C for 10 minutes before SDS-PAGE analysis.

For Ub-IP, a modified protocol previously reported (4, 5) was followed. Cells were treated with 20 μM MG132 (C2211-5MG, Sigma-Aldrich) in DMEM for 4 hours before lysis with 1 mL of RIPA containing PI and 10 mM iodoacetamide (IAA) per 10-cm dish. After lysis and centrifugation, the supernatants were incubated with 20 µL of prewashed anti-HA-agarose beads (A2095, Millipore) for 2 hours and washed with RIPA buffer three times before boiling in sample buffer.

For IP after cell cytosol and membrane fractionation, cells in three 10-cm dishes were transfected with the desired plasmids for 16-18 hours, trypsinized, and reseeded on uncoated or FN-coated dishes for 3 hours. The cells were then subjected to dithiobis(succinimidyl propionate) (DSP; 22585, Thermo Scientific) for cross-linking for 2 hours as described by Zlatic et al (6). After quenching and washing with PBS (0.1 mM CaCl₂ and 1 mM MgCl₂), the cells were scraped and collected in MgCl₂ buffer (25 mM HEPES, pH 7.4; 2.5 mM MgCl₂; and 250 mM sucrose) for subcellular fractionation as previously reported (7) with some modification. Cells were passed through a 30G needle 25 times and centrifuged at 1000 g for 10 minutes before protein quantification and ultracentrifugation at 200,000 g for 1 hour. The supernatant (cytosolic fraction) and the pellet (membrane fraction), after fully resuspended with MgCl₂ buffer containing 1% Triton X-100, were incubated with prewashed Myc-Trap beads (ytma-20, ChromoTek) at 4°C with end-on-end rotation for 1 hour. The beads were washed with IP buffer three times, resuspended in 40 µL of 1X sample buffer, incubated at 37°C for 1 hour, and boiled at 95°C for 10 minutes before SDS-PAGE analysis.

Drug and fibronectin (FN) treatment
Cycloheximide (CHX) (C7698, Sigma), MG-132 (C2211, Sigma), NH₄Cl (A3661, Applichem), and chloroquine (CQ) (C6628, Sigma) were diluted in cell culture medium and applied to the cells in the concentration of 100 µg/mL, 10 µM or 20 µM (Ub-IP), 10 mM, and 200 µM, respectively, for times as indicated. For FN treatment, C33A cells were washed twice with PBS and cultured in serum-free DMEM for 16-18 hours. FN (356008, CORNING) diluted in serum-free DMEM to a concentration of 20 µg/mL was then applied to the cells for the indicated times. For the experiments in HeLa cells subjecting to mass spectrometry and IP, FN diluted in PBS was pre-coated on culture dishes at 37°C for two hours. Cells were then trypsinized and reseeded on FN-coated dishes for 1.5 (mass spectrometry) and 3 hours (IP).

Immunofluorescence (IF) analysis
After transfection, the cells on the 12-mm microscope coverslips (Assistant) were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. After washing with PBS and permeabilized (0.1% Triton X-100 in PBS) for 5 minutes, the cells were washed again before blocking with blocking buffer (1% bovine serum albumin (BSA) in PBS) for 1 hour. Primary antibodies were diluted in blocking buffer and used to stain the cells for 1 hour. After PBS washing, secondary antibodies (Alexa Fluor 594/488 anti-rabbit/mouse) and DAPI (1:5000, BD Biosciences) diluted in blocking buffer were used to stain the cells for additional 40 minutes. Following a final wash, the cells were fixed onto the slides with mounting solution (Mowiol 4-88/DABCO). Fluorescence images were captured with an Axioplan 2 microscope (Carl Zeiss, Inc.), and analyzed with FIJI software (ImageJ2). For quantification of the membrane-targeting ability of the Arl4 proteins, the fluorescence intensity of the plasma membrane:cytosol (PM:C) ratio was calculated using the following equation:

PM/C ratio= (Intensity of the cell body region-Intensity of the cytosol region)/Intensity of the cytosol region. The quantification processes were described previously (3, 8).

Recombinant protein induction and purification
E. coli expression plasmids for glutathione S-transferase (GST) and Arl4 proteins were transformed into BL21(DE3) competent cells, and for HYPK-related proteins were transformed into pLysS competent cells. The cells were grown to log phase as previously described (3), and isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce recombinant proteins under the following conditions: GST, Arl4A and Arl4C at 0.5 mM at 37°C for 3 hours, Arl4D at 0.25 mM at 25°C for 3 hours, and HYPK at 0.25 mM at 25°C for 3 hours.

For purification, cell pellets were suspended in lysis buffer (20 mM Tris-HCl, pH 7.9; 500 mM NaCl; 5 mM imidazole; 10% glycerol; 0.1% Triton X-100; PI cocktail; and 100 µg/mL lysozyme) and maintained on ice for 30 minutes. Suspensions were exposed to a cell disruption bomb (4639, Ashcroft Inc.) at 1500 psi and 4°C for 10 minutes, and the lysates were centrifuged at 14000 g for 10 minutes. For His-tagged proteins, supernatants were incubated with Ni-NTA resin (88222, Thermo Scientific) at 4°C for 1 hour. Immobilized proteins were serially washed with lysis buffer containing 5, 25, and 50 mM imidazole. Elution buffer (20 mM Tris-HCl, pH 7.9; 500 mM NaCl; and 250 mM imidazole) was used to elute purified His-tagged proteins. For obtaining GST-tagged proteins, supernatants were incubated with glutathione-Sepharose beads (17-5132-01, GE Healthcare) at 4°C for 2 hours. The proteins bound to the agarose were washed with PBS three times and resuspended in PBS containing PI cocktail.

In vitro binding and kinase assay
2 μg of GST-tagged fusion proteins bound on glutathione-Sepharose beads (175132-01, GE Healthcare) were incubated with 2 μg of His-tagged Arl4 proteins in 1 mL of in vitro binding buffer (20 mM Tris-HCl, pH 8.0; 100 mM NaCl; 5 mM MgCl₂; 1 mM EDTA; 1 mM DTT; 250 mM sucrose; 10% glycerol; 0.1% Triton X-100; and PI cocktail) at 4°C while rotating for 1 hour. The beads were washed three times with 1 mL of wash buffer (in vitro binding buffer containing 1% Triton X-100), and boiled in sample buffer for western blotting. For kinase assay, HA-Pak1-CAAX was purified from HeLa cells as previously described (3). After 3 times of wash by the lysis buffer and 3 times of wash by the kinase buffer (50 mM HEPES, pH 7.4, 5 mM NaCl, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM DTT), kinase reactions were started by adding 3 µg of eluted GST or GST fusion proteins with 25 μM cold ATP and 5Uci of [32P]-γ-ATP at 30°C for 30 minutes. The reactions were stopped by adding sample buffer and analyzed by SDS-PAGE and autoradiography.
Protein quantification and western blotting

The concentrations of the precleaned lysates or experimental samples were determined with a Bio-Rad DC protein assay kit (500-0116, Bio-Rad) according to the manufacturer’s protocols. Quantified lysates were boiled with sample buffer, separated by 12.5% SDS-PAGE and transferred to 0.45-µm PVDF membranes (Immobilon P, Millipore) in a semidy transfer unit (TE70, Hoefer) or wet transfer tank (TE22, Hoefer). Blotted membranes were processed and detected as previously reported (3).

Yeast two-hybrid screening

Ahr4 related proteins and lamin in pBTM116 vector, and HYPK related proteins in pACT2 vector (Clontech) were co-transformed in the yeast strain L40 (MATa leu2 his3 trpl LYS2::(LexAop) 4-HIS3 URA3::(LexAop) 8-lacZ) (9). The protein-protein interactions through both histidine auxotrophy and β-galactosidase expression assays were described previously (1, 10).

RNA extraction and RT-PCR

To determine the mRNA expression level, total RNA was extracted with a GENEzol Pure Kit (GZX200, Geneaid) through an in-column DNase I digestion procedure. 1 µg of total RNA was reverse transcribed (RT) by PrimeScript RT Reagent Kit (RR037A, TaKaRa) in a T3000 Thermocycler (Biometra). Then, 1 µL of the RT product was used to perform polymerase chain reactions (PCRs) using Raly PreMix, (TM-1101, Ten Giga Bio), with Ahr4A-, Ahr4D-, and HYPK-specific primers for 25 cycles and with GAPDH primers for 20 cycles. The end-point PCR products were run in a 1% agarose EtBr gel for UV detection. For real-time PCR, 2 µL of 5-fold diluted RT product was mixed with Fast SYBR Green Master Mix (4385612, Applied Biosystems) and subjected to StepOnePlus Real-Time PCR System (Applied Biosystems) to gain cycle threshold (Ct) values. The following PCR primers were used:

HYPK (F, 5’- GAATGCAGGCAGGCTGGTG -3’, and R, 5’-TCAGTTGGAATGGGAATACGAC-3’); Ahr4A (F, 5’-ATGGGGAATGGCTGTTACGACC-3’, and R, 5’-CTACTTTTCTTTCCCTCAGGCC-3’); Ahr4D (F, 5’-ATGGGGAACCATTTGACT-3’, and R, 5’-CACCGTCCTTTTCTCGGC-3’); and GAPDH (F, 5’-AGGGCTGCTTTTTAATCTCCTG-3’, and R, 5’-CCCACTGTATTGTGAAGGA-3’).

Stable isotope labeling by amino acids in cell culture (SILAC)

For SILAC experiments, HeLa cells were maintained in arginine- and lysine-depleted DMEM (Thermo Scientific, Rockford, IL, USA) supplemented with 10% dialyzed FBS (Gibco, USA, One Shot format) and 0.1 mg/mL light L-lysine and L-arginine or 0.1 mg/mL heavy [U-13C]L-lysine and [U-13C]L-arginine (Sigma-Aldrich, St. Louis, MO, USA), respectively (11, 12). The cells were passaged every 2–3 days, and the medium was replaced with the corresponding light or heavy labeling medium. By the time the cells doubled approximately eight times, nearly 100% of the isotopic labeling amino acids had been incorporated and cells were then transfected with Ahr4A plasmids.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by liquid chromatography-tandem mass spectrometry (GelLC-MS/MS)

Lysates of the Ahr4A-transfected HeLa cells with or without FN treatments in SILAC culture were separated via 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue G-250. The Ahr4A-containing gel bands were excised from the SDS-PAGE gel and subjected to in-gel tryptic digestion. Briefly, the gel pieces were destained with 50% (vol/vol) acetonitrile/50 mM NH₄HCO₃, reduced by incubation with 25 mM containing 10 mM dithiothreitol at 60°C for 30 minutes, and alkylated with 55 mM IAA at room temperature for an additional 30 minutes. The proteins were digested with trypsin (Promega, Madison, WI, USA) for 16 hours at 37°C, and the resulting peptide mixtures were extracted for two-dimensional liquid chromatography combined with tandem mass spectrometry analysis (2D LC-MS/MS) as described previously (12). For enrichment of the phosphorylated peptides, online TiO2-based liquid chromatography was applied.

Enrichment and identification of phosphopeptides using online TiO2 liquid chromatography with MS/MS

Each peptide mixture was resuspended and acidified in 2% formic acid/40% acetonitrile. The peptide mixtures were loaded onto an in-house-packed TiO2 trap column (GL Sciences, 10 µm, 0.3 × 5 mm) as described previously (13). Online TiO2 liquid chromatography with tandem mass spectrometry experiments were performed on an LTQ-Orbitrap Elite Hybrid MS spectrometer (Thermo Fisher Scientific) operated with Xcalibur 2.2 software (Thermo Fisher Scientific). For the MS scan, the m/z scan range was 350–2000 Da. Neutral loss scanning (98 for +1, 49 for +2, and 32.7 for +3 charged ions) was used to detect phosphopeptides. For the database search, the resulting mass spectra were searched using the Swiss-Prot human sequence database with the Mascot search engine (Matrix Science, London, UK, version 2.2.04). To quantify the identified proteins and phosphopeptides, the raw spectrometry data, including the precursor ion quantifier node for SILAC, were analyzed using Proteome Discoverer software (version 1.4, Thermo Fisher Scientific, San Jose, CA). A phosphosite localization algorithm, phosphoRS Site Probabilities, in Proteome Discoverer software was used to assign the phosphorylation sites on the identified peptides.

Statistical analysis

All data collected from at least three biological replicates are presented as the means±SD. GraphPad Prism 8 software was used for all numerical analyses, with two-sample t-tests, one-way ANOVA performed to identify significant differences between groups, and one-phase exponential decay to identify half-lives (T₁/₂) of Ahr4 proteins. Post hoc Dunnett’s multiple comparison test was performed after a significant one-way ANOVA to calculate the significance between each two groups. P values < 0.05 were considered to be statistically significant (*P≤0.05; **P≤0.01; ***P≤0.001).
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Figure S1. Characterization of Arl4A/D upregulation under FN treatment and Arl4A/D protein degradation. (A) C33A cells were serum starved (SS) for 16-18 hours before retrieval with 20 μg/mL FN for the indicated hours. Mock, the control group cultured in DMEM with FBS. E-cadherin, an FN-responsive protein. α-Tubulin and Coomassie Blue (CB) staining were used as protein loading controls. (B) Cells shown in Fig. 1A were harvested at the indicated time points for RNA extraction and RT for real-time PCR. The ΔCt values for Arl4A/D were derived from the Ct of GAPDH, and the mock group served as a second normalization control for mRNA level comparisons. mRNA levels were analyzed by one-way ANOVA with Dunnett's post-hoc multiple comparison test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; n=5. (C, D) C33A cells (C) and HeLa cells (D) with detectable endogenous Arl4A and Arl4D expression were treated with cycloheximide (CHX) or cotreated with proteasome/lysosome inhibitors (MG132, a proteasome inhibitor; NH₄Cl, a lysosomal inhibitor; chloroquine (CQ), a lysosomal inhibitor) for 6 hours. The protein level of Arl4A/D was normalized to that of α-tubulin, and the quantified results are shown in dot plots, with error bars representing the means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; n=3. The statistical results were analyzed by one-way ANOVA with Dunnett’s post-hoc multiple comparison test. (E) HeLa cells were coexpressed with Myc-Arl4A, Myc-Arl4C, or Myc-Arl4D and hemagglutinin-ubiquitin (HA-Ub) to detect the ubiquitination signals of Arl4s after MG132 treatment.
Figure S2

A

| Arl4A | Arl4C | Arl4D |
|-------|-------|-------|
| WT | Q79L | T34N | T51N |
| WT | Q72L | T27N | T44N |
| WT | Q70L | T35N | T52N |
| Lamin | | | |

Relative pull-downed Arl4A

B

- Full length
- HYPK fragment obtained from Y2H
- N-terminal
- C-terminal
- Reported functional domain
- Predicted coiled coil domain

C

| Arl4A WT | Arl4C WT | Lamin |
|---------|---------|-------|
| FL | 1-69 | 70-129 |
| 89-129 | 89-115 |

D

HYPK mutants

A1: M ... R A A A G N V E A L T N *
A2: M ... R E H M A A A V E A L T N *
A3: M ... R E H M G N V A A A L T N *
A4: M ... R E H M G N V E A A A A L T N *
A5: M ... R E H M G N V E A A A A A L T N *
A6: M ... G E H M G N V E A A A A A L T N *

E

| Arl4A WT | Arl4C WT | Lamin |
|---------|---------|-------|
| FL | A1 | A2 |
| A3 | A4 | A5 |
| A6 | | |

F

| His-Arl4A WT | Arl4A 2 % input |
|-------------|-----------------|
| GST | GST-HYPK |
| CB staining | GST only |

Relative pull-downed Arl4A

G

| His-Arl4D WT | Arl4D 2 % input |
|-------------|-----------------|
| GST | GST-HYPK |
| CB staining | GST only |

Relative pull-downed Arl4D
Figure S2. Characterization of the Arl4-HYPK interaction in the yeast two-hybrid system. (A) Arl4A, Arl4C, and Arl4D fused with the LexA DNA-binding domain (DB) were cotransformed with HYPK19-129 tagged with the hemagglutinin (HA)-GAL4 activation domain (AD) in yeast strain L40. Lamin, the negative control for HYPK. Three transformed yeast colonies were assessed on histidine-deficient (-His) plates for 24 hours and subjected to a β-galactosidase assay with X-gal for 1 hour. (B) Schematic HYPK fragments for determining the Arl4-interacting regions. (C) Arl4A/D-LexA DB and HYPK-HA-GAL4 AD were expressed in yeast L40 cultured on -His plates for 24 hours and in X-gal for 20 minutes and overnight (O/N) to determine β-galactosidase activity. (D) The diagram represents alanine scanning mutations in 114-129 of HYPK. Every three consecutive a.a. were mutated to alanine residues in HYPK 115-129 to produce mutants A1-A5. A6, a missense mutation in the tumor database cBioPortal. (E) HYPK-HA-GAL4 AD WT and alanine mutants were preyed in yeast L40 to interact with Arl4A/D. Yeast transformants were allowed to grow on -His plates for 24 hours and in X-gal for 1 hour. (F, G) In vitro binding of purified His-Arl4A (F) and His-Arl4D (G) with GST-HYPK WT, A1, and A3 mutants. Equal inputs of GST proteins were shown by staining with CB. The His signals of pulled down Arl4 proteins were quantified in the dot plots with the means ± SD. The statistical results were analyzed by one-way ANOVA with Dunnett’s post-hoc multiple comparison test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; n=3.
Figure S3. HYPK translocates to the plasma membrane when coexpressed with Arl4A/D. (A) Subcellular localization of EGFP-HYPK WT, A1, and A3 mutants in HeLa cells. DAPI, the stain of cell nuclei. Scale bar, 20 \( \mu \)m. (B) Subcellular localization of EGFP-HYPK after coexpression with Arl4A, Arl4C, and Arl4D. Scale bar, 10 \( \mu \)m. The Pearson’s correlation coefficients of the Arl4 and EGFP-HYPK signals were calculated with ZEN imaging software, and the number of cells analyzed is indicated in the plot. The coefficients of Arl4A-HYPK, Arl4C-HYPK, and Arl4D-HYPK were compared through one-way ANOVA with Dunnett’s post-hoc multiple comparison test, showing the means ± SD. ***, \( P < 0.001 \). (C, D) The colocalization of EGFP-HYPK WT, A1 and A3 and Arl4A (C) or Arl4D (D) at plasma membrane protrusion sites. Scale bar, 20 \( \mu \)m. The plasma membrane/cytosol ratios of HYPK in each group were calculated and shown in dot plots analyzed by one-way ANOVA with Dunnett’s post-hoc multiple comparison test.
**Figure S4.** HYPK is prone to colocalize with the membrane-bound forms of Arl4A/D on the plasma membrane. (A, B) Arl4A/D WT and variant mutants Q79L/Q80L (GTP-bound mimetics), T34N/T35N nucleotide-free forms), T51N/T52N (GTP-deficient mimetics), and G2A (a nonmyristoylated form) were coexpressed with EGFP-HYPK for IF staining. Arl4A/D and EGFP were coexpressed in HeLa cells as a noncolocalized control on the left. Scale bar, 20 µm. (C, D) The plasma membrane/cytosol ratios of EGFP-HYPK in (A, B) were calculated and shown in dot plots. The ratio differences were analyzed by one-way ANOVA with Dunnett’s post-hoc multiple comparison test. Error bars represent the means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
**Figure S5.** HYPK stabilizes the Arl4A/D proteins during proteasome inhibition. (A-C) C33A (A), A549 (B), and HeLa (C) cells expressing shCtrl or shHYPK#2 RNA were treated with MG132 for 0, 1, 3, and 9 hours. Arl1 and Arl13b are the Arl family proteins for comparison with the Arl4 proteins. α-Tubulin, loading control. (D-F) The protein level changes of Arl4A (D), Arl4C (E), Arl4D (F) and the other Arl proteins were quantified after normalization to α-tubulin and their protein levels at time 0. A two-sample t-test was applied for the results shown in the dot plots, with error bars representing the means ± SD. *, P < 0.05; **, P < 0.01; n=3. (G, H) C33A (G) and HeLa (H) cells expressing shHYPK#2 RNA were rescued with HYPK-WT<sub>Res</sub> before MG132 treatment. Arl4A/D protein was normalized to α-tubulin and quantified in dot plots, showing the means ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.00; n=3. The statistical results were analyzed by one-way ANOVA with Dunnett’s post-hoc multiple comparison test.
Figure S6. HYPK binding facilitates Arl4A/D-mediated cell migration. (A) HeLa cells expressing shCtrl RNA and shHYPK RNA and the cells rescued with the resistant form of HYPK WT, A1, and A3 were allowed to migrate in the Transwell device. The knockdown and rescue efficiency of HYPK was examined by detecting HYPK mRNA levels by RT–PCR. RT+ and RT− represent samples obtained from RT–PCR with and without reverse transcriptase, respectively. GAPDH was used as the internal control for the PCRs. Cells that migrated to the lower side of the Transwell membrane were stained with crystal violet and quantified. (B) Transwell migration after HYPK knockdown and rescue in Arl4A-overexpressing HeLa cells. The expression levels of HYPK and Arl4A were monitored by RT–PCR as described in (A). The number of migrated cells per field in each group was compared through one-way ANOVA with Dunnett’s post-hoc multiple comparison test. The solid bars represent the means ± SD. **, P < 0.01; ***, P < 0.001; n=3.
Table S1. Identification and quantification of phosphorylated S141/S143 in Arl4A upon fibronectin treatment on the basis of two independent experiments with SILAC-based proteomics analysis.

| Accession | Gene name | Sequence | Phosphosite a | Heavy (FN+)/Light (FN−) | Light (FN+)/Heavy (FN−) |
|-----------|-----------|----------|----------------|-------------------------|-------------------------|
| P40617    | Arl4A     | NsLSLSEIKEK NSLSLSEIKEK | S141/S143       | 1.22 1.30               | 1.54 1.32               |

a, Phosphorylation site assignment was based on calculations performed with phosphoRS Site Probabilities, a phospho-site localization algorithm in Proteome Discoverer software (version 1.4, Thermo Fisher Scientific, San Jose, CA).