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

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The Arl4 small GTPases participate in a variety of cellular events, including cytoskeleton remodeling, vesicle trafficking, cell migration, and neuronal development. Whereas small GTPases are typically regulated by their GTPase cycle, Arl4 proteins have been found to act independent of this canonical regulatory mechanism. Here, we show that Arl4A and Arl4D (Arl4A/D) are unstable due to proteasomal degradation, but stimulation of cells by fibronectin (FN) inhibits this degradation to promote Arl4A/D stability. Proteomic analysis reveals that FN stimulation induces phosphorylation at S143 of Arl4A and at S144 of Arl4D. We identify Pak1 as the responsible kinase for these phosphorylations. Moreover, these phosphorylations promote the chaperone protein HYPK to bind Arl4A/D, which stabilizes their recruitment to the plasma membrane to promote cell migration. These findings not only advance a major mechanistic understanding of how Arl4 proteins act in cell migration but also achieve a fundamental understanding of how these small GTPases are modulated by revealing that protein stability, rather than the GTPase cycle, acts as a key regulatory mechanism.

Pak1 | Arf-like GTPase | HYPK | protein stability | cell migration

Significance

Arl4 small GTPases act in cell migration through multiple effector proteins, but how they are regulated in this role has been unclear. We find that Pak1 kinase phosphorylates Arl4A and Arl4D (Arl4A/D), which then enables the chaperone protein HYPK to bind these small GTPases. The resulting complex prevents the proteasomal degradation of Arl4A/D and promotes their targeting to the plasma membrane for cell motility. These findings advance a major understanding of how Arl4 proteins act in cell migration by revealing a novel mechanism of regulating their function.

The Arl4 [ADP ribosylation factor (Arf)-like 4 proteins] small GTPases, comprising Arl4A, Arl4C, and Arl4D, are developmentally regulated. In adults, they have restricted tissue expression and have been found to act in a variety of cellular events. Arl4A recruits ELMO-Dock180, a conserved Rac regulator, to membranes for actin remodeling (1) and also directs cell migration through Robo1 and Pak1 effectors (2, 3). Several studies have reported that Arl4C functions in filopodia formation (4), cancer cell invasion (5, 6), and tumorigenesis (7–9). Arl4D has been found to activate Arf6 by recruiting cytohesin-2/ARNO for actin remodeling to promote cell migration (10, 11). How the Arl4 proteins are regulated in these cellular roles has been enigmatic. Small GTPases are typically activated by guanine nucleotide exchange factors and deactivated by GTPase-activating proteins (12, 13). However, neither class of key regulators has been identified for the Arl4 proteins. Consistent with this current state of knowledge, these small GTPases have been found to undergo rapid nucleotide exchange and appear structurally unable to bind GDP (13, 14). Thus, a major goal has been to elucidate how Arl4 proteins are regulated independent of the canonical mechanism that involves the GTPase cycle.

The p21-activated kinases (Paks) stand at the hub of several signaling pathways for cell proliferation, migration, and survival (15). In cell migration, Pak1 phosphorylates paxillin at the leading edge of migrating cells for the rapid turnover of focal adhesion (16). Pak1 also phosphorylates LIMK1 and myosin light chain for actin reorganization to promote cell migration (17, 18). Pak activation requires cell adhesion through the interaction between integrins and the extracellular matrix (19). A well-characterized example has been the stimulation of cells by fibronectin (FN), which recruits Pak1 to the plasma membrane, where it then interacts with other molecules to coordinate downstream events (20, 21). In this role, FN acts in a variety of physiologic and pathologic circumstances, with wound healing being an example of the former (22) and cancer cell invasion being an example of the latter (23). With respect to the latter, alternatively spliced forms of FN have been found to act in collective tumor migration and are predictive of adverse outcomes for patients with cancer (24–26).

We have recently found that FN promotes the cooperative recruitment of Arl4A and Pak1 to the plasma membrane, which contributes to sustained Pak1 activation needed for cell migration (3). In this study, we find that Arl4A and Arl4D (Arl4A/D) undergo rapid proteasomal degradation. FN stimulation activates Pak1 to phosphorylate these small GTPases, which leads to their binding by the chaperone-like protein HYPK

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(Huntingtin-interacting protein K). This binding stabilizes the targeting of Arl4A/D to the plasma membrane to promote cell migration.

Results

FN Stimulation Stabilizes Arl4A/D Protein Level through Pak1. In considering how Arl4 small GTPases can be regulated independent of their GTPase cycle, we considered that protein turnover is a general mechanism of regulating protein function. Thus, to explore whether this mechanism regulates Arl4 proteins, we first observed that Arl4A protein level decreased upon serum starvation of cells, which rebounded after 3-h exposure to FN (*SI Appendix*, Fig. S1*A*). As a technical note, we initially examined C33A cells, because the endogenous level of all three Arl4 proteins can be readily detected in these cells (3). However, because these cells do not readily attach to FN-coated surfaces, we sought to achieve FN stimulation by adding FN to serum-free medium. Validating this approach, we found that this approach resulted in E-cadherin down-regulation (*SI Appendix*, Fig. S1*A*), which is a known effect of FN stimulation (27–29). We next found that the levels of endogenous Arl4A/D, but not those of Arl4C and Arl1, increased upon FN stimulation (Fig. 1*A* and *B*). Thus, FN-triggered protein increase was specific for Arl4A/D.

To further define this effect, we next found that it could not be attributed to increased messenger RNA levels of Arl4A/D (*SI Appendix*, Fig. S1*B*). Thus, we sought insight into how regulation could be achieved at the protein level. Arl4A/D protein levels were reduced when protein synthesis was inhibited by cycloheximide treatment, while the protein level of Arl4C remained relatively steady (Fig. 1*C*). We then used pharmacologic inhibitors and found that the degradation of Arl4A/D was likely a proteasomal rather than a lysosomal process (*SI Appendix*, Fig. S1*C* and *D*). Further supporting this conclusion, we detected

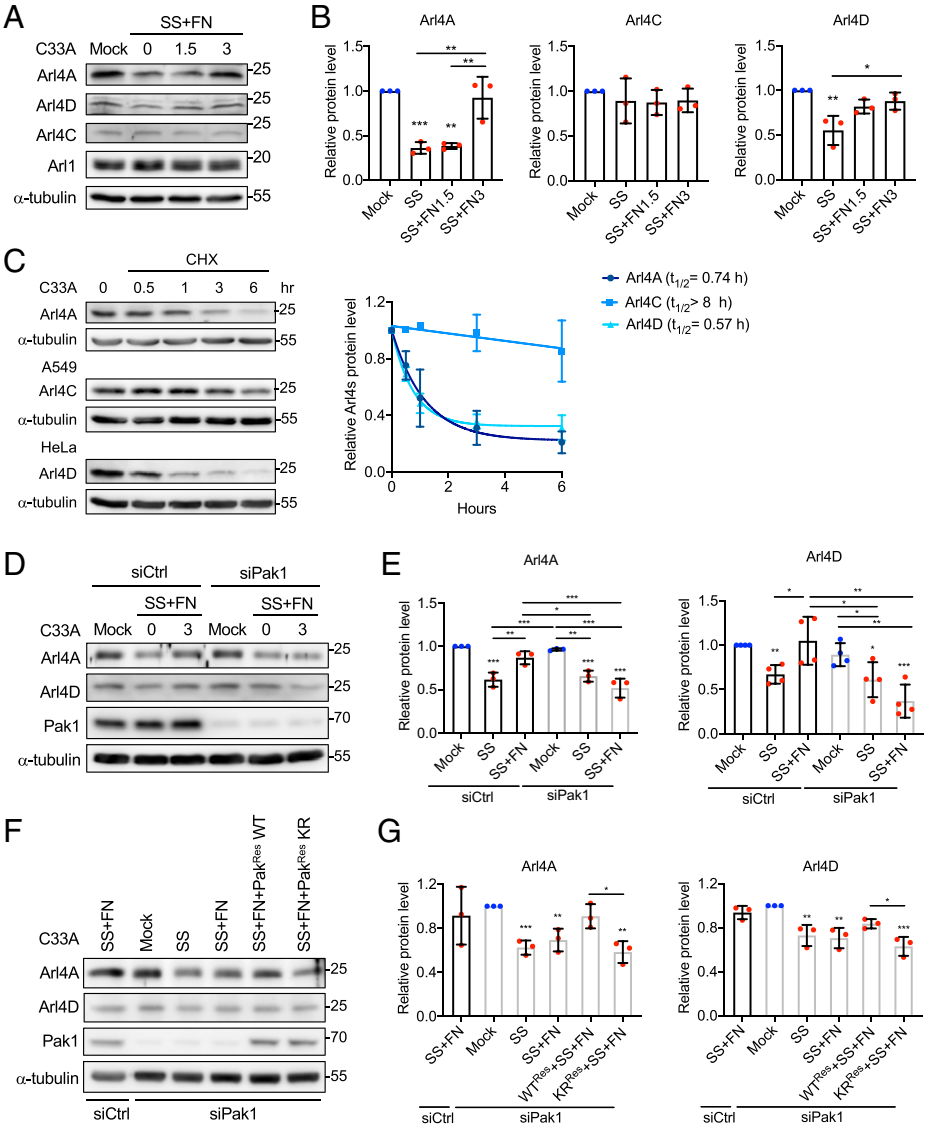

Fig. 1. Pak1 kinase is required to stabilize fast-degrading Arl4A/D under FN stimulation. (*A*) C33A cells were either kept in complete Dulbecco's modified Eagle's medium (mock) or were serum starved (SS) for 16 h before treatment with FN at 20 μg/mL for the indicated hours. (*C*) Cell lines with relatively abundant endogenous Arl4A (C33A), Arl4C (A549), and Arl4D (HeLa) were treated with CHX for the indicated times. The Arl4 protein level at each time point was normalized to the protein level at time 0 and is shown in the line graph, with error bars representing the mean ± SD; $n = 3$. (*D*) Mock and FN treatments were conducted in short interfering control (siCtrl) and short interfering Pak1 (siPak1) C33A cells. (*F*) Mock and FN treatments were further conducted in siCtrl-, siPak1-, and Pak1-rescued C33A cells. Res, siRNA-resistant form. (*B*, *E*, *G*) Quantification of Arl4 protein levels as determined by Western blotting in (*A*, *D*, *F*). The statistical results are shown in dot plots with error bars representing the means ± SD; $n = 3$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. One-way ANOVA with Dunnett's post hoc multiple comparison test was applied.

higher ubiquitination levels on Arl4A/D after blocking proteasomal degradation (*SI Appendix*, Fig. S1*E*).

As Pak1 is activated by FN stimulation (19), and we had found previously that Pak1 interacts with Arl4A/D (3), we next treated cells with small interfering RNA (siRNA) against Pak1 and found that FN stimulation could no longer stabilize the protein levels of Arl4A/D (Fig. 1*D* and *E*). Furthermore, wild-type (WT), but not a kinase-dead mutant of Pak1 (K299R, Pak1 KR), was able to rescue the reduced Arl4A/D protein levels that had been induced by knocking down Pak1 (Fig. 1*F* and *G*). Thus, these results revealed that FN stimulation likely enhanced the protein levels of Arl4A/D through the kinase activity of Pak1.

Pak1 Enhances Arl4A/D Stability by Phosphorylating S143/S144 Residues of Arl4A/D. To elucidate whether Pak1 activity leads to Arl4A/D phosphorylation, we next performed stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomics analysis. For these studies, we used HeLa cells, as they can be readily transfected to achieve a high level of Arl4A expression, which facilitates the detection of its phosphorylation in cells. Furthermore, we have previously used HeLa cells for studies on the function and regulation of exogenously expressed Arl4A (3). Two independent SILAC analyses detected increased phosphorylation of Arl4A at S141 and S143 upon FN stimulation (*SI Appendix*, Table S1 and Fig. 2*A*). As phosphorylation at S143 is the conserved residue to that of Arl4D, we next

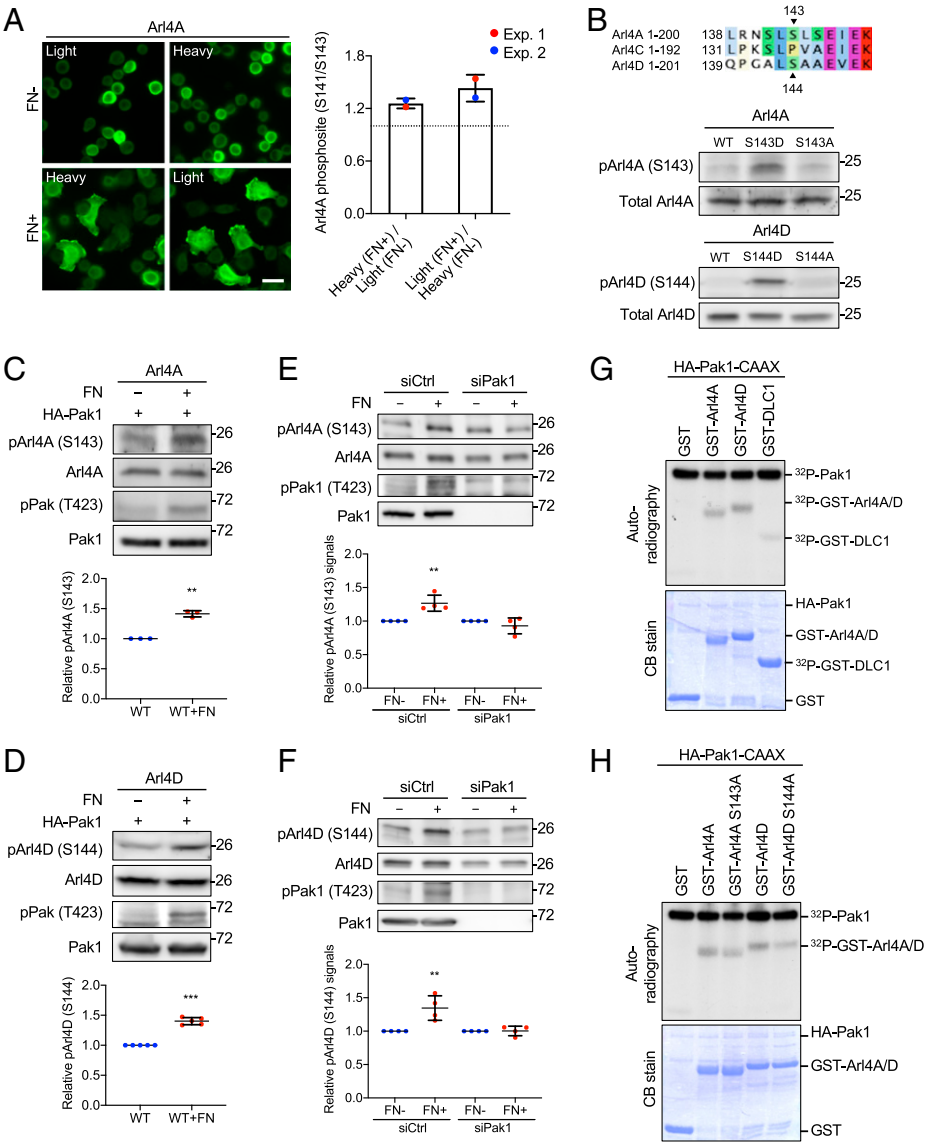

Fig. 2. Pak1 phosphorylates Arl4A/D on S143/S144 under FN signaling. (*A*) SILAC phosphoproteomic analysis of Arl4A upon FN stimulation. The [U-$^{13}C_6$]–labeled "heavy" and unlabeled "light" HeLa cells were transfected with Arl4A and reseeded on FN-uncoated (FN−) or -coated (FN+) coverslips for 1.5 h before SILAC liquid chromatography–tandem mass spectrometry. (*Left*) The immunofluorescence images indicate the same phenotype of Arl4A (green) in the two swapping groups upon reseeding on FN. Scale bar, 25 μm. (*Right*) The phosphorylation ratios of FN+/FN− of S141/S143 in Arl4A. The dots represent two biological replicates in which the heavy and light Arg/Lys-labeled chains were swapped. (*B*) HeLa cells equally overexpressing Arl4A/D WT, S143D/S144D, and S143A/S144A mutants were harvested for immunoblotting with anti–phospho-Arl4A/D (pArl4A/D) (S143/S144) antiserum. The anti-Arl4A/D antiserum was used to indicate the total Arl4A/D protein expression levels. (*C* and *D*) HeLa cells transfected with the indicated proteins were seeded on FN− or FN+ surfaces for 1.5 h and harvested to detect pArl4A/D (S143/S144) by Western blotting. pPak1 (T423) indicated the activation of Pak1 under FN treatment. (*E* and *F*) Experiments in (*C* and *D*) were conducted in short interfering control (siCtrl) and short interfering Pak1 (siPak1) cells. The pArl4A/D signals were quantified by normalizing to total Arl4A/D and are shown in the dot plots with error bars representing the means ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3$ to 5. The statistical results were analyzed by two-sample *t* test. (*G*) The in vitro kinase assay was performed by incubating HA-Pak1-CAAX purified from HeLa cells and GST and GST-Arl4A/D purified from *Escherichia coli* at 30 °C for 30 min. GST-DLC1 served as positive controls. (*H*) The kinase assay was performed as described for *G*, with equal amounts of GST, GST-Arl4A/D WT, and GST-Arl4A/D S143A/S144A phospho-defective mutants. CB, Coomassie blue; Exp, experiment.

generated phospho-specific antibodies against these sites. To confirm that the antibody recognized phosphorylation of Arl4A at S143, we mutated this site to aspartate (S143D) to mimic constitutive phosphorylation and to alanine (S143A) to mimic constitutive dephosphorylation, and then found that the antibody recognized S143D much better than S143A in transfected cells (Fig. 2*B*). We also mutated the corresponding site in Arl4D, generating S144D and S144A, and found that the antibody recognized S144D much better than S144A (Fig. 2*B*). Using these antibodies, we then confirmed that FN stimulation induced the phosphorylation of Arl4A/D at S143/S144 residues (Fig. 2*C* and *D*), respectively, while knockdown of Pak1 compromised the phosphorylation (Fig. 2*E* and *F*). We also confirmed Pak1 activation in this setting by tracking the phosphorylation of the T423 residue in Pak1 using a phospho-antibody specific for this site (Fig. 2*C–F*). To assess whether Pak1 can directly phosphorylate Arl4A at S143 and Arl4D at S144, we next performed the in vitro kinase assay. Pak1 can be constitutively activated by appending the CAAX motif for stable membrane localization. We isolated Pak1-CAAX from transfected cells and confirmed its activity by incubating with DLC1 (30), which is a known substrate of Pak1. Incubation with purified Arl4A/D then confirmed that Pak1 can directly phosphorylate these Arl4 proteins (Fig. 2*G*). We also confirmed that Pak1 targeted the S143/S144 residues of Arl4A/D, as mutating these residues to alanines, S143A/S144A, reduced the phosphorylation of Arl4A/D by Pak1 (Fig. 2*H*).

We next compared the stability of the Arl4A/D phosphorylation mutants. Performing cycloheximide (CHX) chase studies, we found that the D mutants of Arl4A/D were degraded less rapidly than the corresponding A mutants (Fig. 3*A* and *B*). In addition, the D mutants had weaker polyubiquitination signals than the A mutants, as assessed by the ubiquitination–immunoprecipitation (Ub-IP) assay (Fig. 3*C* and *D*). Thus, the results altogether revealed that Pak1 enhances the protein stability of Arl4A/D by phosphorylating the S143 residue of Arl4A and the S144 residue of Arl4D.

The Chaperone-Like Protein HYPK Interacts with Arl4A/D. We next sought insight into how phosphorylation of Arl4A/D enhances their protein stability. RABIF/MSS4 had been found previously to act as a Rab-stabilizing holdase chaperone in insulin-stimulated GLUT4 exocytosis (31). In this regard, we had previously performed a yeast two-hybrid screen using the GTP-bound form of Arl4D and identified HYPK as an interactor (32, 33). HYPK, an interactor of polyQ-expanded Huntingtin (Htt) (34), was reported to reduce Htt aggregation via chaperone-like activity mediated through its conserved nascent polypeptide-associated alpha subunit (NPAA) domain (35, 36). Furthermore, HYPK is up-regulated in the unfolded protein responses (37, 38). Thus, we explored whether HYPK could have a role in stabilizing Arl4A/D.

We first performed the yeast two-hybrid system and found that HYPK exhibited stronger interaction with Arl4A/D than with Arl4C (*SI Appendix*, Fig. S2*A*). We also found that Arl4A/D interacted with residues 115 to 129 of HYPK, which are within its NPAA domain that is responsible for its chaperone activity (36) (*SI Appendix*, Fig. S2*B* and *C*). We next confirmed the interaction between Arl4A/D and HYPK in cells, through coprecipitation studies (Fig. 4*A*). We also confirmed a direct interaction between Arl4A/D and HYPK through pulldown studies using purified components (Fig. 4*B*). We then considered that the yeast two-hybrid studies had revealed HYPK to interact with both the GTP-bound and the GTP-deficient forms of Arl4A/D: Q79L/Q80L and T51N/T52N, respectively. However, performing coprecipitation studies on cell lysates, we found that HYPK preferentially interacted with the GTP-bound mutants of Arl4A/D rather than the GTP-deficient mutants (Fig. 4*C* and *D*). Thus, as GTP-binding promotes membrane recruitment of Arl4A/D, a likely explanation was that the lack of membrane context in the yeast two-hybrid studies enabled HYPK to bind both GTP-bound and GTP-deficient forms of Arl4A/D.

In support of this explanation, we performed immunofluorescence microscopy and found that transfected HYPK showed a

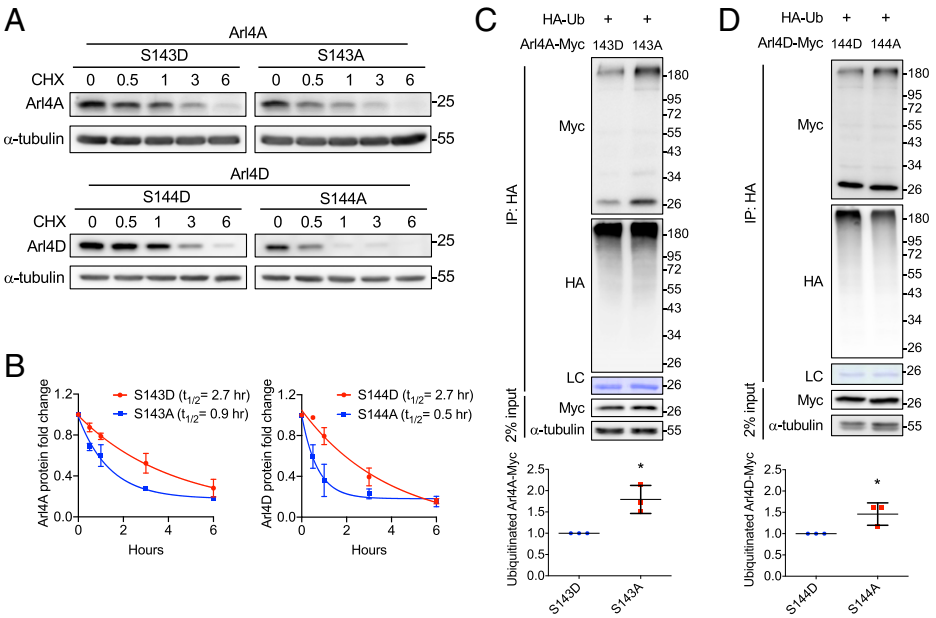

Fig. 3. Arl4A/D S143/S144 phosphorylation influences protein stability and ubiquitination. (*A*) Arl4A S143D/S143A and Arl4D S144D/S144A mutants were transfected into HeLa cells for the CHX chase assay for the indicated times. (*B*) One-phase exponential decay and $T_{1/2}$ of each phosphomutant assayed in *A* are shown separately; $n = 3$. (*C* and *D*) HeLa cells were transfected with the indicated proteins and treated with MG132 for 4 h before Ub-IP. The Myc signals of phosphomimetic mutants (S143/144D) were compared with the signals of phosphodefective mutants (S143/144A) after immunoprecipitation (IP). A two-sample *t* test was applied for the results shown in the dot plots, with error bars representing the means ± SD; $n = 3$. *$P < 0.05$. HA, hemagglutinin; LC, light chain of HA antibody.

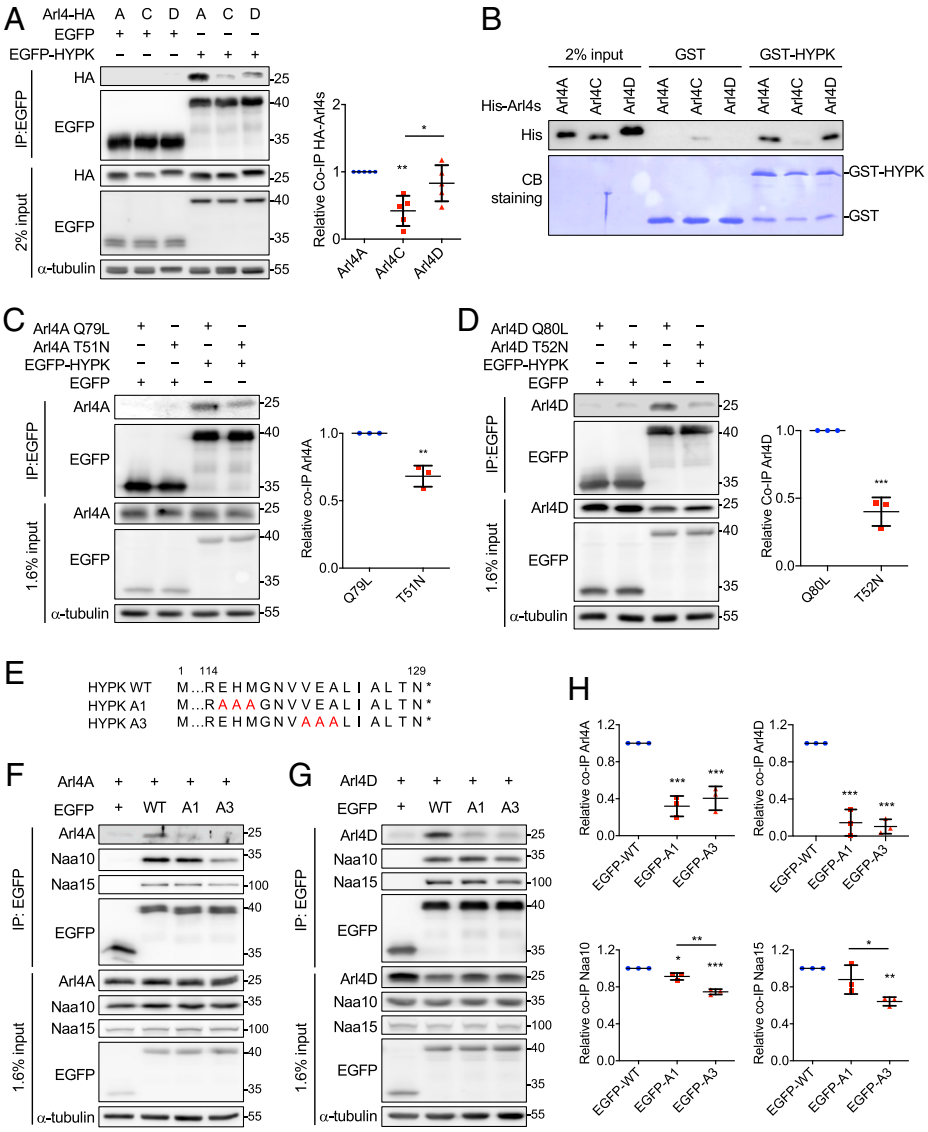

Fig. 4. The chaperone-like protein HYPK specifically interacts with Arl4A/D. (*A*) Arl4s-HA was equally expressed with enhanced green fluorescent protein (EGFP) or EGFP-HYPK in HeLa cells and treated with dithiobis(succinimidyl propionate) at 1 mM for 2 h before coimmunoprecipitation (co-IP) and Western blotting analysis. (*B*) In vitro binding of His-tagged Arl4A, Arl4C, and Arl4D with GST or GST-HYPK. His-Arl4 proteins pulled down by GST-fusion proteins were analyzed by Western blotting. (*C* and *D*) HeLa cells expressing either Arl4A Q79L and T51N (*C*) or Arl4D Q80L and T52N (*D*) were coexpressed with EGFP or EGFP-HYPK for co-IP. QL/TN, GTP-bound/GTP-deficient mimics. (*E*) Mutated residues of Arl4A/D binding defective HYPKs A1 and A3. (*F* and *G*) HeLa cells expressing either Arl4A (*F*) or Arl4D (*G*) were coexpressed with EGFP, EGFP-HYPK, EGFP-A1, or EGFP-A3 for co-IP. Naa10 and Naa15, which are components of the NatA complex, were the positive controls for co-complexation with HYPK. The co-IP signals of Arl4 proteins and NatA components were quantified and are shown in the dot plots of *A*, *C*, *D* and *H*, with error bars indicating the mean ± SD; $n = 3$ to 5. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The statistical results were analyzed by two-sample *t* test or one-way ANOVA with Dunnett's post hoc multiple comparison test for groups larger than two. CB, Coomassie blue; HA, hemagglutinin.

diffuse pattern consistent with cytosolic distribution (*SI Appendix*, Fig. S3*A*). However, the overexpression of Arl4A/D, but not Arl4C, resulted in a population of HYPK colocalizing with Arl4A/D at the plasma membrane (*SI Appendix*, Fig. S3*B*). Moreover, these colocalizations were more prominent when the GTP-bound mutants of Arl4A/D were expressed than when the GTP-deficient mutants were expressed (*SI Appendix*, Fig. S4).

To identify residues in HYPK that are critical for its interaction with Arl4A/D, we next performed alanine-scanning mutagenesis followed by yeast two-hybrid analysis to further define within the region of HYPK that we had found to be critical for its direct interaction with Arl4A/D (*SI Appendix*, Fig. S2*D* and *E*). We then generated two Arl4 binding–deficient HYPK mutants, A1 and A3, and confirmed that they had markedly reduced direct interaction with Arl4A/D, as assessed by pulldown studies using purified components (*SI Appendix*, Fig. S2*F* and *G*). We also performed cell-based studies, which confirmed that the A1 and A3 mutants exhibited markedly reduced interactions with Arl4A/D (Fig. 4*E*–*H*); however, their membrane recruitment seemed to not only depend on the direct interaction with Arl4A/D (*SI Appendix*, Fig. S3*C* and *D*). Furthermore, the A1 and A3 mutants retained better interaction with Naa10 and Naa15 (Fig. 4*E*–*H*), which are known binding partners of HYPK (39). Thus, the A1 and A3 mutations were relatively specific in abrogating the interaction between HYPK and Arl4A/D.

Binding by HYPK Stabilizes the Protein Levels of Arl4A/D. We next examined whether the interaction between HYPK and Arl4A/D promotes the stability of Arl4A/D. Performing the CHX chase assay, we found that the overexpression of WT HYPK, but not the A3 mutant, reduced the degradation of Arl4A/D (Fig. 5*A*–*D*). We also sought to examine the effect of

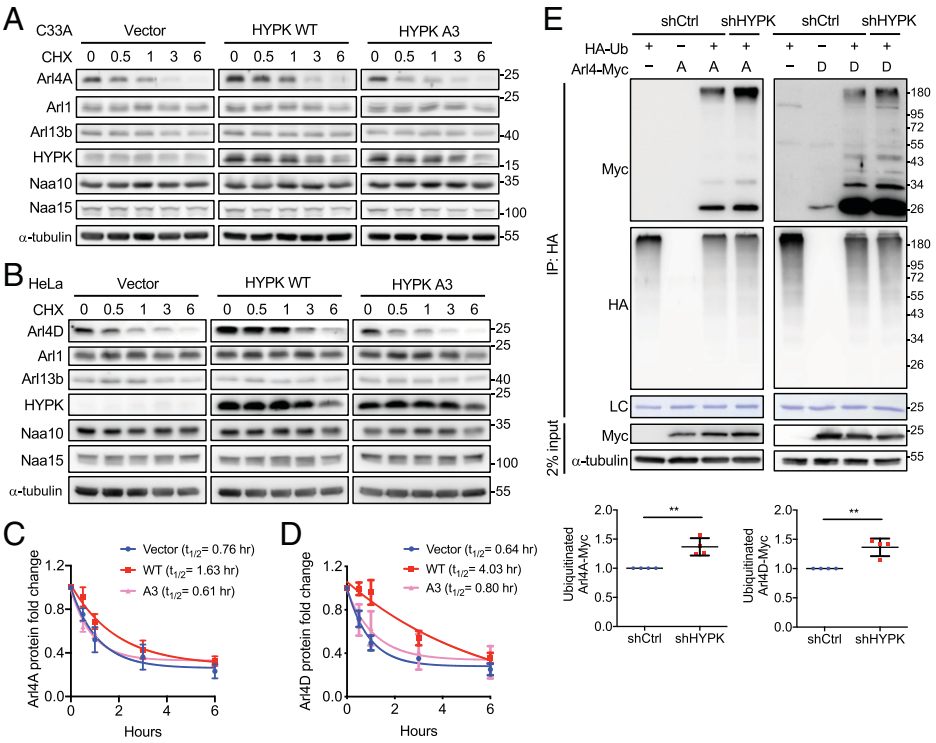

Fig. 5. The HYPK–Arl4A/D interaction contributes to Arl4A/D protein stabilization. (*A* and *B*) CHX was applied to C33A and HeLa cells expressing empty vector, HYPK WT, and HYPK A3 for the indicated times, and endogenous Arl4A in C33A cells (*A*) and Arl4D in HeLa cells (*B*) were examined by Western blotting. (*C* and *D*) The protein levels of the Arl4s from *A* and *B* were normalized to α-tubulin and the protein level at time 0. The one-phase exponential decay of each group was plotted; $n = 3$. The half-lives ($t_{1/2}$) of groups expressing the empty vector, HYPK WT, and the HYPK A3 mutant are shown. (*E*) Short hairpin control (shCtrl) RNA– and short hairpin HYPK (shHYPK) RNA–expressing HeLa cells were transfected with the indicated proteins and treated with MG132 before Ub-IP and Western blotting. Arl4A/D-Myc signals from immunoprecipitation (IP) were quantified by normalizing to immunoprecipitated hemagglutinin–ubiquitination (HA-Ub) signals and Arl4 inputs. The results are shown in dot plots, with error bars representing the mean ± SD; $n = 4$. $^{**}P < 0.01$. The statistical results were analyzed by two-sample *t* test.

knocking down HYPK. Because the Arl4A/D proteins have relative short half-lives, we took two approaches to detect a potential increase in Arl4A/D degradation upon knocking down HYPK.

In one approach, we found that knocking down HYPK increased the polyubiquitination levels of Arl4A/D (Fig. 5*E*). In another approach, we treated cells with MG132 to inhibit the proteasomal degradation of Arl4A/D and then found that knocking down HYPK decreased their protein levels, but not that of Arl4C, Arl1, or Arl13b (*SI Appendix*, Fig. S5*A–F*). We also confirmed the specificity of the shRNA treatments in targeting against HYPK, as rescue with WT HYPK restored the protein levels of Arl4A/D toward that of the control condition (*SI Appendix*, Fig. S5*G* and *H*).

Phosphorylation of Arl4A/D Enhances Their Interaction with HYPK on the Plasma Membrane. We next examined whether their interaction with HYPK contributes to how phosphorylation of Arl4A/D enhances their localization at the plasma membrane. We initially performed coprecipitation studies and found that HYPK interacted better with the D mutants of Arl4A/D S143/S144 as compared with the corresponding A mutants (Fig. 6*A* and *B*). We then fractionated cells into total membrane versus cytosol and found that FN stimulation enhanced the interaction between HYPK and Arl4A/D on the membrane but not in the cytosol (Fig. 6*C* and *D*). The membrane fraction of Arl4A/D also showed increased phosphorylation at S143/S144, as assessed by the phospho-antibodies directed against these residues (Fig. 6*C* and *D*). We next found that knocking down HYPK reduced the levels of Arl4A/D on the plasma membrane while not affecting that of Arl4C (Fig. 7*A*). Moreover, rescue using the siRNA-resistant form of WT HYPK (HYPK WT^{Res}) but not the interaction-deficient mutant (HYPK-$A3^{Res}$) was able to restore the plasma membrane levels of Arl4A/D (Fig. 7*B* and *C*). In the same context, Arl4A/D protein levels were also rescued (Fig. 7*D* and *E*). Thus, the collective results revealed a key role for HYPK in stabilizing Arl4A/D on the plasma membrane.

HYPK Binding to Arl4A/D Promotes Their Role in Cell Migration. As Arl4A/D acts in cell migration (2, 3, 10), which requires their targeting to the plasma membrane (3), we next further interrogated the role of HYPK binding to Arl4A/D in this context. We first found that knocking down HYPK inhibits cell migration, as assessed by treating cells with two different targeting sequences against HYPK in the Transwell migration assay (*SI Appendix*, Fig. S6*A*). This inhibition was rescued by HYPK WT^{Res} but not the mutant forms deficient in binding to Arl4A/D (i.e., $A1^{Res}$ and $A3^{Res}$) (*SI Appendix*, Fig. S6*A*). In a complementary analysis, we examined the effect of targeting against HYPK in the context of Arl4A overexpression, which enhances cell migration. In this situation, knocking down HYPK also inhibited cell migration, which was again rescued by the expression of WT HYPK but not mutant forms that are deficient in binding to Arl4A (*SI Appendix*, Fig. S6*B*). Thus, these results confirmed the importance of HYPK binding to Arl4A/D for their role in cell migration.

Discussion

Our detailed elucidation of how Arl4A/D act in cell migration has advanced a fundamental understanding of how these small GTPases are regulated. Whereas small GTPases are typically regulated by their GTPase cycle, we have found that Arl4A/D are regulated through protein stability. One of the best characterized

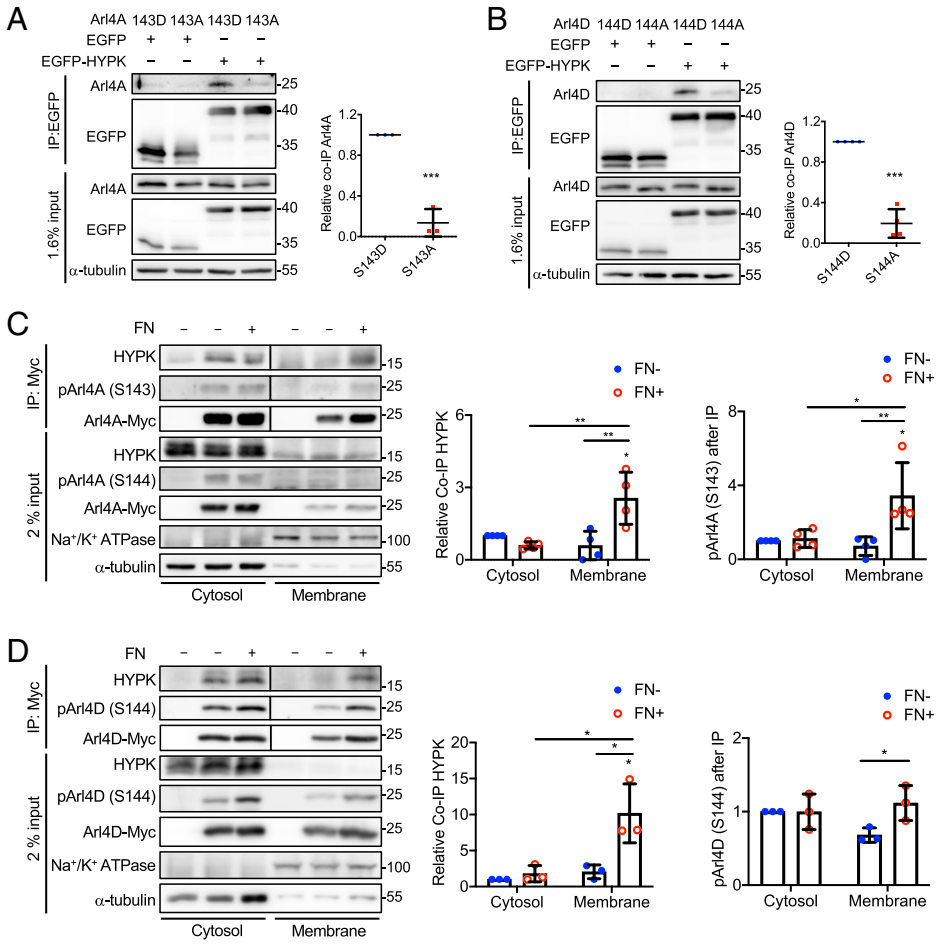

Fig. 6. HYPK efficiently binds to Arl4A/D through the recognition of phospho-S143/S144 under FN stimulation. (*A* and *B*) HeLa cells were transfected with phospho-memetic or -defective Arl4A/D S143/S144 mutants for coimmunoprecipitation (co-IP) by enhanced green fluorescent protein (EGFP) or EGFP-HYPK. (*C* and *D*) Co-IP of Arl4A/D and HYPK in the cytosolic and membrane fractions upon FN treatment. HeLa cells were transfected with the indicated proteins before reseeding on FN− or FN+ dishes for 3 h. Dithiobis(succinimidyl propionate) cross-linking was performed before cell fractionation and immunoprecipitation (IP). α-Tubulin, cytosolic fraction protein; Na^+/K^+ ATPase, membrane fraction protein. (*Right*) The co-IP levels of HYPK and pArl4A/D (S143/S144) are quantified. The results, after normalization, are presented in dot plots with error bars showing the mean ± SD; $n = 3$ to 4. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The statistical results were analyzed by two-sample *t* test or one-way ANOVA with Dunnett's post hoc multiple comparison test when more than two groups were compared.

cellular roles of Arl4A/D is their involvement in cell migration. Previously, FN stimulation was found to recruit Arl4A/D to the plasma membrane to promote cell migration (3). To understand at the mechanistic level how this occurs, we have revealed in the present study that FN stimulation induces Pak1 to phosphorylate Arl4A/D to promote their protein stability by preventing their proteasomal degradation. Further defining this mechanism, we found that HYPK plays a key role, as it binds to the phosphorylated Arl4A/D to stabilize their localization at the plasma membrane, which is needed for their role in cell migration. A model summarizing our mechanistic findings is shown in Fig. 8.

Although Arl4C does not share the same mechanism of regulation as Arl4A/D, it also plays an important role in cell migration. Arl4C seems likely to act also independent of guanine nucleotide exchange factor activation, as it can be loaded with GTP spontaneously (14). Arl4C expression has been found to be induced at the transcriptional level under cholesterol and Wnt/EGF signaling (40, 41). Another study showed that Arl4C function in the developing hippocampus relies on the tight control of CRL5 E3 ubiquitin ligase for dendritic outgrowth in neurons (42). Thus, although not sharing the identical mechanism of regulation as Arl4A/D, Arl4C is also likely to be regulated at the level of its expression rather than by the GTPase cycle.

FN has been reported to control translational machinery through eukaryotic initiation factors 4F and 2 and to have no synergistic effect with serum-stimulated translation (43). We have found that FN stimulation functions differently in the case of Arl4A/D, acting instead to prevent their protein degradation. Furthermore, whereas the traditional way of examining FN stimulation involves plating them as a solid matrix to trigger cell adhesion signaling through integrin receptors, we find that the presence of soluble plasma FN was sufficient to stabilize the Arl4A/D protein levels. This revelation suggests that, whereas FN stimulation in the context of solid matrix promotes cancer cell invasion through tissue, FN in its soluble form in serum could stimulate cancer cells to migrate in the circulatory system for their metastatic dissemination.

Similar to the case of Arl4 small GTPases, Rnd proteins are Rho family GTP-binding proteins whose GTPase activities are not detectable (44). Rnd proteins comprise Rnd1, Rnd2, and Rnd3 (RhoE). RhoE has been found to bind GTP constitutively in vivo (45), and its protein turnover is similarly as rapid as that of Arl4A/D (46). RhoE function was reported to be regulated by phosphorylation by the Rock1 kinase, which is induced by PDGF stimulation. This phosphorylation promotes the protein stability of RhoE and correlates with RhoE activity in stress fiber disruption (47). As such, our elucidation of how Arl4A/D are stabilized through binding by a chaperone-like protein could be relevant to understanding how RhoE is regulated through protein turnover.

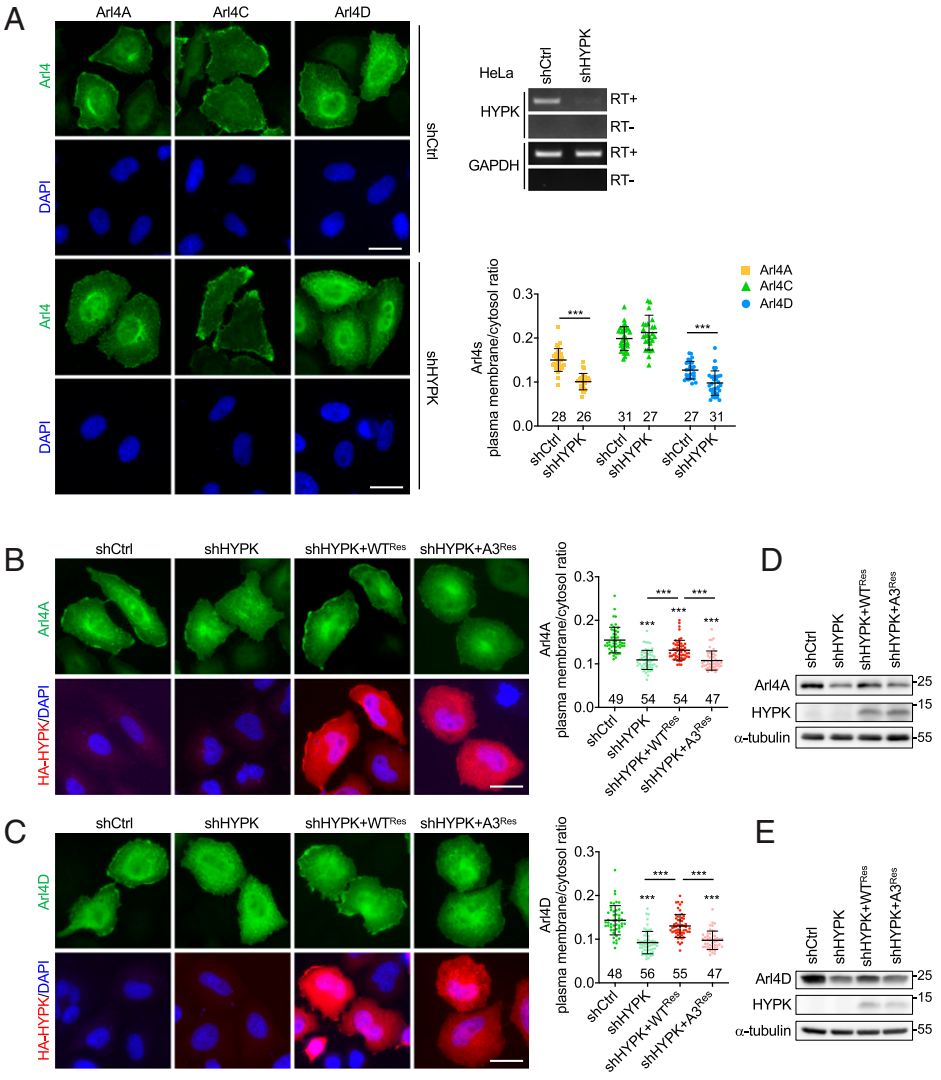

Fig. 7. HYPK consolidates the membrane targeting signal of Arl4A and Arl4D. (*A*) Arl4 proteins expressed in short hairpin control (shCtrl) or short hairpin HYPK (shHYPK) HeLa cells were stained with Arl4A-, Arl4C-, and Arl4D-specific antibodies (green) and DAPI (blue; stains the nuclei). Scale bar, 25 μm. The efficiency of HYPK knockdown was determined by RT-PCR. GAPDH was used as the internal control. The plasma membrane to cytosol ratios of Arl4A, Arl4C, and Arl4D were quantified as described in *SI Appendix, Materials and Methods*. The ratios in the shCtrl- and shHYPK-expressing cells were compared by two-sample *t* test, with error bars showing the means ± SD. ***$P < 0.001$. The cell numbers analyzed are marked in the plot. (*B* and *C*) HYPK knockdown cells were rescued with the resistant form (Res) of HA-HYPK WT or the HA-HYPK A3 mutant (red) in HeLa cells. Scale bar, 25 μm. The plasma membrane to cytosol ratios of Arl4A/D in each group were calculated and are 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. ***$P < 0.001$. (*D* and *E*) Immunoblotting of the protein expression levels in *B* and *C*.

We further note that RABIF/MSS4 has been found to act as a Rab8a-, Rab10-, and Rab13-stabilizing holdase chaperone, and depletion of RABIF abolished Rab10-mediated GLUT4 exocytosis upon insulin stimulation (31). BAG6, an ATP-independent molecular chaperone, is also required for the elimination of a number of Rab small GTPases for the balance of

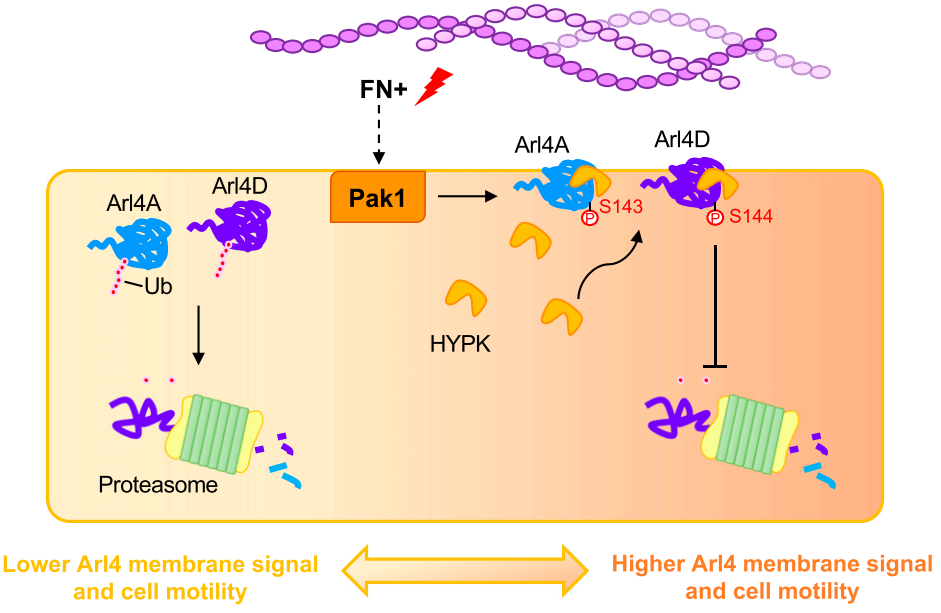

Fig. 8. The model of FN-Pak1–triggered signaling for Arl4A/D protein stabilization. In Arl4A/D-expressing cells, Arl4A/D undergo rapid degradation. Under FN stimulation, Pak1-dependent phosphorylation at S143/S144 of Arl4A/D on the plasma membrane potentiates local and efficient HYPK binding for protein protection. HYPK-mediated protection not only stabilizes Arl4A/D from fast proteasome degradation but also strengthens their membrane targeting for cell migration. Ub, ubiquitination.

endosomal trafficking pathways (48). Thus, regulation through protein stability may be a more prevalent mechanism of modulating small GTPases than currently appreciated.

Materials and Methods

Detailed descriptions of antibody and plasmid preparation; cell culture, transfection, knockdown, migration assay, and coimmunoprecipitation in mammalian cells; drug and FN treatment; immunofluorescence analysis; recombinant protein induction and purification; in vitro binding and kinase assay; protein quantification and Western blotting; yeast two-hybrid screening; RNA extraction and RT-PCR; SILAC liquid chromatography–tandem mass spectrometry experiments; and statistical analysis are provided in *SI Appendix, Materials and Methods*.

Data Availability. The phosphoproteomic analysis data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (https://www.ebi.ac.uk/pride/) (49) with the project name entitled "Quantitative phosphoproteomic analysis of human Arl4A upon fibronectinstimulation" (data set identifier:PXD032397).

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