RESEARCH ARTICLE

Analysis of biological networks in the endothelium with biomimetic microsystem platform

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INTRODUCTION

Posttranslational protein modifications (PTMs), such as phosphorylation, can “switch on” and “switch off” the biological function of proteins. Often, by modulating the binding affinities between different proteins, PTMs control the activity as well as intracellular distribution of proteins involved in signal transduction in biological systems (8, 35, 36). Understanding how PTMs control a protein’s biological network or “interactome” can reveal how specific PTMs regulate adaptive cellular responses associated with phenotypic changes.

One of the most common techniques for detecting changes in protein-protein interactions is an affinity purification (AP) assay, whereby capture of biological material from the cell lysate is performed using an enriched bait protein coupled to a solid support (30). It serves as a library-independent alternative to the commonly used yeast two-hybrid (Y2H; 45) and mammalian two-hybrid (M2H; 39) assay. It can be used to isolate protein complexes in primary cells by covalently attaching purified His-tagged proteins to a solid microscale support. Using this Bio-MSP, we have analyzed the interactomes of unphosphorylated and phosphomimetic end-binding protein-3 (EB3) in endothelial cells. Pathway analysis of these interactomes demonstrated the novel role of EB3 phosphorylation at serine 162 in regulating the biological role of specific PTMs (Fig. 2).

Using the Bio-MSP, we analyzed the effect of phosphorylation of the microtubule-associated end-binding protein-3 (EB3) at S162. We have previously demonstrated that dephosphorylation of this residue promotes microtubule growth and decreases the assembly of vascular endothelial cadherin (VE-cadherin) junctions, thus identifying this site as an important “phosphoswitch” of biological function (24). To determine how changes in EB3 phosphorylation alter downstream signaling pathways, we isolated proteins bound to EB3 in phosphorylated and phosphomimetic end-binding protein-3 (EB3) in endothelial cells. Pathway analysis of these interactomes demonstrated the novel role of EB3 phosphorylation at serine 162 in regulating the biological role of specific PTMs (Fig. 2).

MATERIALS AND METHODS

Cell culture. Primary human lung microvascular endothelial cells (HMVEC-L; cat. no. CC-2527, lot no. 0000582655; Lonza) and human pulmonary arterial endothelial cells (HPAECs; cat. no. CC-2530, lot no. 0000662151; Lonza) were grown in Endothelial Basal Medium (cat. no. CC-3121; Lonza) supplemented with 10% FBS (cat. no. 35-015-CVX; Mediatech) and either Endothelial Growth Medium Microvascular (EGM-MV) BulletKit (cat. no. CC-3125; Lonza) or

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Endothelial Growth Medium (EGM) BulletKit (cat. no. CC-3124; Lonza). Lonza supplies endothelial cells isolated from donor lung tissue. Each donor has a unique lot number. Lot no. 000058265 was derived from a 17-yr-old Hispanic male, and lot no. 0000662151 was derived from a 48-yr-old Caucasian man. The cells were characterized by positive immunofluorescence staining for endothelial markers VE-cadherin and platelet endothelial cell adhesion molecule (PECAM-1). Cell lines tested negative for mycoplasma, bacteria, yeast, and fungi. Primary cells were used at passages 4–8. Human embryonic kidney 293 (HEK-293) cells (cat. no. 85120602; Sigma-Aldrich) were grown in Dulbecco’s modified Eagle’s medium (DMEM; cat. no. 11995065; Gibco). All cells were maintained at 37°C and 5% CO₂.

Transfection of cells with mammalian vectors. HPAECs were transected using electroporation with the Amaxa primary endothelial cell Nucleofector kit (cat. no. VPI-1001; Lonza) according to the manufacturer’s instructions using code M-003. HEK-293 cells were transfected using X-tremeGENE HP (Roche Applied Science) according to the manufacturer’s instructions.

RNA interference. HPAECs (cat. no. CC-2530, lot no. 0000662151; Lonza) were transfected with ON-TARGETplus SMARTpool (Dharmacon, Lafayette, CO) containing a mixture of four small interfering RNAs (siRNAs) targeting human platelet-activating factor acetylhydrolase IB subunit-α (PAFAH1B1; L-010330-00-0005) or a nontargeting SMARTpool (D-001810-10-05) using GeneSilencer siRNA transfection reaction (cat. no. T-500750). Cells were used at 72-h posttransfection for Western blot analysis to validate specificity of PAFAH1B1 antibody (sc-374586; Santa Cruz Biotechnology).

Antibody validation. Anti-EB3 (0103020-D-02; Absea Biotechnology) and anti-VE-cadherin (sc-6458; Santa Cruz Biotechnology) antibodies were previously validated by us (14, 16); anti-PECAM-1 (sc-1506-R; Santa Cruz Biotechnology) and anti-proliferation marker protein Ki-67 (Ki-67; 14-5698-82; Thermo Fisher Scientific) antibodies were validated by Dergilev et al. (7) and Wu et al. (48). Cortactin antibody (GTX-113681; GeneTex) was validated by the manufacturer using clustered regularly interspaced short palindromic repeats (CRISPR) cortactin knockout cell lines. Representative full-length blots for PAFAH1B1 antibody (sc-374586; Santa Cruz Biotechnology) are presented in Fig. 1.

Preparation of (His6)-tagged EB3 constructs. Preparation of (His6)-tagged EB3 was described previously (24). (His6)-tagged recombinant proteins were expressed in Escherichia coli strain BL21(DE3) (Stratagene). Bacteria were grown at 37°C in Luria-Bertani medium containing 50 μg/ml kanamycin. When the optical density measured at 600-nm wavelength (OD600) reached 0.6–0.7, protein synthesis was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 250 μM. After 4 h at 30°C, bacterial pellets were isolated and sonicated (4 × 1 min) in medium comprising 150 mM NaCl, 5 mM 2-mercaptoethanol, 2 mM CaCl₂, 10 mM imidazole, 2 mM PMSF, and 25 mM Tris, pH 7.4.

Cross-linking of EB3 to beads. Purified 6xHis-tagged EB3 and 6xHis-tagged EB3-S162E were added to HisPur nickel-nitrilotriacetic acid (Ni-NTA) magnetic beads (cat. no. 88831; Thermo Fisher Scientific) in solution (0.1 mg/ml) for 1 h at room temperature. The beads were extensively washed with 150 mM NaCl and 20 mM HEPES (pH 7.0). To covalently link the proteins to beads, beads were incubated with 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-hydroxysuccinimide (NHS) in 150 mM NaCl and 20 mM HEPES, pH 7.5) and finally washed with 150 mM NaCl and 20 mM EDTA (pH 7.0) for 3 h at 4°C. The isolated protein complexes were cross-linked to the bait protein using the reversible cross-linker dithiobis(succinimidy1 propionate) (DTSP) in PBS containing 2 mM Ca²⁺ (pH 7.4) for 5 min at room temperature. The reaction was quenched with 1 M Tris (pH 7.5). The cross-linked protein complexes were washed four times with PBS containing 2 mM Ca²⁺ to remove nonspecific binding and eluted by incubating with buffer containing 25 mM Tris·HCl (pH 7.5), 10 mM NaCl, 0.1% SDS, and 100 mM dithiothreitol (DTT) for 1 h at 60°C. The protein complexes were spun down using 0.22-μm tubes (Corning) to remove beads while allowing proteins to pass through. Proteins were then concentrated using 10K Amicon Ultra centrifuge tubes and reconstituted in sample buffer.

Avi-green fluorescent protein-EB3 BirA streptavidin pulldown. Protein complexes bound to biotinylated Avi-green fluorescent protein (GFP)-EB3 were collected using avidin-biotin affinity-based separation as described (24). Briefer, HEK-293 cells were cotransfected with Avi-GFP-EB3 and BirA, pretreated with 1 μM cyclosporin A for 30 min, and lysed in buffer containing 20 mM Tris·Cl (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40, phosphatase inhibitor cocktail I and II (1:100; Sigma-Aldrich), 10 mM glycerol 2-phosphate, and protease inhibitor (Roche Applied Science). Lysates were incubated with NeutrAvidin agarose beads (Thermo Fisher Scientific) for 2 h at 4°C.

SDS-PAGE. Samples were run on a 4–20% Tris-glycine gel for 90 min at 100 V in Tris-glycine running buffer. The gel was removed and stained using Coomassie blue, and the protein band was excised for mass spectrometry. The samples were digested with trypsin and analyzed on an LTQ Velos ion trap mass spectrometer, and the resulting peptide precursor intensities were used for analysis.

Gene set enrichment analyses. MS data were processed using base R tools. Precursor intensity values were log₁₀ transformed. Proteins without at least one nonredundant (multimatching) peptide were filtered from the analyses. Average intensity was computed as the mean precursor intensity, weighted by the level of redundancy, such...
that peptides matching several proteins (1-to-many) were given lower weights than peptides matching single proteins (1:1) in proportion to the number of matches. Enrichment tests of the 1,302 “canonical pathways” and Gene Ontology (“GO”) gene sets of the Molecular Signatures Database (MsigDB v5.1) were conducted for EB3- and EB3-S162E-binding proteins using Fisher’s exact test after filtering for proteins detected within the MS experiments.

**SxIP and LxxPTPh motif enrichment analysis.** Protein sequences for proteins identified in experimental conditions Avi-GFP-EB3, His-EB3, or His-EB3-S162E were downloaded from Universal Protein Resource (UniProt), first matching gene symbols to UniProt IDs using the UniProtweb tool “Retrieve/ID mapping” ([https://www.uniprot.org/uploadlists/](https://www.uniprot.org/uploadlists/)). Motif searches were performed using regular expression: SxIP motif with “[ST][IL]P” and LxxPTPh motif with “LPT[AVILMFY]”. Statistical enrichment of this motif in different protein lists was computed using Fisher’s exact test.

A protein interaction network was created using Network Analyst v3.0. Only proteins with strong signals from the MS experiments (>10^4 mean peptide intensity) and only strongly supported interactions (>900 confidence score, Search Tool for the Retrieval of Interacting Genes/Proteins, or STRING) were included in a zero-order network, and the largest network was identified.

**FICT-dextran permeability.** To measure endothelial permeability, HPAECs expressing either wild-type EB3 (EB3-WT)-GFP, EB3-S162A-GFP, or EB3-S162E-GFP were grown in 12-mm Transwell inserts with 0.4-μm pores separating the upper and lower wells (Corning Life Sciences) for 2 days to establish a confluent endothelial monolayer. A solution of 0.5 mg/ml FICT-dextran (70,000 average mol wt) in phenol red free medium was added to the upper well. Phenol red free medium without tracer was added to the lower wells. Samples were taken in triplicate from the lower well at the beginning of the experiment and at every hour for 4 h. The relative fluorescence intensity was normalized to the starting value for each well, and the normalized fluorescence signal was plotted over time. Linear regression was used to calculate the permeability rates.

**Immunofluorescence staining.** Cells expressing EB3-GFP and the EB3 mutants were fixed in 4% formaldehyde for 20 min, washed once with PBS, and then permeabilized for 15 min using 0.01% Triton X-100 in PBS. Nonspecific sites were blocked using 3% bovine serum albumin (BSA) for 2 h at room temperature. Cells were incubated with primary and then with secondary antibodies using 1:100 dilution for 1 h at room temperature.

**Analysis of Ki-67.** To measure the number of proliferating cells, HPAECs expressing either EB3-WT-GFP, EB3-S162A-GFP, or EB3-S162E-GFP were fixed and stained for Ki-67 and DAPI as described above. The number of Ki-67-positive nuclei was divided by the total number of nuclei in GFP-expressing cells and expressed as a percentage.

**Quantification of PECAM-1 junction area at adherens junctions.** To quantify the relative amount of PECAM-1 at adherens junctions, a z-projected image was thresholded to eliminate background fluorescence. The threshold area within a 5-μm-wide band around the inner edge of the cell was calculated. The data were expressed as the PECAM-1-positive area divided by the total junction area.

**Statistical analyses.** Statistical analyses were performed using GraphPad Prism. An unpaired t-test was used for experiments with two experimental groups and ANOVA for more than two experimental groups. *P* < 0.05 was considered statistically significant.

**RESULTS**

To identify novel proteins that differentially bind either dephosphorylated or phosphorylated EB3, we first established the method for isolating endogenous protein complexes using a novel Bio-MSP (Fig. 2, A–C). To create a Bio-MSP that carries a bait protein, we used a surface chemistry approach (Fig. 2A). Purified human 6xHis-tagged EB3 or phospho-

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**Fig. 2. Schematic representation of work flow for identification of “interactome” using biomimetic microsystem platform (Bio-MSP). A:** Covalent attachment of protein to microsystem carrier. The 6xHis-tagged protein end-binding protein-3 (EB3) is attached to nickel-nitrilotriacetic acid (NiNTA) carrier in an oriented manner through the high binding affinity of the 6xHis tag to the Ni2+ atom. The protein is covalently linked to the carrier by using the cross-linking agents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to create a Bio-MSP. B: comparison of protein capture steps for covalently linked EB3 vs. nonlinked EB3. Specific binding partners bind to the His-tagged bait protein, while nonspecific proteins are washed away during capture. During elution of specific binding partners for analysis, the covalently bound bait protein will remain attached to the carrier. The non-covalently linked bait protein will be abundant in the sample, which may prevent further analysis. C: work flow for identification of interactome. Proteins are first captured by Bio-MSP. Eluted protein complexes are then submitted for liquid chromatography-mass spectrometry (LC-MS) analysis. The Gene Ontology association and pathway enrichment analyses are used to determine protein signaling networks. The networks are validated using functional assays. D: Western blot analysis for 6xHis-EB3 either covalently bound to magnetic microscale carrier using EDC/NHS (+) or not covalently linked (−). Note that the absence of EB3 in the sample indicates it was covalently bound to the carrier. E: Coomassie-stained gel for protein complexes isolated using either His-EB3 or His-EB3-S162E Bio-MSP or overexpressed Avi-green fluorescent protein-EB3. MW, molecular mass.
metic EB3-S162E were covalently linked to Ni-NTA beads using EDC and NHS (Fig. 2A). The Ni\(^{2+}\) atom coordinates with the polyhistidine tag of the bait protein to provide the correct orientation of the protein on the bead (19). Cross-linking leads to formation of a covalent bond between the carboxylic groups of the Ni-NTA moiety and the primary amines of the protein near the 6xHis tag (4). As a result, EB3 remains attached to the beads after denaturation of the sample before electrophoresis (Fig. 2D).

To determine the difference in binding affinities between the phosphomimetic and dephosphorylated proteins, we sought to minimize all other variables such as cell confluence and passage number. Therefore, we divided the lysate prepared from a single flask of HMVEC-L into two samples. The beads were incubated with the cell lysate for 3 h allowing formation of the complexes. The isolated protein complexes were separated by SDS-PAGE (Fig. 2E) and submitted for mass spectrometry analysis. To compare this novel technique with the present standard, we also coexpressed Avi-GFP-EB3 with BirA in HEK-293 cells and isolated complexes using streptavidin beads (Fig. 2E; 5).

We have identified 1,282 proteins bound to ectopically expressed Avi-GFP-EB3 in HEK-293 cells and significantly fewer proteins, 478 and 487, bound to S126E and EB3, respectively, in HMVEC-L (Fig. 3A). These results are consistent with the known limitations of overexpression systems that often lead to identification of false-positive hits (10, 37). There was a greater number of common proteins present in all three samples with a lower number of unique proteins that were specific for either dephosphorylated or phosphomimetic proteins (Fig. 3B). To quantify the relative abundance of proteins in each sample, the five peptide sequences with the greatest precursor identities were selected for further analysis (Fig. 3C).

Highlighting the most abundant proteins in each sample identified well-known EB3 binding partners such as EB3 and EB1, which form homodimers and heterodimers in cells and in vitro (6, 23); tubulin (18, 43); actin; and actin-binding proteins (12; Fig. 3A) indicating that our method reproduces previously published data. In primary endothelial cells, we detected several novel EB3 partners including VE-cadherin [cadherin 5 (CDH5); 27], intercellular adhesion molecule 1 (ICAM1; 3), and platelet endothelial cell adhesion molecule (PECAM1; 28),...
specific markers of endothelial cells. The sample prepared from HEK-293 cells contained biotin-binding and biotin-containing proteins [methylcrotonyl-CoA carboxylase 1 (MCCCI), propionyl-CoA carboxylase (PCC), and acetyl-CoA carboxylase-alpha (ACACA)] suggesting contamination of the sample with proteins interacting with the affinity reagent (Fig. 3, A and B). On the basis of these data we conclude that our novel method provides an accurate isolation of bona fide interacting proteins with fewer background contaminants.

After filtering the data through the Contaminant Repository for Affinity Purification (CRAPome; 32) and plotting the abundance of each protein found interacting with either EB3 or EB3-S162E in HMVEC-L, the results showed a triphasic distribution of proteins that specifically bound either EB3 or EB3-S162E or both (Fig. 3C). There was no difference in the number of proteins containing either SxIP (20) or LxxPTPhe (26) motifs between these two groups, suggesting that phosphorylation of S162, which is located within the linker, has no effect on the binding of proteins to the COOH terminus of EB3. To validate the presence of unique proteins identified by MS in each sample, the interactions were examined by Western blot (Fig. 3, D–H). The results confirmed that VE-cadherin (CDH5) and cortactin (CTTN) were bound to both EB3 and EB3-S162E (Fig. 3, D and E), whereas neuron navigator 1 (NAV1) and PECAM-1 were specifically bound to EB3-S162E (Fig. 3, F and G), and PAFAH1B1, also known as lissencephaly-1 homolog (LIS1), displayed a higher affinity to dephosphorylated EB3 (Fig. 3H).

Furthermore, Gene Ontology analysis showed the association of dephosphorylated and phosphomimetic EB3 proteins with specific signal transduction networks (Figs. 3C and 4). Particularly, phosphomimetic EB3 (EB3-S162E) was bound to a significantly higher proportion of proteins involved in cell-to-cell adhesion [endothelial cell adhesion molecule (ESAM), CDH2, CDH5, PECAM1, ICAM1, and integrin subunit-b1 (ITGB1)], while dephosphorylated EB3 was bound to proteins involved in the negative regulation of cell proliferation [transforming growth factor-b1 (TGFb1)], hepatocyte growth factor-regulated tyrosine kinase substrate (HGS); protein kinase, interferon-inducible double-stranded RNA-dependent inhibitor, repressor of (P58 repressor) (PRKRIR); protein activator of interferon-induced protein kinase EIF2AK2 (PRKRA); and PAFAH1B1 (Figs. 3C and 4A)]. Further analysis of protein interaction networks for proteins detected in the MS experiment (Fig. 4, B and C) indicated the specific interactions of dephosphorylated EB3 with the Akt/mechanistic target of rapamycin (mTOR)/40S ribosomal protein S6 (RPS6) signaling pathway, known to be critical for cell proliferation (47). This analysis further indicates that phosphorylation of EB3 in endothelial monolayers (24) might provide a phosphoswitch inhibiting cell proliferation.

Since endothelial cells undergo contact-dependent inhibition of cell proliferation upon VE-cadherin trans-dimerization in a confluent monolayer and intact vessels (17), we tested next whether this EB3-S162 phosphoswitch may be required for cell transition from a proliferative to an adherent phenotype. EB3-WT-GFP, phosphodefective EB3-S162A-GFP, or phosphomimetic EB3-S162E-GFP mutants were overexpressed in HPAECs and stained for Ki-67, a common marker for cellular proliferation that is highly expressed in the nucleus during the S phase (13, 38). The number of Ki-67-positive nuclei was significantly higher in EB3-S162A-GFP-expressing cells compared with cells overexpressing WT or EB3-S162E-GFP (Fig. 5, A and B) indicating the role of dephosphorylated EB3 in positively regulating the proliferation of endothelial cells within a confluent monolayer, which is consistent with its interactome.

In contrast to dephosphorylated EB3, the phosphomimetic EB3 mutant interacted with several endothelial-specific adhesion molecules suggesting that phosphorylation may be required for assembly of cell-to-cell adhesion. The permeability of the endothelial monolayer to a 70-kDa fluorescent dextran tracer (41) was measured after overexpressing either EB3-WT-GFP, EB3-S162A-GFP, or EB3-S162E-GFP. Cells overexpressing the phosphodefective EB3 mutant showed increased permeability compared with EB3-WT- or EB3-S162E-expressing cells (Fig. 6, A and B). Additionally, a marker of endothelial adherens junctions, PECAM-1 (1), which interacts specifically with the phosphomimetic EB3-S162E mutant (Fig. 3G), showed decreased junctional localization in cells expressing EB3-S162A compared with EB3-WT-GFP or EB3-S162E-GFP (Fig. 6, C and D). These results indicate that the increase in permeability seen in EB3-S162A-expressing cells was associated with decreased cell-to-cell adhesion. Taken together, these results indicate that phosphorylation of EB3 on S162 acts as a phosphoswitch between proliferative and adhesive phenotypes of endothelial cells.

DISCUSSION

This work describes a simple and robust method for identifying the biological function of protein phosphorylation on the basis of differential protein-protein interactions. We used this method to determine the role of the phosphoswitch at S162 of EB3 (24). Since protein phosphorylation can modulate the binding affinity between proteins, it often leads to activation or inhibition of a specific biological function (21, 25). We therefore tested whether phosphorylation of EB3 changes its bona fide interactions in endothelial cells. Using this novel approach, we discovered a set of proteins that differentially bind EB3 in phosphorylated and dephosphorylated states, indicating that phosphorylation switches EB3 function by modulating its association with proteins involved in distinct biological networks. As differential protein-protein interactions may not always regulate a specific biological process, it is critical to test the biological consequences of these interactions using functional assays.

We demonstrated that EB3 phosphorylation acts as a phosphoswitch that modulates adhesion and proliferation of endothelial cells. When EB3 is in a phosphorylated state, it increases the assembly of adherens junctions and thereby decreases endothelial permeability by binding proteins that are involved in cell-to-cell adhesion. Dephosphorylated EB3 sequesters negative regulators of cell proliferation, thereby increasing cell proliferation while decreasing cell-to-cell adhesion. Although the inverse relationship between cell proliferation and adhesion has been established for several decades (46), how intracellular signaling coordinates the transition between two alternative cellular functions remains unclear. Previous reports indicate that confluent cells demonstrate higher membrane phosphatase activity as the result of the junctional accumulation of high cell density-enhanced phos-
Fig. 4. Pathway analyses of end-binding protein-3 (EB3) and EB3-S162E “interactome.” A: enrichment analysis for “canonical pathways” and Gene Ontology (“GO”) gene sets of EB3 and EB3-S162E interactomes. Dashed vertical lines represent the total proportion of proteins detected in either or both experiments. Colored bars represent the proportion of proteins from a given gene set that were detected in the two experiments. Dendrogram is based on gene set composition using Jaccard’s similarity index. Numbers in parentheses represent numbers of genes in each gene set. Fisher’s exact test was used for statistical significance: *P < 0.01, **P < 0.001. B and C: protein interaction network for proteins detected in the mass spectrometry experiment as EB3 (B) or EB3-S162E (C) binding partners. High-confidence protein interactions [Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), ≥900] are shown for proteins detected with strong signal intensities (>10^4 peptide intensity). These proteins are colored on the basis of whether they bound to EB3 (B; green), EB3-S162E (C; red), or both (B and C; gray). Only zero-order interactions for the largest network are shown. ACTB, β-actin; ACTR1A, actin-related protein-1A; APP, amyloid-β precursor protein; CCT2, chaperonin-containing TCP1 subunit 2; CLPB, ClpB homolog, mitochondrial AAA ATPase chaperonin; DNAJA1, DnaJ heat shock protein family (Hsp40) member A1; EEF1A1, eukaryotic translation elongation factor 1-α1; FN1, fibronectin 1; FUS, FUS RNA-binding protein; GRB2, growth factor receptor-bound protein-2; HNRNP, heterogeneous nuclear ribonucleoprotein; PAFAH1B1, platelet-activating factor acetylhydrolase IB subunit-α; PCBP, poly(rC)-binding protein; PDIA3, protein disulfide isomerase family A member 3; PSMC3, proteasome 26S subunit, ATPase 3; PTBP1, polypyrimidine tract-binding protein-1; PTK2, protein tyrosine kinase 2; RACK1, receptor for activated C kinase 1; RPL, ribosomal protein L; RPS, ribosomal protein S; SF3, splicing factor 3; SRSF9, serine and arginine-rich splicing factor 9; UBA52, ubiquitin A-52 residue ribosomal protein fusion product 1; YWHA, tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein. See Fig. 3 legend for additional names.
phatase 1 (DEP-1) that likely attenuates key mitogenic pathways (17, 44). Here, we described another signaling mechanism involving changes in EB3 phosphorylation state as modulated by cell-to-cell adhesion.

Our data demonstrate that the assembly of VE-cadherin adhesion provides a feedforward regulation by inducing phosphorylation of EB3 at S162 (24) that switches on the EB3 interactome to further support the assembly of cell-to-cell adhesion, whereas dephosphorylation of EB3 switches on cell proliferation signals. Our results are consistent with previous findings indicating that EB3 exists in a mainly phosphorylated state in endothelial monolayers in culture and blood vessels (14), since expression of EB3-WT generally mimicked the EB3-S162E phenotype. While our results cannot exclude an indirect effect of EB3 phosphorylation on cell proliferation via the assembly of stable cell-to-cell adhesion, the fact that dephosphorylated EB3 specifically interacts with negative regulators of cell proliferation indicates a potential direct mechanism.

While we used this biomimetic microsystem platform to identify the biological function of EB3 phosphorylation, this method could be expanded to any PTM or point mutation associated with or causing a disease (Fig. 7). For example, in rare autosomal dominant human diseases such as fibrodysplasia ossificans progressiva (FOP), a single point mutation of activin A receptor (ACVR1) triggers aberrant ACVR1 signaling, which transforms connective tissue into a secondary skeleton (2, 31, 40). Whereas the gain-of-function R206H mutation causes aberrant signaling due to a change in binding affinity of the receptor to the immunophilin 12-kDa FK506-binding protein (FKBP12; 42), it remains mainly unknown how another mutation (G328 to tryptophan, glutamic acid, or arginine) that is present in a small subset of patients with FOP (22) induces receptor signaling. Therefore, our method can be applied to
determine how a specific mutation within ACVR1 affects the native protein (Fig. 7A). It could also be used to determine whether a protein has distinct functions in health and disease by comparing the protein interaction network in cancer tissues and normal tissues, thus changing the background environment (a healthy vs. a diseased tissue) affects protein networks. Example.

There are several limitations to this methodology that should be taken into consideration. This method utilizes purified, recombinant proteins with a short His-tag allowing for covalent attachment of the bait protein. As with any type of protein modification, it is possible that addition of the tag or simply the use of recombinant proteins changes the way that an endogenous protein interacts with other proteins inside the cell, leading to either false-positive or false-negative results. In addition, the methodology described here does not allow assessment of interactomes within the specific intracellular compartments. Confirmation of possible targets via functional assays is therefore critical. Despite these limitations, this method offers a unique improvement on similar techniques. Therefore, this biomimetic microsystem platform may become a valuable tool in identifying the biological function of various PTMs and mutations and their implications in human disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.K. and Y.A.K. conceived and designed research; K.K., M.S., Z.C., D.S., and F.H. performed experiments; K.K., J.K., M.S., P.K., M.M.-C., and Y.A.K. analyzed data; K.K., J.K., and Y.A.K. interpreted results of experiments; K.K., J.K., and Y.A.K. prepared figures; K.K. and Y.A.K. drafted manuscript; K.K. and Y.A.K. edited and revised manuscript; Y.A.K. approved final version of manuscript.
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