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Phosphoproteomic analysis of tumor-endothelial signaling identifies EPHA2 as a negative regulator of transendothelial migration

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Abstract

Exit of metastasizing tumor cells from the vascular system is regulated by their dynamic interactions with the endothelial cells that line the internal surface of vessels. While extravasation is a key event within the metastatic cascade, the signals controlling tumor cell adhesion to the endothelium and their subsequent transendothelial migration are poorly understood. By combining Stable Isotope Labeling by Amino acids in Cell culture (SILAC) and phosphoproteomic analysis,

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we mapped cell-specific changes in protein phosphorylation that were triggered by contact between breast cancer cells and endothelial cells. From the 2,669 unique phosphorylation sites identified, 77 and 43 were differentially phosphorylated in the tumor cells and endothelial cells, respectively. Among the phospho-regulated proteins within the tumor cells, the receptor tyrosine kinase Ephrin type-A receptor 2 (EPHA2) was found to inhibit transendothelial migration of breast cancer cells. We demonstrated that this was dependent on EPHA2-Tyr⁷⁷² phosphorylation, which was decreased in the breast cancer cells upon endothelial contact. Importantly, comparison of isogenic breast cancer cell lines with different metastatic capacities revealed that EPHA2-Tyr⁷⁷² dephosphorylation was specifically associated with higher lung metastatic potential. Altogether, our cell-specific phosphoproteomic analysis provides the first bidirectional map of contact-initiated signaling between tumor and endothelial cells, which has led to the characterization of a novel EPHA2-based regulatory mechanism of transendothelial migration.

Introduction

Metastasis is a multistep process where tumor cells must first enter and survive in the circulation before exiting the vascular system for colonization of secondary organs (1). Extravasation requires metastasizing cells to cross the endothelial barrier of blood vessel walls. The initial steps of extravasation are governed by dynamic interactions between tumor and endothelial cells. For example, live cell image analyses have shown that tumor cells first adhere to and then migrate along the vessel before traversing the endothelium — a highly interactive process that involves morphological changes and cytoskeletal reorganization in both cancer cells as well as endothelial cells (2–4). The value of understanding these cell-cell interactions on a molecular level has recently been demonstrated, where targeting lectin-dependent tumor-endothelial adhesion effectively reduces metastatic burden (5). Efforts to dissect tumor cell extravasation have uncovered a variety of regulatory molecules such as E-selectin and Vascular Endothelial Growth Factor Receptor (VEGFR) in endothelial cells (6–8), and integrin- β 1 (9–11) and CD82 (12, 13) in tumor cells, which can promote or hinder cancer cell escape from the circulation. However, despite these advances, the dynamic signaling pathways that drive tumor-endothelial interactions remain poorly characterized, which limits the development of therapeutic strategies targeting disseminating cancer cells.

A major technical constraint in studying dynamic tumor-endothelial signaling is the requirement for direct cell-cell contact between two different cell populations. Systems-wide analysis of these heterotypic interactions is a challenge since co-cultured cells must be lysed together for biochemical processing, and ultimately, the distinct molecular changes occurring in each cell type are lost. Studies of molecular players that govern the interplay between tumor and endothelial cells have thus been largely dependent on candidate-based approaches, which are limited by existing knowledge of extravasation.

Mass spectrometry (MS) provides a sensitive methodology for detection and quantification of proteins and post-translational modifications, which enables unbiased, system-wide study of cell signaling. Additionally, we and others have described how stable isotopomeric versions of amino acids can be introduced into the cellular proteomes (*i.e.* SILAC) to

facilitate MS analysis of contact-initiated signaling between different cell types in a cell-specific manner (14–16).

Here, we employed a SILAC-based phosphoproteomic strategy to uncover bidirectional signaling pathways that are regulated upon contact between human breast cancer cells and endothelial cells. Based on our findings, we describe a novel role for Ephrin type-A receptor 2 (EPHA2) in negatively regulating tumor-endothelial adhesion and transendothelial migration of breast cancer cells. Strikingly, phosphorylation of EPHA2-Tyr⁷⁷² was found to be downregulated in highly metastatic cancer cells upon endothelial contact. We further demonstrate that phosphorylation of EPHA2-Tyr⁷⁷² is critical for EPHA2-mediated inhibition of transendothelial migration and becomes deregulated in cancer cells with increased lung metastatic potential. Identification of such EPHA2-based regulation of transendothelial migration highlights the value of using cell-specific phosphoproteomics to provide novel mechanistic insight into the signaling events underpinning complex cell-cell interactions.

Results

SILAC-labeling enables cell-specific analysis of contact-initiated signaling between tumor cells and endothelial cells

To elucidate the dynamics of the signaling pathways underlying tumor cell extravasation, we conducted a cell-specific phosphoproteomic analysis of contact-initiated signaling between tumor and endothelial cells. To this end, an *in vivo*-selected lung metastatic derivative of MDA-MB-231 cells (subpopulation LM2-4175 (17), hereafter referred to as LM2) was utilized as a cancer cell model system. *In vitro* characterization has shown that LM2 cells adhere to and transmigrate through an endothelial cell monolayer more efficiently than the parental population from which they were derived (fig. S1A, B, and (18)), indicating that signaling pathways driving extravasation are enriched within this selected cell population. Having monitored the kinetics of LM2 cell attachment to a monolayer of Human Umbilical Vein Endothelial Cells (HUVECs), we decided to perform our analysis of tumor-endothelial signaling after 15 min of co-culture thereby capturing early signaling events following initial LM2-HUVEC cell contact.

Using SILAC, cell-specific labels were introduced into LM2 cells and HUVECs to ensure each cell type had a distinct and traceable proteome when tumor and endothelial cells were co-cultured. To probe regulatory signaling events triggered specifically in cancer cells following contact with endothelial cells, we labeled LM2 cells with medium or heavy isotopomers of arginine and lysine (Arg+6 Da, Lys+4 Da and Arg+10 Da, Lys+8 Da respectively) and HUVECs with “light” arginine and lysine (Arg+0 Da, Lys+0 Da). Heavy-labeled LM2 cells were collected by enzyme-free cell dissociation buffer, thereby preserving membrane proteins and adhesion receptors, and seeded onto a monolayer of light-labeled HUVECs to simulate early interactions between disseminating tumor cells and the vascular endothelium (Fig. 1A, left panel). After 15 min of co-culture, non-adherent LM2 cells were gently removed and cancer cells that had attached to the endothelial layer were lysed together with the HUVECs. In parallel, medium-labeled LM2 cells were collected under the same conditions and maintained as suspension cells in monoculture to represent circulating

tumor cells prior to any contact with the endothelium. These were then added to the harvested LM2-HUVEC co-culture in a 1:1 ratio of heavy:medium-labeled cells to provide a point of reference. Cytoplasmic- and membrane-enriched fractions were prepared, digested and enriched for phosphopeptides (Fig. 1B). To normalize changes in phosphopeptide abundance to differences in total protein abundance, samples of digested cell lysate were fractionated in parallel. Peptides and phosphopeptides were analyzed by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). Based on the SILAC labeling of the different cell populations, light-labeled peptides were assigned to HUVECs, medium-labeled peptides to LM2 cells in suspension, and heavy-labeled peptides to LM2 cells that had made contact with HUVECs. As such, the heavy/medium ratio for each phosphopeptide was normalized and used to quantify phosphorylation-dependent signaling changes occurring specifically in the LM2 cells upon contact with HUVECs.

Conversely, to elucidate signaling events in HUVECs that were initiated by contacting cancer cells, light-labeled LM2 cells were seeded on top of a confluent monolayer of heavy-labeled HUVECs, while medium-labeled HUVECs were maintained in monoculture (Fig. 1A, right panel). Following 15 min of co-culture, the unattached LM2 cells were removed. Cells were lysed, mixed in a 1:1 ratio of heavy:medium HUVECs, followed by membrane fractionation, proteomic and phosphoproteomic analysis as described above (Fig. 1B).

To generate a high confidence dataset, we conducted a minimum of four independent biological experiments for each cell type, which identified a total of 5,291 unique phosphopeptides (see fig. S2 for a flow diagram of MS data processing steps). Identified phosphopeptides were $\log_2$ -transformed ($\log_2(H/M)$) and normalized for gross differences in protein quantity between control and co-cultured cells. This was followed by combining phosphopeptides that represented the same phosphorylation site (*i.e.* peptides occurring in various charge states, or arising from incomplete digest), which mapped to 2,669 unique phosphorylation sites across 1,387 unique proteins. Based on the two separate SILAC co-culture experimental setups, we assigned 2,279 unique phosphorylation sites to proteins from the LM2 cells and 1,080 to proteins from the HUVECs (Fig. 2A). To focus on proteins that were modulated by either an increase or decrease in phosphorylation following tumor-endothelial cell co-culture, we filtered identified phosphosites based on a $\log_2(H/M)$ fold change over 0.5 or below -0.5. We subsequently manually inspected the raw MS data to eliminate mixed MS/MS spectra, unresolved extracted ion chromatograms, and low SILAC signal intensity thereby avoiding confounding peptide identifications and quantification to ensure a high quality dataset. Together, this refined dataset provides confident relative quantification of phosphorylation sites for 107 serine, 10 threonine and 5 tyrosine phosphorylation sites, where 77 and 43 phosphorylation sites were modulated in the LM2 cells and HUVECs respectively (Fig. 2B and fig. S3; see tables S1 and S2 for values of $\log_2(H/M)$ in each biological replicate and overall average fold change). For phosphorylation sites quantified in a minimum of two independent biological experiments, a two-sided unpaired t-test was performed ($\alpha = 0.05$; $-0.5 > \log_2(H/M) > 0.5$). This led to the identification of a set of “core” regulated phosphorylation sites presented with red bars in Figure 2B (see fig. S4 for raw values from individual experiments).

Global quantification of changes occurring at the protein level highlighted that only 7.9% and 9.6% of identified proteins displayed a $\log_2(H/M)$ fold change over 0.5 at 15 min of direct co-culture in LM2 cells and HUVECs respectively (fig. S5). Within these samples, we were able to identify and quantify the protein abundance for 33 of the 92 phospho-regulated proteins (displayed as yellow circles in Fig. 2B; tables S3, S4, and S5). Importantly, only 3 phosphoproteins displayed changes in abundance that closely corresponded with the degree of regulation observed at their specific phosphorylation sites. Overall, this suggests that the majority of identified and quantified changes in site phosphorylation were a consequence of altered kinase or phosphatase activity rather than a change in protein abundance.

Phosphoproteomic analysis of tumor-endothelial contact provides bidirectional map of signaling pathways underlying cancer cell extravasation

Regulated phosphorylation sites from our analysis of tumor-endothelial contact were put into biological context by mapping the identified phospho-regulated proteins to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, with further refinement from relevant literature review. Based on these analyses, we constructed a map of bidirectional signaling and interaction pathways that featured changes in phosphorylation upon tumor-endothelial contact (Fig. 3). This highlighted that a considerable number of the identified phosphorylation changes were occurring on proteins central to cell-cell adhesion and transendothelial migration. For example, the cell surface receptor CD44, which showed a decrease in phospho-Ser⁷⁰⁶, is known to participate in cell-cell adhesion along with tumor-endothelial interaction (19). We further identified phospho-regulation of membrane receptors controlling homo- and heterotypic cell-cell contact like EPHA2 (20) and integrin- β 4 (ITGB4) (21), as well as members of integrin pathways (*e.g.* Epidermal Growth Factor Receptor (EGFR), paxillin (PAXI), talin (TLN1), nestin (NEST), zyxin (ZYG), kinectin (KTN1) catenin- β 1 (CTNB1), p120-catenin (CTND1) (22–26).

In addition to regulators of cell-cell contact, our analysis highlighted proteins that have previously been implicated in diapedesis. Endothelial filamins, such as filamin A (FLNA), are involved in the formation of docking structures surrounding transmigrating neutrophils (27). Serine/threonine-protein kinase 10 (STK10) and platelet endothelial cell adhesion molecule (PECAM1), which both showed changes in serine phosphorylation in the LM2 cells, are also known to regulate leukocyte transmigration (28–30), suggesting shared signaling pathways between tumor cell and immune cell extravasation.

Importantly, our analysis allows the precise localization of regulated phosphorylation sites, which offers insight into specific functions carried out by individual proteins. For instance, we identified a decrease in phosphorylation of TLN1-Ser⁴²⁵ in the LM2 cells upon contact with HUVECs. This particular phosphorylation site has been shown to regulate integrin- β 1 activity, in vitro cell adhesion and motility, as well as bone metastasis of prostate cancer cells (31). Furthermore, we observed decreased phosphorylation of PAXI-Ser⁸⁵, which has been shown to be involved in protrusion formation via regulation of talin-paxillin interaction (32).

We additionally observed the regulation of phosphorylation sites that have not previously been characterized in the context of extravasation. For example, while the involvement of

Protein phosphatase 1 regulatory subunit 12A (MYPT1) in cell adhesion is known to be governed through phosphorylation sites Ser⁴⁴⁵, Ser⁴⁷² and Ser⁹¹⁰ (33), we identified an alternative phosphorylation site in MYPT1, Ser⁵⁰⁹, that showed a significant 4.4-fold decrease in phosphorylation during LM2-HUVEC co-culture. This dataset thus provides a valuable resource of signaling pathways that are triggered upon initial tumor-endothelial contact and offers a useful tool for further studying cancer cell extravasation leading to metastasis.

EPHA2 is identified as an inhibitor of tumor-endothelial adhesion and transendothelial migration

We hypothesized that the proteins undergoing regulation by phosphorylation in our co-culture experiments may modulate tumor-endothelial interactions, thereby controlling tumor cell extravasation. Among the many processes comprising extravasation, tumor-endothelial cell adhesion can be quantitatively assessed in a simple setup that allows for medium-throughput screening. We systematically silenced 20 of the regulated phosphoproteins in the LM2 cells using small interfering RNA (siRNA) and measured the impact on tumor cell adhesion to a HUVEC monolayer. Knockdown of *EGFR*, *MYPT1*, *CLIP1*, *CLIP2* and *EPHA2* affected tumor-endothelial cell adhesion in a statistically significant manner (red bars in fig. S6A; ratio paired t-test against control, $\alpha = 0.05$).

This demonstrates that our phosphoproteomic analysis of contact-initiated signaling can identify specific proteins involved in tumor-endothelial adhesion.

Silencing *EPHA2* produced the most acute phenotype with a 1.5-fold increase in tumor-endothelial adhesion (phenotype further validated using two individual siRNA sequences and siRNA of alternative chemistry; Fig 4A,B and fig. S6B,C). Interestingly, *EPHA2* has previously been linked with breast cancer metastasis and poor patient prognosis (34–36). However, a specific role for *EPHA2* in the process of extravasation has yet to be explored in breast cancer, thus offering an interesting candidate for further study.

Since *EPHA2* is known to regulate the cell-cell adhesion/repulsion balance as well as cell-substrate attachment (37), we also performed parallel adhesion assays on tissue culture plastic to verify that this increase in tumor-endothelial adhesion was not simply due to an increase in general cell adhesiveness. *EPHA2*-silenced LM2 cells did not display increased attachment to plastic (fig. S6D), suggesting that *EPHA2* acts to specifically block breast cancer cell adhesion to HUVECs.

To determine whether *EPHA2* also regulates LM2 cancer cell migration through the endothelium, we tested the effect of *EPHA2* depletion in a transendothelial migration assay. HUVECs were grown to a confluent monolayer in transwell inserts and the ability of *EPHA2*-silenced LM2 cells to migrate through the endothelial layer was assessed. Silencing *EPHA2* in LM2 cells with pooled siRNA and two individual siRNA sequences of a different chemistry significantly increased cancer cell transendothelial migration (Fig. 4C and fig. S6B,E), suggesting that *EPHA2* inhibits both tumor-endothelial cell adhesion and transendothelial migration. To examine the impact of *EPHA2* knockdown on the metastatic behavior of tumor cells in vivo, we injected *EPHA2*-silenced LM2 cells into the tail vein of

nude mice and analyzed the number of tumor cells residing in the lung 20 hours post injection (Fig. 4D). In keeping with our observations in cultured cells, reduction of EPHA2 significantly increased the retention of LM2 cells in the lungs (Fig. 4E). Overall, this supports that EPHA2 in breast cancer cells impairs early stages of lung colonization.

EPHA2 inhibits transendothelial migration in several breast cancer cell lines

Having established that EPHA2 is an inhibitor of tumor-endothelial cell interactions in LM2 cancer cells, we subsequently tested whether the proposed role of EPHA2 was shared across other breast cancer cells. Transendothelial migration assays were repeated in additional cell lines, where *EPHA2* was either silenced or stably expressed. In SUM149PT cells, where EPHA2 abundance is comparable to the LM2 cells (Fig. 4F), silencing *EPHA2* significantly increased transendothelial migration (Fig. 4G and H). Conversely, in cell lines MDA-MB-468 and MDA-MB-453, with low amounts of endogenous EPHA2 (Fig. 4F), stable expression of *EPHA2* significantly decreased cancer cell ability to cross an endothelial monolayer (Fig. 4G, I and J). Together, these results indicate an inhibitory role of EPHA2 in the transendothelial migration of breast cancer cells.

EPHA2-mediated inhibition of transendothelial migration is dependent on interaction with endothelial ephrin-A1

Eph receptor tyrosine kinases interact with their cognate membrane-tethered ephrin ligands on apposing cells, which elicits a bidirectional response governing the adhesion/repulsion balance (20, 38, 39). It is known that EPHA2 activation by its A-type ephrins instigates a cell-cell repulsive response, which suggests a putative mechanism for the EPHA2-mediated inhibition we observed in transendothelial migration. As such, ablating the interaction between EPHA2 on tumor cells and its cognate ephrin ligand on endothelial cells should recapitulate the effect of *EPHA2* silencing. We first confirmed the presence of ephrin-A1 in HUVECs by affinity purification and MS analysis of EPHA2 ectodomain-interacting proteins (fig. S7A). We further verified that EPHA2 receptors could be blocked from engaging ephrin-displaying surfaces by using soluble, recombinant ephrin-A1-Fc as a competitive ligand (fig. S6F and G). Notably, when EPHA2 receptor ligand-binding sites were treated with saturating levels of recombinant ephrin-A1-Fc prior to the transendothelial migration assay, the ability of the cancer cells to cross the endothelial layer was significantly increased (Fig. 5A). Staining of recombinant ephrin-A1 bound to EPHA2 in intact LM2 cells highlighted that ligand-receptor complexes remained at LM2 cell surfaces, suggesting that the increase in transendothelial migration was due to blocking EPH-ephrin interactions rather than receptor internalization (fig. S6G). This indicates that engagement of the EPHA2 receptor by endothelial ephrin-A1 is required for negative regulation of transendothelial migration.

Phosphorylation of EPHA2-Tyr⁷⁷² is critical for EPHA2-mediated inhibition of transendothelial migration

Based on existing models of Eph receptor function, engagement of EPHA2 in LM2 cells by ephrin-A1 on HUVECs is predicted to initiate EPHA2 phosphorylation and receptor activation, resulting in cell-cell repulsion. Interestingly, our phosphoproteomic analysis of contact-initiated signaling showed that while total EPHA2 abundance remained constant,

phosphorylation at EPHA2-Tyr⁷⁷² was significantly decreased by 2.2-fold in LM2 cells following interaction with endothelial cells (Fig. 2B, Fig 5B; representative MS2 spectrum identifying the phospho-Tyr⁷⁷² EPHA2 peptide shown in Fig. 5C). This decrease in phosphorylation within the receptor activation loop would correspond to Eph receptor inactivation, favoring a cell-cell adhesive response over a repulsive response (40–42). We therefore speculated that dephosphorylation of EPHA2-Tyr⁷⁷² exists as a mechanism for highly metastatic cells to subvert EPHA2-mediated inhibition of transendothelial migration (see model in Fig. 6A). To investigate the importance of the Tyr⁷⁷² phosphorylation site in EPHA2-mediated regulation of transendothelial migration, an EPHA2-Y772F mutant was stably expressed in breast cancer cells with low endogenous expression of EPHA2. Loss of phosphorylation at Tyr⁷⁷² through mutation of the tyrosine to a non-phosphorylatable phenylalanine significantly increased transendothelial migration ability of *EPHA2*-expressing cells when compared to the wild-type control (Fig. 6B). This indicates that EPHA2-Tyr⁷⁷² phosphorylation in cancer cells is indeed required for EPHA2-dependent inhibition of transendothelial migration.

We further assessed the function of EPHA2-Y772F in an in vitro kinase assay to determine whether phosphorylation of Tyr⁷⁷² is important for EPHA2 kinase activation. Compared to the wild-type receptor, we found that kinase activity of EPHA2-Y772F was impaired. This suggests that Tyr⁷⁷² phosphorylation drives EPHA2-mediated inhibition of transendothelial migration through receptor kinase activation. Notably, EPHA2-Y772F still demonstrated substantial kinase activity over a kinase-dead K646M mutant version of EPHA2. This discrepancy between the two EPHA2 mutants demonstrates that changes in EPHA2-Tyr⁷⁷² phosphorylation do not directly equate to changes in EPHA2 kinase activity, and therefore implies that EPHA2-Tyr⁷⁷² phosphorylation may also modulate transendothelial migration through kinase-independent mechanisms.

One possible mechanism whereby EPHA2 tyrosine phosphorylation can be downregulated to promote transendothelial migration is through recruitment of phosphatases (see model in Fig. 6A). Low molecular weight protein tyrosine phosphatase (LMW-PTP) has previously been shown to alter the repulsive response of EPHA2 to ephrin-A1 stimulation, and EPHA2 desphosphorylation by LMW-PTP has further been linked to increased oncogenic potential (43–45). Moreover, it has been shown using recombinant protein domains in vitro that LMW-PTP can dephosphorylate the EPHA2-Tyr⁷⁷² site (46). To assess LMW-PTP as a candidate for regulating tumor cell extravasation through EPHA2, we firstly verified that EPHA2 and LMW-PTP interact by co-immunoprecipitation (fig. S8A). Pertinently, we further demonstrated that depletion of LMW-PTP reduced LM2 cell adhesion to, and transmigration through an endothelial monolayer (fig. S8). This highlights that LMW-PTP is required for effective tumor-endothelial interactions and may therefore be a potential phosphatase mediating of EPHA2-Tyr⁷⁷² dephosphorylation to enable tumor cell transendothelial migration.

EPHA2-Tyr⁷⁷² phospho-regulation is altered in highly metastatic LM2 cells

Having identified that phosphorylation of EPHA2-Tyr⁷⁷² is critical for EPHA2-mediated inhibition of transendothelial migration, we then sought to verify if altered regulation of

Tyr⁷⁷² phosphorylation in EPHA2 is a feature of highly metastatic cells. LM2 cells display an enhanced metastatic capacity relative to the parental MDA-MB-231 cells from which they were derived (17, 18). Whilst originating from the same genetic background, the LM2 population showed increased tumor-endothelial adhesion and transendothelial migration *in vitro* compared to their less aggressive parental counterpart population (fig. S1, A and B). Since we confirmed that the two cell populations displayed the same amounts of endogenous EPHA2 (fig. S1, C-E), we investigated underlying differences in the regulation of EPHA2 Tyr⁷⁷² phosphorylation between the two cell populations.

LM2 and parental populations were individually stimulated with clustered recombinant ephrin-A1-Fc and immunoblotted for the activation loop phosphorylation (p-Tyr⁷⁷²) in EPHA2 (Fig. 6E). As expected, EPHA2 stimulation by ephrin-A1 initially increased Tyr⁷⁷² phosphorylation in both cell lines. Notably, whilst total EPHA2 abundances were constant, densitometry analysis of western blots from multiple independent experiments showed that Tyr⁷⁷² phosphorylation was significantly decreased in the LM2 cells at 10 and 15 min following receptor activation (Fig. 6F and fig. S9C). In contrast, Tyr⁷⁷² phosphorylation in the parental population remained stable. Interestingly, extending our analysis to include phosphorylation of EPHA2-Tyr⁵⁸⁸ and -Ser⁸⁹⁷ revealed similar kinetics at these regulatory sites in both parental and LM2 cells, where phosphorylation was maintained without significant change following ephrin-A1 stimulation (fig. S9A,B).

Given the importance of EPHA2 Tyr⁷⁷² phosphorylation in inhibition of transendothelial migration, the observed decrease of phospho-Tyr⁷⁷² in the LM2 cells could facilitate tumor cell extravasation and contribute to their increased metastatic potential.

In addition to the LM2 lung-metastatic derivative, MDA-MD-231 subpopulations that preferentially metastasize to the brain (BrM2a) and bone (BoM1) have also been established (47, 48). These have been used to profile and characterize the molecular basis for different metastasis tissue tropisms (47) and provide us with additional model cell lines to probe whether deregulation of EPHA2 phospho-Tyr⁷⁷² is a common occurrence in metastatic breast cancer cells or specific to lung-selective dissemination. Interestingly, time course analysis of EPHA2 phospho-Tyr⁷⁷² across the different MDA-MB-231 populations showed that, in contrast to the LM2, BrM2a and BoM1 metastatic cell lines displayed similar EPHA2 phospho-Tyr⁷⁷² kinetics to the Parental population, where increased phospho-Tyr⁷⁷² was maintained at 10 and 15 min time points (Fig. 6E,F). This suggests that the early downmodulation of EPHA2-Tyr⁷⁷² phosphorylation we identified is a distinct regulatory mechanism that occurs in highly lung metastatic LM2 cells. Altogether, this emphasizes site-specific phospho-regulation of EPHA2 and distinct phospho-Tyr⁷⁷² dynamics in cells with different metastatic capacity.

Discussion

Using cell-specific phosphoproteomic analysis, we present for the first time a map of bidirectional phosphorylation-dependent signaling pathways initiated between interacting breast cancer (LM2) and endothelial cells (HUVECs). Through this approach, we identified regulation of proteins with known roles in cell-cell adhesion, cytoskeletal remodeling and

leukocyte transmigration, such as EPHA2, CD44, MYPT1, PECA1, and PAXI. Overall, these findings support the notion that signaling pathways relevant to cellular processes within cancer cell extravasation can be identified through the presented workflow.

We observe quantitative changes in key phosphorylation sites upon tumor-endothelial contact, such as TLN1-Ser⁴²⁵ and MYPT1-Ser⁵⁰⁹, which provides specific insight into the dynamic modulation of signaling pathways and further highlights regulation occurring through hitherto uncharacterized phosphorylation sites. Similarly, increased phosphorylation of proteins governing membrane trafficking, such as Polymerase I and Transcript Release Factor (PTRF), Multivesicular body subunit 12A (F125A) and Tumor Protein D52 (TPD52), reveals additional cellular processes that are regulated upon tumor-endothelial contact, which could be further investigated.

Moreover, our analysis also provides unbiased insights into phosphorylation-dependent signaling pathways regulated in the endothelial cells. Consistent with previous *in vivo* studies showing their active reorganization upon contact with cancer cells (2–4), our phosphoproteomic approach identified several proteins in the HUVECs that are known to regulate cytoskeletal remodeling. Among them are Microtubule-associated protein 4 (MAP4), stathmin (STMN1), and SUN domain-containing protein 2 (SUN2) specifically involved in nuclear migration. Interestingly, FLNA-Ser²¹⁵² was significantly phosphorylated in the HUVECs upon cancer cell contact. This provides a novel biological context in which this phosphorylation site may be regulating FLNA-mediated actin cytoskeletal assembly (49) and ERK signaling (50) in endothelial cells. These results, together with other proteins identified in our dataset, provide new avenues to decipher the endothelium involvement in extravasation.

We additionally confirmed that our approach could be used to identify regulators of tumor-endothelial interaction by demonstrating the functional relevance of several phospho-regulated proteins such as MYPT1, EGFR and EPHA2 in tumor cell adhesion to HUVECs. While tumor-endothelial cell adhesion was utilized as a basic phenotype for us to assess proteins of interest, extravasation of tumor cells involves numerous processes beyond the initial attachment of tumor cells to the endothelium, which are all coordinated through the concerted action of multiple molecular players. Future studies may therefore reveal how phosphoproteins identified in our analysis of tumor-endothelial signaling contribute to other aspects of tumor-endothelial interactions such as transendothelial migration. In support of this, our continued investigations into EPHA2 highlighted a further role for the tumor cell receptor in inhibiting the transendothelial migration and early stages of lung colonization by breast cancer cells. While it is known that Eph receptor family members regulate cell-cell contact (20, 38, 39) our findings present a novel function for EPHA2 in regulating tumor cell interaction with and passage through an endothelial barrier.

A role for EPHA2 in inhibiting transendothelial migration is strikingly paradoxical, given that EPHA2 is extensively reported with tumor-promoting activities (51–54) and elevated EPHA2 expression correlates with increasing metastatic behavior and poor patient prognosis in breast cancer (34–36). This discrepancy in EPHA2 function could possibly be rationalized by reported correlations between tumor aggressiveness and a decreased phospho-tyrosine

content of EPHA2 (51, 52, 55, 56), which suggest a potential deregulation of the receptor as cancer progresses. Indeed, when comparing MDA-MB-231 populations of different metastatic capacity, we showed that rapid dephosphorylation of the EPHA2-Tyr⁷⁷² phosphorylation site occurred selectively in the more highly metastatic LM2 cancer cell population. Interestingly, our phosphoproteomic analysis of tumor-endothelial signaling identified a significant decrease in EPHA2 phospho-Tyr⁷⁷² upon LM2 cancer cell contact with endothelial cells, and we further demonstrated that ablating phosphorylation at Tyr⁷⁷² compromised EPHA2-mediated inhibition of transendothelial migration.

Importantly, although Tyr⁷⁷² is the key activation loop tyrosine residue in EPHA2, mutation to prevent phosphorylation at position 772 only partially impaired kinase activity of the receptor. Moreover, in the highly metastatic LM2 cancer cells, phospho-regulation of the activation loop Tyr⁷⁷² appeared to be uncoupled from Tyr⁵⁸⁸, a key juxtamembrane phosphorylation site in EPHA2 receptor activation. We further observed that in addition to the rapid dephosphorylation of Tyr⁷⁷² in the LM2 cells following ephrin-A1 stimulation, significant downregulation of the EPHA2 receptor also occurred in the LM2 cells compared to the parental cells following prolonged stimulation (fig. S9C). Given that Tyr⁷⁷² phosphorylation is tightly associated with juxtamembrane tyrosine phosphorylation, receptor kinase activation and subsequent degradation, these data together suggest that EPHA2-Tyr⁷⁷² phosphorylation may have additional regulatory roles that are independent from EPHA2 kinase activity.

Based on our characterization of EPHA2-Tyr⁷⁷² we propose that targeted Tyr⁷⁷² dephosphorylation exists as a molecular switch by which aggressive cancer cells can overcome ligand-driven EPHA2 inhibition of transendothelial migration to facilitate their metastasis. This dynamic reduction in phospho-Tyr⁷⁷² is likely to result from phosphatase activity, and we highlight LMW-PTP as a potential tyrosine phosphatase for modulating the switch in EPHA2 function to promote tumor-endothelial interactions.

A similar molecular mechanism based on Akt phosphorylation of EPHA2-Ser⁸⁹⁷ has previously been implicated in regulating the switch between EPHA2-mediated inhibition and promotion of cancer cell migration (57). Incidentally, phosphorylation of EPHA2-Ser⁸⁹⁷ and ligand-independent EPHA2 signaling has also been associated with increased prostate cancer cell adhesion to, and transmigration through bone marrow endothelial monolayers (58). These examples of site-specific regulation emphasize the importance of considering individual regulatory sites in specific cellular contexts. In contrast to general correlations that have been made between gross phosphotyrosine content of EPHA2 and cancer cell behavior, our findings provide insight into specific regulatory mechanisms underlying the dichotomous pro- and anti-oncogenic functions of EPHA2.

Notably, comparison of EPHA2 phospho-Tyr⁷⁷² kinetics between the highly metastatic MDA-MB-231 subpopulations suggested that Tyr⁷⁷² dephosphorylation specifically occurs in the lung-selective LM2 cells. These differences in Tyr⁷⁷² phospho-regulation underscore the importance of cellular context in studying receptor signaling. Indeed, we observe disparities in the regulation of EPHA2 phosphorylation in LM2 cells between our co-culture model system and in ephrin-A1-stimulated cells. This may in part be due to differences in

signal intensity as we previously reported for ephrin-B1 dependent activation of EphB2 (16). Moreover, cell to cell contact-initiated signaling elicits a response that is coordinated through multiple receptors and soluble factors providing several input cues. Ultimately, this highlights the elegance of the SILAC-based phosphoproteomic approach we present, as we can more effectively model these multivariate biological systems in a co-culture setting, thus enabling the study of complex cell-cell interactions.

Overall we provide a valuable resource of signaling pathways initiated upon tumor-endothelial contact, which reveals new regulators of tumor-endothelial interaction and highlights the importance of identifying dynamic signaling events in both a cell- and phosphorylation site-specific manner to understand complex cellular behavior.

Materials and Methods

Reagents

Tissue culture media were provided by Life Technologies together with the following reagents: OLIGO R3 (1-1339-03), insulin (12585014), zeocin (R25001), Lipofectamine RNAiMAX Transfection Reagent (13778-150), Lipofectamine 2000 (11668027), enzyme-free cell dissociation buffer (13151-014), dialysed FBS (26400), CellTracker Green CMFDA (5-Chloromethylfluorescein Diacetate) (C7025), CellTracker Orange CMRA (C34551) and DAPI (D1306). Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich.

Cell lines

Parental MDA-MB-231-TGL and sub-populations 4175 (LM2), BrM2a, and 1833 (BoM1), were kindly provided by Joan Massagué (Memorial Sloan Kettering Cancer Centre) (47, 48, 59). MDA-MB-468 and SUM149PT cell lines were a kind gift from Rachel Natrajan (The Institute of Cancer Research), MDA-MB-453 cells were a kind gift from Jorge Reis-Filho (Memorial Sloan-Kettering Cancer Center), Human Umbilical Vein Endothelial Cells (HUVECs) pooled were purchased from Promocell, and EA.hy926 cells were from ATCC.

Tissue culture

MDA-MB-231 (Parental, LM2, BrM2a and BoM1), MDA-MB-453, MDA-MB-468, EA.hy926 and HEK 293FT cells were cultured in Dulbecco's Modified Eagle Medium DMEM (41966-052) supplemented with 10% FBS and antibiotic/antimycotic solution. SUM149PT cells were cultured in DMEM/F-12 (31331-028) supplemented with 5% FBS, 10 mM HEPES, 5 μ g/mL insulin, 1 μ g/mL hydrocortisone (H0888) and antibiotic/antimycotic solution. HUVECs were cultured in medium 199 GlutaMAX (41150-087) supplemented with 20% FBS, 0.01 mg/mL heparin (H3393), 50 μ g/mL endothelial cell growth supplement (AbD Serotec 4110-5004) and antibiotic/antimycotic solution. They were grown on tissue culture dishes, coated with 0.1% gelatin (G1890) and kept in culture for a maximum of 7 passages. All cells were grown in humidified incubators at 37°C, 5% CO₂. Cells from non-commercial sources were authenticated by STR profiling. All cell line stocks were routinely tested to confirm the absence of mycoplasma with e-Myco Mycoplasma PCR Detection Kit (iNtRON 25235).

SILAC labeling

Basal SILAC medium, pH 7.2, was made up from sodium bicarbonate and DMEM or RPMI deficient in *L*-arginine and *L*-lysine (Caisson Labs DMP49 and RPP21 respectively) according to manufacturer's instructions. For labeling cells, basal SILAC medium was supplemented with 10% dialyzed FBS, antibiotic/antimycotic solution and isotopomeric versions of *L*-lysine and *L*-arginine, each at a final concentration of 50 $\mu\text{g/mL}$. Isotopomeric versions of amino acids for SILAC labeling were all supplied by Sigma-Aldrich: *L*-arginine- $^{13}\text{C}_6$, $^{15}\text{N}_4$ hydrochloride (Arg+10 Da) (608033) and *L*-lysine- $^{13}\text{C}_6$, $^{15}\text{N}_2$ hydrochloride (Lys+8 Da) (608041) were used for heavy-labeling; *L*-arginine- $^{13}\text{C}_6$ hydrochloride (Arg+6 Da) (643440) and *L*-lysine-4,4,5,5-d $_4$ hydrochloride (Lys+4 Da) (616192) were used for medium-labeling; and *L*-arginine (A8094) and *L*-lysine (L5501) were used for light-labeling. LM2 cells were grown for a minimum of 6 passages in DMEM-based SILAC labeling media before use in co-culture experiments. For SILAC labeling of HUVECs, RPMI-based SILAC medium was conditioned for 16 hours with fully labeled immortalized human endothelial cells EA.hy926. Conditioned medium was sterile filtered, diluted 1:1 with fresh RPMI SILAC medium, and supplemented with heparin and endothelial cell growth supplement. HUVECs were grown for two passages in conditioned SILAC media before use in co-culture experiments. Labeling efficiency of LM2 cells and HUVECs was assessed for a minimum of 3 independent cell cultures for each label.

SILAC co-culture for analysis of contact-initiated signaling

HUVECs were grown to confluent monolayers in 15 cm dishes. Prior to co-culture, confluent monolayers of HUVECs were serum-starved for 1.5 hours and a reserved dish of HUVECs was used for counting. To study the LM2 cells phosphoproteome, fully labeled LM2 cells (~70% confluency) were serum-starved for 16 hours, harvested with enzyme-free cell dissociation buffer and washed twice in serum-free, basal SILAC DMEM before counting. Heavy-labeled LM2 cells (Arg+10/Lys+8) were plated onto light-labeled HUVECs in a 2:1 LM2:HUVECs cell ratio in a volume of 3 mL per dish. Medium-labeled LM2 cells (Arg+6/Lys+4) were collected and kept in suspension in parallel to the co-culture. Cells were incubated for 15 min at 37°C, 5% CO $_2$. After incubation, unattached cells were removed from the dishes and counted to estimate the number of LM2 cells remaining attached to HUVEC monolayers. Heavy-labeled LM2 cells and HUVECs were lysed with medium-labeled LM2 cells in a 1:1 heavy:medium cell ratio with ice-cold 0.1 M Na $_2$ CO $_3$ pH 11, supplemented with protease inhibitor cocktail (P8340), phosphatase inhibitor cocktail 3 (P0044) and 10 mM sodium orthovanadate (S6508). To investigate the endothelial cells phosphoproteome, the same protocol for co-culture and cell lysis was followed using heavy- and medium-labeled HUVECs with light-labeled LM2 in a 2:1 LM2:HUVECs cell ratio.

Sample preparation and phosphoproteomic analysis

Membrane fractionation and sample preparation were adapted from (60). Samples were snap-frozen in liquid nitrogen. MgCl $_2$ was added to 1 mM final concentration for DNA digestion with Benzonase (Novagen 70746) for 15 min on ice. Cell lysates were then centrifuged at 100,000 *g* for 30 min at 4°C to separate soluble proteins from membrane-

associated proteins (pellet). Pellets were washed twice by two rounds of resuspension in lysis buffer followed by 30 min centrifugation before resuspension in 6 M urea, 2 M thiourea, 40 mM ammonium bicarbonate (ABC). Protein concentrations of both cytoplasmic and membrane-enriched fractions were assessed by Bradford protein assay (Bio-Rad). Reduction, alkylation and digestion were performed by Filter Aided Sample Preparation (61) (FASP; Vivacon 10 K) with 1 mg total protein per unit. After buffer exchange to 40 mM ABC, proteins were reduced in 20 mM dithiothreitol (DTT), 40 mM ABC for 45 min and subsequently alkylated in 50 mM iodoacetamide (IAA) for 50 min in the dark at room temperature. Following, CaCl_2 was added to a final concentration of 1 mM and samples were incubated with sialidase A / NANase III (Europa Bioproducts PZGK80040) and PNGase F (New England Biolabs P0704) overnight at 37°C to remove sialylations and N-glycosylations. Digestion was performed with Lys-C (Wako 125-05061) (1% w:w enzyme:substrate) for 3 hours at 37°C, followed by addition of trypsin (Worthington) (1% w:w enzyme:substrate) for overnight digestion. Digested peptides were recovered by collecting the flow-through and subsequent washes of the FASP column membrane in ABC, and then 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile (MeCN). Recovered peptides were dried under vacuum in a Savant SC250 express SpeedVac concentrator (Thermo Scientific).

All phosphopeptide enrichments were conducted as described in (62). Dried peptide pellets were resuspended in TiO_2 loading buffer (80% MeCN, 5% TFA, 1 M glycolic acid) and 3 mg Titansphere TiO_2 beads (10 μm ; GL-Sciences 5020 75010) were added per 500 μg peptides for 15 min shaking incubation at room temperature. Beads were then sequentially washed with i) loading buffer; ii) 80% MeCN, 1% TFA; and iii) 10% MeCN, 0.2% TFA. Phosphopeptides were eluted by two incubations with 1% ammonium hydroxide, pH 11.3. The flow through was then subjected to two more TiO_2 enrichment steps and the three elutions were pooled. Samples were acidified with a mix of TFA / Formic acid (FA), desalted with OLIGO R3 micro-columns packed in-house, and dried under vacuum.

For intra-experiment normalization based on global protein H/M ratios, samples of digested cell lysate prior to phospho-enrichment were fractionated and analyzed in parallel.

Discovery LC-MS/MS of phosphopeptide enriched samples

Samples were resuspended in 0.1% FA and run on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) coupled to a cHiPLC-Nanoflex chromatography system (Eksigent). Reversed-phase chromatographic separation was carried out on a 200 μm inner diameter (i.d.) x 0.5 mm trap column packed with C18 (3 μm bead size, 120 Å, Eksigent), a 75 μm i.d. x 15 cm column packed with C18 (3 μm bead size, 120 Å, Eksigent) with a linear gradient of 5-50% solvent B (100% MeCN + 0.1% FA) against solvent A (100% H_2O + 0.1% FA) with a flow rate of 300 nL/min. The mass spectrometer was operated in the data-dependent mode. Survey scans were performed in the Orbitrap with a resolution of 60,000 at m/z 400 and a Fourier Transform target value of 10^6 ions. The 10 most abundant ions were selected for fragmentation by higher-energy collisional dissociation and scanned in the Orbitrap at a resolution of 7,500 at m/z 400. Selected ions were dynamically excluded for 8 s. For accurate mass measurement, the lock mass option was enabled using the

polydimethylcyclsiloxane ion (m/z 455.120025) as an internal calibrant. For peptide identification, raw data files produced in Xcalibur software (Thermo Scientific) were processed in Proteome Discoverer V1.3 (Thermo Scientific) and searched using Mascot (v2.2). Searches were performed with a precursor mass tolerance of 20 ppm, a fragment mass tolerance of 0.05 Da, and a maximum of two missed cleavages. Static modifications were limited to carbamidomethylation of cysteine, and variable modifications used were SILAC labels (Lys+4.025 Da; Lys+8.050 Da; Arg+6.020 Da; Arg+10.008 Da); oxidation of methionine; deamidation of asparagine and glutamine; and phosphorylation of serine, threonine, and tyrosine residues. The database used for searching was SwissProt (04/2013, number of entries 89601). Peptides were further filtered using a Mascot significance threshold < 0.05 and a False Discovery Rate < 0.01 (q -value from Percolator 1).

Quantitative analysis of phosphorylation site regulation

For relative phosphopeptide quantification, heavy/medium ratios were determined by Proteome Discoverer (1.3). All further data analysis was performed using the statistical package R (R Development Core Team, 2012; <http://www.R-project.org/>). $\log_2$ (heavy/medium) ratios ($\log_2(H/M)$) were compiled for each quantified phosphopeptide and normalized to the median of the $\log_2(H/M)$ value of the input samples, not subjected to phospho-enrichment. Peptide phospho-positions were filtered based on a phosphoRS site probability $> 60\%$, where only the best scoring peptide was kept before mapping the position on the protein sequence. UniProt entry names are used in the text and figures to refer to identified proteins. Corresponding UniProt Accessions are listed in tables S1, S2, S3 and S4, and more information can be found on <http://www.uniprot.org>. One of the identified regulated phosphopeptides could not be uniquely attributed to either CAP-Gly domain-containing linker protein 1 (CLIP1) or 2 (CLIP2) due to sequence similarity and has thus been referred to as “CLIP1/2-S348” (with Ser³⁴⁸ in CLIP1 corresponding to Ser³⁵² in CLIP2). Phosphopeptides containing multiply phosphorylated residues have been referenced with all sites featuring phosphorylation (*i.e.* “AHNK-S210-S212-S216” which is phosphorylated on Ser²¹⁰, Ser²¹² and Ser²¹⁶). Spectra were manually assessed to remove mixed spectra, unresolved extracted ion chromatograms, and low SILAC signal intensity to avoid erroneous quantification before further analysis. Phosphorylation sites were then considered regulated if their average $\log_2(H/M)$ was above 0.5 or under -0.5. For each regulated phosphorylation site quantified in a minimum of two independent experiments, an unpaired two-sided t -test was performed ($\alpha = 0.05$). Significant results based on these criteria are referred to as the “core” set of regulated phosphorylation sites.

Fractionation and LC-MS for quantitative analysis of cellular proteome

Peptide samples were prepared as described above without deglycosylation step. High pH reverse phase chromatographic separation was performed on a Agilent Zorbax 300 extend – C18 column (4.6 X 150 mm, 3.5 μ m) using a 21 min stepped linear gradient of 0.5–40% solvent B (acetonitrile) against solvent A (H₂O; 0.1% NH₂O) and 40–85% solvent B against solvent A with a flow rate of 0.4 ml/min. Individual 10 second fractions were collected from 3 to 34 min. The 186 resulting fractions were pooled with equidistance across the gradient (checkboard) into 19 final fractions for LC-MS analysis.

Samples were run on a Q-Exactive Plus mass spectrometer (Thermo Scientific) coupled to a Dionex Ultimate 3000 RSLC nano system (Thermo Scientific). Reverse phase chromatographic separation was performed on a C18 PepMap 300 Å trap cartridge (0.3mm i.d. x 5 mm, 5 µm bead size; loaded in a bi-directional manner), a 75 µm i.d. x 50 cm column (5 µm bead size) using a 120 min linear gradient of 0-50% solvent B (acetonitrile; 0.1% formic acid) against solvent A (H₂O; 0.1% FA) with a flow rate of 300 nL/min. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 400-2000) were acquired in the Orbitrap with a resolution of 70,000 at m/z 400 and FT target value of 1×10^6 ions. The 10 most abundant ions were selected for fragmentation using higher-energy collisional dissociation (HCD) and dynamically excluded for 30 seconds. Fragmented ions were scanned in the Orbitrap at a resolution of 17,500 at m/z 400. For peptide identification, raw data files were produced in Xcalibur 2.1 (Thermo Scientific) and processed in Proteome Discoverer V1.4 (Thermo Scientific). Searches against SwissProt human database using Mascot (v2.2) were performed with settings and filters as described above, including only peptide ion Scores >20 and unique, proteotypic peptides. For relative protein quantification, the threshold intensity for calculating SILAC ratio was set to the 5% quantile for each sample. For proteins quantified in 3 or more independent experiments, the relative log₂(heavy/medium) ratios of their 3 most intense peptides were averaged to obtain the protein relative quantity between the heavy and medium channels for each experiment.

siRNA knockdown

Primary RNAi experiments were performed using siGENOME SMARTpool siRNA (Thermo Scientific). Secondary validation of silencing phenotypes was conducted with individual siRNA oligonucleotides of siGENOME or ON-TARGETplus design (Thermo Scientific), or pooled siRNA from FlexiTube GeneSolution (QIAGEN). Details of individual siRNA sequences as well as non-targeting/negative control siRNA used for normalization and statistical comparisons are provided as supplementary information (tables S6 and S7). Transfections were carried out with Lipofectamine RNAiMAX according to manufacturer's instructions. Cells were transfected with siRNA at a final concentration of 10 nM (siGENOME), 15 nM (QIAGEN) or 25 nM (ON-TARGETplus) 72 hours prior to assaying the impact of silencing.

Tumor-endothelial cell adhesion assay

Cancer cells were detached with enzyme-free cell dissociation buffer, washed and resuspended in medium 199, 20% FBS before 1×10^5 cancer cells were plated onto HUVEC monolayers that had been grown to confluency in a 96-well plate. Cells were co-cultured at 37°C, 5% CO₂ for 30 min (unless otherwise specified), before wells were washed to remove unattached cells. The remaining adherent cancer cells were quantified by the Bright-Glo Luciferase Assay System (Promega). Luminescence was measured on a SpectraMax M5 microplate reader and mean signal intensity was calculated from four replicate wells for each condition. Luminescence of adhering cells was normalized to luminescence from control wells containing the starting number of cancer cells that were plated in parallel.

Transendothelial migration assay

Cancer cells were detached with enzyme-free cell dissociation buffer, washed and resuspended in medium 199 GlutaMAX, 2.5% FBS at 5×10^5 cells/mL. 5×10^4 tumor cells were seeded onto HUVEC monolayers, grown to confluency in the upper chambers of 6.5 mm Transwell inserts with 8.0 μ m pore polycarbonate membrane (Corning). Lower chambers were filled with 600 μ L medium 199, 20% FBS to establish a FBS gradient. Cells were incubated at 37°C, 5% CO₂ for 8 hours before transwells were emptied and washed with PBS. Cells on the lower surface of the transwell membrane were detached with trypsin and collected by centrifugation. FBS was added to neutralize trypsin before collected cancer cells were quantified by luciferase assay, measured on a VICTOR X5 multilabel plate reader (PerkinElmer). Mean signal intensity was calculated from four technical replicates of each condition and normalized to luminescence from control wells containing the starting number of cancer cells that were plated in parallel.

In vivo lung retention assay

All animal work was conducted in accordance with UK Home Office guidelines, as established in the Animals (Scientific Procedures) Act 1986, under project license PPL70/7413. No adverse events were observed during the experiments. Cancer cells for in vivo assay were pre-stained for 45 min with CellTracker dyes made up in serum-free media to a final concentration of 8 μ M. “Experimental” cells, transfected with siRNA (*EPHA2* or non-targeting control), were stained with CellTracker Green CMFDA dye. In parallel, non-transfected cells were stained with CellTracker Orange CMRA to serve as an “injection control”. Stained cells were allowed to recover in normal growth medium for 6 hours before harvesting using enzyme-free cell dissociation buffer. Experimental (green) cells were mixed in a 1:1 ratio with injection control (orange) cells. Cell mixes were analyzed on a BD LSRII flow cytometer to verify starting green/orange cell ratios for subsequent normalization of acquired data. Twelve Female CD-1 nude mice (Charles River), aged between 6-8 weeks old, were injected in the tail vein with 8×10^5 cells in a volume of 100 μ L PBS (6 animals/group). After 20 hours, mice were sacrificed by CO₂ asphyxiation and whole lungs were excised. Lungs were fixed in 4% formaldehyde/PBS and imaged on a Zeiss 710 confocal microscope under a 20x objective with pinhole diameter set to 1 AU. Images were taken at a 512x512 resolution as 3x3 tiles (1,024 μ m²), randomly selected on both sides of each lung to give a total of 10 fields of view per lung. Fluorescent cell area quantification was performed using Volocity Image Analysis Software (PerkinElmer).

Identifying expressed ephrins in HUVECs

To identify ligands of *EPHA2* from the HUVEC lysate, HUVECs were grown to confluent monolayers and lysed in PLC buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM NaPPi, 10% glycerol and 1% Triton X-100; supplemented with sodium orthovanadate, and protease and phosphatase inhibitor cocktails). Affinity pull-downs were performed with a recombinant EphA2-Fc chimera (R&D Systems 639-A2-200), using 1-2 μ g bait per 500 μ g protein lysate. Samples were rotated overnight at 4°C before complexes were captured on Protein A-Sepharose 4B (P9424). Parallel pull-downs were performed using an Fc fragment to control for identification of non-specific interactants. To

ensure biological reproducibility, pull-downs were performed with 4 independently cultured samples of HUVECs. Proteins isolated on sepharose beads were separated by SDS-PAGE for in-gel digestion as previously described (63). Digestions were carried out with either 100 ng sequence-grade trypsin (Promega) or 250 ng AspN (P3303) overnight at 37°C. Extracted peptides were pooled and dried for discovery LC-MS/MS analysis, as described above.

Blocking native EPHA2-ephrin-A1 interactions

Cancer cells were harvested by enzyme-free cell dissociation buffer and incubated for 45 min on ice with non-clustered, recombinant ephrin-A1-Fc chimera (R&D Systems) at a concentration of 3 μg per 10^6 cells. As a negative control, cells were separately treated in parallel with IgG Fc (Bethyl Laboratories). Cells were washed to remove excess ligand/antibody before use in in vitro cellular assays. For verifying surface localization of ephrin-A1-EPHA2 complexes, cells were fixed with 4% formaldehyde for 15 minutes before washing and staining with Goat IgG DyLight 650 conjugate antibody (Thermo Scientific SA510121; 2.5 $\mu\text{L}/10^6$ cells) for 30 minutes at room temperature. Flow cytometry analysis was conducted on a BD LSR II flow cytometer and FlowJo vX 10.0.7 (TreeStar Inc) software.

To test blockade of EPHA2 ligand binding sites, soluble ephrinA1-Fc was made up in PBS and 50 μL was added to wells of a 96-well plate to provide a 2 $\mu\text{g}/\text{cm}^2$ coating per well. The plate was coated at 37 °C for 2 hours before wells were washed with cold PBS and blocked with a PBS/1% BSA solution for 1 hour at 37 °C. Wells were washed with medium prior to adhesion assay.

Cloning

For generation of luciferase-expressing cancer cell lines, a bicistronic expression construct encoding luciferase and EGFP was created by excising and subcloning the luciferase reporter gene from pCLucf (Addgene 37328) into pcDNA3.1 (+) zeo-IRES-EGFP (gift from Beatrice Howard, The Institute of Cancer Research) using *EcoRI* and *BamHI* restriction sites. Human EPHA2-Y772F in vector pTargetT (gift from Anton Bennett, Yale University) was PCR-amplified with a forward primer containing a *HindIII* restriction site (5'-TGACAAAGCTTATGGAGCTCCAGGCAGC-3') and a reverse primer containing a *XhoI* site (5'-ATCTGAACTCGAGTCAGATGGGGATCCCCAC-3') for subcloning into vector pcDNA3 at the *HindIII*-*XhoI* sites. Site-directed mutagenesis was performed to generate an EPHA2 K646M mutant expression construct using a QuikChange II Site-Directed Mutagenesis Kit, according the manufacturer's instructions. Human wild type EPHA2 in vector pcDNA3 (kind gift from Dr. Tony Pawson) was used a template for PCR with mutagenesis primers 5'-GTGCCGGTGGCCATCATGACGCTGAAAGCC-3' and 5'-GGCTTTCAGCGTCATGATGGCCACCGGCAC-3'. The pcDNA3-EPHA2-Y772F and pcDNA3-EPHA2-K646M constructs were verified by DNA sequencing.

Transfections and generations of stable cell lines

All transfections with cDNA were carried out using Lipofectamine 2000 according to manufacturer's instructions. Cell lines transfected for luciferase/GFP expression were grown under Zeocin selection and high luciferase-expressing cells were isolated as GFP^{high}

populations by fluorescence-activated cell sorting (FACS) on a BD FACS Aria flow cytometer.

Luciferase-expressing MDA-MB-468 and MDA-MB-453 cells were transfected with constructs for expression of wild-type EPHA2 (EPHA2-WT) (kind gift from Dr Tony Pawson), mutant EPHA2-Y772F or pcDNA3 vector control (Invitrogen) and grown under selection by G418 (A1720). Resistant colonies were pooled to create polyclonal populations for each cell line. Stably transfected cells were stained with mouse anti-EPHA2-Phycoerythrin (PE) conjugate [371805] (R&D Systems FAB3035P) at $8 \mu\text{L}/10^6$ cells, according to manufacturer's instructions. Cells were counterstained with $0.2 \mu\text{g}/\text{mL}$ DAPI to discern live cells before cell populations were isolated by FACS based on their quantity of EPHA2 at the surface.

Targeted analysis of EPHA2 abundance by mass spectrometry

Selected Reaction Monitoring (SRM) was used to analyze the expression of wild-type EPHA2 or mutant EPHA2-Y772F in cell lines (figs. S1E and S7). Total cell lysates were resolved by SDS-PAGE, and gel bands at 130 kDa molecular weight were excised for in-gel digestion and targeted analysis by LC-MS as previously described (63). Characteristic peptides and transitions monitored for EPHA2 and EPHA2-Y772F are provided as supplementary information (see table S8). Raw data files produced in Xcalibur were analyzed using Skyline v2.1 (MacCoss Lab of Biological Mass Spectrometry) (64).

Relative peptide abundance was determined by summing the area under the curve from extracted ion chromatograms of at least three transitions. Abundance of EPHA2 was deduced from the total SRM signal intensity of three quantotypic peptides. For relative comparison of EPHA2 quantity in LM2 and parental MDA-MB-231 cell populations, LM2 and parental cells were SILAC labeled with medium and heavy amino acids, respectively, to allow simultaneous SRM analysis of both populations. Total intensity of EPHA2 peptides was normalized to total protein quantities in each cell population.

For relative quantification of EPHA2 in the LM2 from SILAC-based co-culture experiments, lysates were incubated with an antibody recognizing human EPHA2 (Santa Cruz sc-924; $1 \mu\text{g}$ antibody per $500 \mu\text{g}$ protein) under rotation at 4°C overnight. Protein A-conjugated Sepharose was washed and equilibrated in PLC buffer before addition to complexes for a further rotation of 1-2 hours to immunoprecipitate EPHA2. Beads were collected by centrifugation and washed in PLC buffer before isolated complexes were resolved by SDS-PAGE for in-gel digest, discovery LC-MS/MS analysis and total protein relative quantification as described above. Normalization was carried out on based on the $\log_2(\text{H/M})$ ratio of the input samples prior to EPHA2-enrichment.

In vitro kinase activity assay

HEK 293FT cells were transfected with pcDNA3 expression vectors for EPHA2-WT, EPHA2-Y772F, EPHA2-K646M or empty vector control and grown for 48 hours before lysis in PLC buffer. EPHA2 antibody (sc-924) was conjugated onto MagReSyn beads (ReSyn Biosciences MR-PRA002) with overnight rotation at 4°C . Immunoprecipitation of EPHA2 was carried out using $0.5 \mu\text{g}$ antibody per $500 \mu\text{g}$ protein lysate. The kinase reaction

was carried out as described previously (65) with minor modifications. Briefly, the beads-EPHA2 complexes were washed twice in the Kinase Reaction buffer (KRB; 20 mM HEPES pH 7.5, 2.5 mM MgCl₂, 4 mM MnCl₂, 0.1 mM Na₃VO₄ and 100 μ M ATP) before incubation in 30 μ L KRB supplemented with 5 μ g human α -Enolase (SRP6109) for 1 hour at room temperature. Supernatants were collected and transferred onto a plate for the kinase activity readout using an ADP-Glo kit (Promega V9101), according to manufacturer's instructions.

EPHA2 stimulation with ephrin-A1

Cancer cells at ~70% confluency in 6-well plates, were serum-starved overnight before stimulation with artificially clustered ephrin-A1. Clustered ligand was freshly prepared by mixing ephrin-A1-Fc with antibody recognizing human IgG Fc (Jackson ImmunoResearch 109-001-008-JIR) in a 1:1.2 μ g ratio with rotation for a minimum of 1 hour at 4°C.

Clustered ephrin-A1 was made up to 2 μ g/mL in serum-free media and 700 μ L was used to stimulate each well of cancer cells. After incubation with ephrin-A1 at 37°C, 5% CO₂ for specified time points, cells were washed and lysed for western blot analysis.

Western blotting

Cells were lysed on ice in PLC buffer and equal amounts of total protein were resolved by SDS-PAGE before transfer onto nitrocellulose membranes (LI-COR 92631092) by a wet transfer system (Bio-Rad). Membranes were blocked in Roti-Block (Carl Roth A151.2) for 30 min at room temperature before probing for target proteins. Primary antibodies used in this study were mouse β -ACTIN [AC-15] (Abcam ab6276; 1/5,000), rabbit GAPDH [FL-335] (Santa Cruz sc-25778; 1/2,000), rabbit EPHA2 [C-20] (Santa Cruz sc-924; 1/1,000), rabbit phospho-EPHA2 (Tyr⁷⁷²) (Cell Signalling Technology #8244S; 1/1,000), rabbit phospho-EPHA2 (Tyr588) (Cell Signalling Technology #12677; 1/1,000), rabbit phospho-EPHA2 (Ser897) (Cell Signalling Technology #6347; 1/1,000), and rabbit LMW-PTP (Abcam ab166896; 1/2,000). IRDye-conjugated anti-Rabbit IgG, DyLight 800 (Cell Signalling Technology #5151) and anti-Mouse IgG, DyLight 680 (Cell Signalling Technology #5470) were used as secondary antibodies at 1/15,000. Visualization of western blots and densitometry analyses based on raw integrated intensity values were conducted using the Odyssey infrared imaging system and software (LI-COR Biosciences).

Statistical analysis

Unless otherwise indicated, data are expressed as mean values $\pm$ SEM calculated from a minimum of three independent experiments. Number of independent experiments are indicated on respective graphs (*N*) or represented as individual points. Statistical differences between two groups were assessed by two-sided, ratio paired or unpaired *t*-tests using GraphPad Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Reymond N, d'Água BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer*. 2013; 13:858–870. [PubMed: 24263189]
2. Leong HS, Robertson AE, Stoletov K, Leith SJ, Chin CA, Chien AE, Hague MN, Ablack A, Carmine-Simmen K, McPherson VA, Postenka CO, et al. Invadopodia Are Required for Cancer Cell Extravasation and Are a Therapeutic Target for Metastasis. *CellReports*. 2014:1–13.
3. Kienast Y, Baumgarten von L, Fuhrmann M, Klinkert WEF, Goldbrunner R, Herms J, Winkler F. Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med*. 2010; 16:116–122. [PubMed: 20023634]
4. Stoletov K, Kato H, Zardoujian E, Kelber J, Yang J, Shattil S, Klemke R. Visualizing extravasation dynamics of metastatic tumor cells. *J Cell Sci*. 2010; 123:2332–2341. [PubMed: 20530574]
5. Murugaesu N, Iravani M, van Weverwijk A, Ivetic A, Johnson DA, Antonopoulos A, Fearn A, Jamal-Hanjani M, Sims D, Fenwick K, Mitsopoulos C, et al. An in vivo functional screen identifies ST6GalNAc2 sialyltransferase as a breast cancer metastasis suppressor. *Cancer Discov*. 2014; 4:304–317. [PubMed: 24520024]
6. Biancone L, Araki M, Araki K, Vassalli P, Stamenkovic I. Redirection of tumor metastasis by expression of E-selectin in vivo. *Journal of Experimental Medicine*. 1996; 183:581–587. [PubMed: 8627169]
7. Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene*. 1997; 15:2169–2177. [PubMed: 9393975]
8. Tremblay P-L, Auger FA, Huot J. Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases. *Oncogene*. 2006; 25:6563–6573. [PubMed: 16715142]
9. Miles FL, Pruitt FL, van Golen KL, Cooper CR. Stepping out of the flow: capillary extravasation in cancer metastasis. *Clin Exp Metastasis*. 2007; 25:305–324. [PubMed: 17906932]
10. Wang H. Tumor cell $\alpha 3$ 1 integrin and vascular laminin-5 mediate pulmonary arrest and metastasis. *J Cell Biol*. 2004; 164:935–941. [PubMed: 15024036]
11. Klemke M, Weschenfelder T, Konstandin MH, Samstag Y. High affinity interaction of integrin $\alpha 4\beta 1$ (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) enhances migration of human melanoma cells across activated endothelial cell layers. *J Cell Physiol*. 2007; 212:368–374. [PubMed: 17352405]
12. Iizumi M, Bandyopadhyay S, Watabe K. Interaction of Duffy Antigen Receptor for Chemokines and KAI1: A Critical Step in Metastasis Suppression. *Cancer Res*. 2007; 67:1411–1414. [PubMed: 17308076]
13. Khanna P, Chung C-Y, Neves RI, Robertson GP, Dong C. CD82 and KAI expression prevents IL-8-mediated endothelial gap formation in late-stage melanomas. 2013; 33:2898–2908.
14. Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics*. 2002; 1:376–386. [PubMed: 12118079]
15. Zhu H, Pan S, Gu S, Bradbury EM, Chen X. Amino acid residue specific stable isotope labeling for quantitative proteomics. *Rapid Commun Mass Spectrom*. 2002; 16:2115–2123. [PubMed: 12415544]
16. Jorgensen C, Sherman A, Chen GI, Pasculescu A, Poliakov A, Hsiung M, Larsen B, Wilkinson DG, Linding R, Pawson T. Cell-specific information processing in segregating populations of Eph receptor ephrin-expressing cells. *Science*. 2009; 326:1502–1509. [PubMed: 20007894]

17. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massagué J. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005; 436:518–524. [PubMed: 16049480]
18. Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, Gomis RR, Manova-Todorova K, Massagué J. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature*. 2007; 446:765–770. [PubMed: 17429393]
19. Dunon D, Piali L, Imhof BA. To stick or not to stick: the new leukocyte homing paradigm. *Curr Opin Cell Biol*. 1996; 8:714–723. [PubMed: 8939652]
20. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. 2010:1–16.
21. Mercurio AM, Rabinovitz I. Towards a mechanistic understanding of tumor invasion—lessons from the $\alpha 6 \beta 4$ integrin. *Seminars in Cancer Biology*. 2001; 11:129–141. [PubMed: 11322832]
22. Boeri Erba E, Bergatto E, Cabodi S, Silengo L, Tarone G, Defilippi P, Jensen ON. Systematic analysis of the epidermal growth factor receptor by mass spectrometry reveals stimulation-dependent multisite phosphorylation. *Mol Cell Proteomics*. 2005; 4:1107–1121. [PubMed: 15901825]
23. Miao H, Burnett E, Kinch M, Simon E, Wang B. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat Cell Biol*. 2000; 2:62–69. [PubMed: 10655584]
24. Beaty BT, Wang Y, Bravo-Cordero JJ, Sharma VP, Miskolci V, Hodgson L, Condeelis J. Talin regulates moesin-NHE-1 recruitment to invadopodia and promotes mammary tumor metastasis. *J Cell Biol*. 2014; 205:737–751. [PubMed: 24891603]
25. Hyder CL, Lazaro G, Pylvanainen JW, Roberts MWG, Qvarnstrom SM, Eriksson JE. Nestin regulates prostate cancer cell invasion by influencing the localisation and functions of FAK and integrins. *J Cell Sci*. 2014; 127:2161–2173. [PubMed: 24610946]
26. Tran H, Pankov R, Tran SD, Hampton B, Burgess WH, Yamada KM. Integrin clustering induces kinectin accumulation. *J Cell Sci*. 2002; 115:2031–2040. [PubMed: 11973345]
27. van Rijssel J, Kroon J, Hoogenboezem M, van Alphen FPJ, de Jong RJ, Kostadinova E, Geerts D, Hordijk PL, van Buul JD. The Rho guanine nucleotide exchange factor Trio controls leukocyte transendothelial migration by promoting docking structure formation. *Mol Biol Cell*. 2012; 23:2831–2844. [PubMed: 22696684]
28. Belkina NV, Liu Y, Hao J-J, Karasuyama H, Shaw S. LOK is a major ERM kinase in resting lymphocytes and regulates cytoskeletal rearrangement through ERM phosphorylation. *Proceedings of the National Academy of Sciences*. 2009; 106:4707–4712.
29. Muller WA, Weigl SA, Deng X, Phillips DM. PECAM-1 is required for transendothelial migration of leukocytes. *Journal of Experimental Medicine*. 1993; 178:449–460. [PubMed: 8340753]
30. Liao F, Ali J, Greene T, Muller WA. Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. *Journal of Experimental Medicine*. 1997; 185:1349–1357. [PubMed: 9104821]
31. Jin J-K, Tien P-C, Cheng C-J, Song JH, Huang C, Lin S-H, Gallick GE. Talin1 phosphorylation activates $\beta 1$ integrins: a novel mechanism to promote prostate cancer bone metastasis. 2014; 34:1811–1821.
32. Kwak TK, Lee MS, Ryu J, Choi YJ, Kang M, Jeong D, Lee JW. Cell Adhesion-dependent Serine 85 Phosphorylation of Paxillin Modulates Focal Adhesion Formation and Haptotactic Migration via Association with the C-terminal Tail Domain of Talin. *J Biol Chem*. 2012; 287:27499–27509. [PubMed: 22761432]
33. Zagórska A, Deak M, Campbell DG, Banerjee S, Hirano M, Aizawa S, Prescott AR, Alessi DR. New roles for the LKB1-NUAK pathway in controlling myosin phosphatase complexes and cell adhesion. *Sci Signal*. 2010; 3:ra25–ra25. [PubMed: 20354225]
34. Fox BP, Kandpal RP. Invasiveness of breast carcinoma cells and transcript profile: Eph receptors and ephrin ligands as molecular markers of potential diagnostic and prognostic application. *Biochemical and Biophysical Research Communications*. 2004; 318:882–892. [PubMed: 15147954]

35. Martin KJ, Patrick DR, Bissell MJ, Fournier MV. Prognostic Breast Cancer Signature Identified from 3D Culture Model Accurately Predicts Clinical Outcome across Independent Datasets. *PLoS ONE*. 2008; 3:e2994–9. [PubMed: 18714348]
36. Brantley-Sieders DM, Jiang A, Sarma K, Badu-Nkansah A, Walter DL, Shyr Y, Chen J. Eph/Ephrin Profiling in Human Breast Cancer Reveals Significant Associations between Expression Level and Clinical Outcome. *PLoS ONE*. 2011; 6:e24426–9. [PubMed: 21935409]
37. Hu M, Carles-Kinch KL, Zelinski DP, Kinch MS. EphA2 induction of fibronectin creates a permissive microenvironment for malignant cells. *Molecular Cancer Research*. 2004; 2:533–540. [PubMed: 15498927]
38. Kullander K, Klein R. Mechanisms and functions of eph and ephrin signalling. *Nat Rev Mol Cell Biol*. 2002; 3:475–486. [PubMed: 12094214]
39. Wilkinson DG. How attraction turns to repulsion. *Nat Cell Biol*. 2003; 5:851–853. [PubMed: 14523393]
40. Binns KL, Taylor PP, Sicheri F, Pawson T, Holland SJ. Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors. *Mol Cell Biol*. 2000; 20:4791–4805. [PubMed: 10848605]
41. Wybenga-Groot LE, Baskin B, Ong SH, Tong J, Pawson T, Sicheri F. Structural Basis for Autoinhibition of the EphB2 Receptor Tyrosine Kinase by the Unphosphorylated Juxtamembrane Region. *Cell*. 2001; 106:745–757. [PubMed: 11572780]
42. Wiesner S, Wybenga-Groot LE, Warner N, Lin H, Pawson T, Forman-Kay JD, Sicheri F. A change in conformational dynamics underlies the activation of Eph receptor tyrosine kinases. *EMBO J*. 2006; 25:4686–4696. [PubMed: 16977320]
43. Parri M. EphrinA1 Repulsive Response Is Regulated by an EphA2 Tyrosine Phosphatase. *Journal of Biological Chemistry*. 2005; 280:34008–34018. [PubMed: 16051609]
44. Chiarugi P, Taddei ML, Schiavone N, Papucci L, Giannoni E, Fiaschi T, Capaccioli S, Rauegi G, Ramponi G. LMW-PTP is a positive regulator of tumor onset and growth. *Oncogene*. 2004; 23:3905–3914. [PubMed: 15021900]
45. Kikawa KD, Vidale DR, Van Etten RL, Kinch MS. Regulation of the EphA2 kinase by the low molecular weight tyrosine phosphatase induces transformation. *J Biol Chem*. 2002; 277:39274–39279. [PubMed: 12167657]
46. Balasubramaniam D, Paul LN, Homan KT, Hall MC, Stauffacher CV. Specificity of HCPTP variants toward EphA2 tyrosines by quantitative selected reaction monitoring. *Protein Science*. 2011; 20:1172–1181. [PubMed: 21538645]
47. Bos PD, Zhang XH-F, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn J, van de Vijver MJ, Gerald WL, Foekens JA, Massagué J. Genes that mediate breast cancer metastasis to the brain. *Nature*. 2009; 459:1005–1009. [PubMed: 19421193]
48. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Córdón-Cardo C, Guise TA, Massagué J. A multigenic program mediating breast cancer metastasis to bone. *CCELL*. 2003; 3:537–549.
49. Vadlamudi RK, Li F, Adam L, Nguyen D, Ohta Y, Stossel TP, Kumar R. Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. *Nat Cell Biol*. 2002; 4:681–690. [PubMed: 12198493]
50. Woo MS, Ohta Y, Rabinovitz I, Stossel TP, Blenis J. Ribosomal S6 Kinase (RSK) Regulates Phosphorylation of Filamin A on an Important Regulatory Site. *Mol Cell Biol*. 2004; 24:3025–3035. [PubMed: 15024089]
51. Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. *Cancer Res*. 2001; 61:2301–2306. [PubMed: 11280802]
52. Zantek ND, Walker-Daniels J, Stewart J, Hansen RK, Robinson D, Miao H, Wang B, Kung HJ, Bissell MJ, Kinch MS. MCF-10A-NeoST: a new cell system for studying cell-ECM and cell-cell interactions in breast cancer. *Clin Cancer Res*. 2001; 7:3640–3648. [PubMed: 11705887]
53. Fang WB, Brantley-Sieders DM, Parker MA, Reith AD, Chen J. A kinase-dependent role for EphA2 receptor in promoting tumor growth and metastasis. *Oncogene*. 2005; 24:7859–7868. [PubMed: 16103880]

54. Brantley-Sieders DM, Zhuang G, Hicks D, Fang WB, Hwang Y, Cates JMM, Coffman K, Jackson D, Bruckheimer E, Muraoka-Cook RS, Chen J. The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. *J Clin Invest.* 2008; 118:64–78. [PubMed: 18079969]
55. Zantek ND, Azimi M, Fedor-Chaiken M, Wang B, Brackenbury R, Kinch MS. E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. *Cell Growth Differ.* 1999; 10:629–638. [PubMed: 10511313]
56. Wykosky J. EphA2 as a Novel Molecular Marker and Target in Glioblastoma Multiforme. *Molecular Cancer Research.* 2005; 3:541–551. [PubMed: 16254188]
57. Miao H, Li D-Q, Mukherjee A, Guo H, Petty A, Cutter J, Basilion JP, Sedor J, Wu J, Danielpour D, Sloan AE, et al. EphA2 Mediates Ligand-Dependent Inhibition and Ligand-Independent Promotion of Cell Migration and Invasion via a Reciprocal Regulatory Loop with Akt. *Cancer Cell.* 2009; 16:9–20. [PubMed: 19573808]
58. Tawadros T, Brown MD, Hart CA, Clarke NW. Ligand-independent activation of EphA2 by arachidonic acid induces metastasis-like behaviour in prostate cancer cells. 2012; 107:1737–1744.
59. Minn AJ, Gupta GP, Padua D, Bos P, Nguyen DX, Nuyten D, Kreike B, Zhang Y, Wang Y, Ishwaran H, Foekens JA, et al. Lung metastasis genes couple breast tumor size and metastatic spread. *Proceedings of the National Academy of Sciences.* 2007; 104:6740–6745.
60. Palmisano G, Lendal SE, Engholm-Keller K, Leth-Larsen R, Parker BL, Larsen MR. Selective enrichment of sialic acid-containing glycopeptides using titanium dioxide chromatography with analysis by HILIC and mass spectrometry. *Nat Protoc.* 2010; 5:1974–1982. [PubMed: 21127490]
61. Wi niewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods.* 2009; 6:359–362. [PubMed: 19377485]
62. Engholm-Keller K, Hansen TA, Palmisano G, Larsen MR. Multidimensional strategy for sensitive phosphoproteomics incorporating protein prefractionation combined with SIMAC, HILIC, and TiO(2) chromatography applied to proximal EGF signaling. *J Proteome Res.* 2011; 10:5383–5397. [PubMed: 21955146]
63. Worboys JD, Sinclair J, Yuan Y, rgensen CJO. Systematic evaluation of quantotypic peptides for targeted analysis of the human kinome. *Nature Publishing Group.* 2014:1–6.
64. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics.* 2010; 26:966–968. [PubMed: 20147306]
65. Holland SJ, Gale NW, Gish GD, Roth RA, Songyang Z, Cantley LC, Henkemeyer M, Yancopoulos GD, Pawson T. Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J.* 1997; 16:3877–3888. [PubMed: 9233798]

One Sentence Summary

Mass spectrometry reveals novel mechanisms regulating metastatic cancer cell extravasation

Fig. 1.

Tumor-endothelial co-culture and cell-specific phosphoproteomic analysis.

(A) Co-culture setup developed to study contact-initiated phospho-signaling between highly metastatic LM2 cancer cell population and Human Umbilical Vein Endothelial Cells (HUVECS). The cell populations of interest were SILAC-labeled prior to co-culture to enable cell-specific analysis. Following co-culture of heavy labeled and light labeled cells, medium labeled cells in monoculture were combined as point of reference. Left and right panels depict the two parallel SILAC-labeling strategies used to analyze signaling changes

in the LM2 and endothelial cells, respectively. **(B)** Schematic representation of MS sample preparation. Cell lysates were fractionated, digested and phosphopeptides were then enriched before LC-MS/MS analysis. **(C)** Peptides were assigned to cell of origin based on their SILAC label. Changes to the abundance of identified phosphopeptides were determined from the relative abundance between heavy (co-culture) and medium (control) label. Sites were considered to be regulated by an increase or decrease in phosphorylation where $\log_2(H/M)$ ratios were above 0.5 or below -0.5, respectively.

Fig. 2.

Regulated phosphorylation sites in co-cultured LM2 cells and HUVECs.

(A) Distribution of all identified phosphorylation sites with a q -value ≤ 0.01 . Venn diagrams show the number of phosphorylation sites identified in the dataset represented by a minimum of one phosphopeptide, quantified from a minimum of one $\log_2(H/M)$ SILAC ratio in the analysis and regulated (average $\log_2(H/M) > 0.5$, or < -0.5). Orange and green represent cytoplasmic and membrane-enriched fractions, respectively. (B) Phosphorylation sites with increased (up) or decreased (down) abundance in co-cultured LM2 cells and

HUVECs. Bars display average $\log_2(H/M)$ for each modulated phosphorylation site (see tables S1 and S2, and figs. S2 and S3 for experimental values and UniProt Accession numbers). All phosphorylation sites were quantified from singly phosphorylated peptides except for Neuroblast differentiation-associated protein AHNAK Ser²¹⁰, Ser²¹², Ser²¹⁶ (“AHNK-S210-S212-S216”). Red bars indicate the “core” set of phosphorylation sites quantified in a minimum of two independent experiments, with an unpaired two-sided t-test P-value ≤ 0.05 . Yellow circles present the median $\log_2(H/M)$ of the corresponding protein where available (raw values in tables S3, S4 and S5). Proteins are listed by their UniProt entry names and phosphorylation sites (S, Serine; T, Threonine; Y, Tyrosine) are indicated followed by their position within the protein sequence.

Overview of initiated signaling and interaction pathways upon tumor-endothelial contact between LM2 cancer cells and HUVECs. This schematic presents a subset of regulated phosphoproteins mapped onto Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and further refined using literature review. Several proteins involved in cytoskeletal remodeling and adhesion are modified by phosphorylation in co-cultured LM2 cells and HUVECs. Proteins in LM2 cells (top) and HUVECs (bottom) are listed with their UniProt entry names and regulated phosphorylation sites (S, Serine; T, Threonine; Y, Tyrosine) are indicated followed by their position within the protein sequence. Fold changes measured upon tumor-endothelial interaction and UniProt Accession numbers are presented in table S1 and S2.

Fig. 4.

EPHA2 inhibits tumor-endothelial interaction and transendothelial migration.

(A) LM2 cells were transfected with *EPHA2*-targeting siRNA pools, and their adhesion to HUVEC monolayers was assessed after a 30-min co-culture. (B) Representative western blot of *EPHA2* silencing in LM2 cells. (C) Impact of *EPHA2* silencing on LM2 transendothelial migration (TEM). (D) Schematic overview of in vivo lung retention assay. (E) Impact of *EPHA2* silencing on early stage lung colonization by LM2 cells. Graph shows mean transfected/injection control ratio $\pm$ SEM, dots represent average ratios from 10 fields of view per lung. $N=6$ mice per group, P -value by unpaired two-sided t-test. (F) Western blot comparison of endogenous *EPHA2* in breast cancer cell lines SUM149PT, MDA-MB-468 and MDA-MB-453 against LM2 cells. (G) Representative western blots of silencing or ectopic expression of wild-type (WT) *EPHA2*. (H-J) TEM assays were performed with *EPHA2*-silenced SUM149PT cells (H), as well as MDA-MB-468 (I) and MDA-MB-453 (J) cell lines where *EPHA2* was stably overexpressed. All bar charts show mean $\pm$ SEM, relative to mock-transfected, lipofectamine (LF) control treatment (unless otherwise

specified). *P*-values indicated on bar charts by two-sided ratio paired t-test against cells transfected with non-targeting siRNA or empty pcDNA3 vector controls.

Fig. 5.

EPHA2 function in tumor-endothelial interaction is modulated by both ligand engagement and changes in in Tyr⁷⁷² phosphorylation. **(A)** Impact of blocking native EPHA2-ligand engagement on transendothelial migration (TEM) of LM2 cells. Prior to TEM assay, LM2 cells were pre-incubated with soluble, non-clustered ephrin-A1-Fc to saturate EPHA2 ligand-binding sites, or IgG Fc negative control. *P*-value calculated by two-sided ratio paired t-test. **(B)** Box plot of relative abundances of total EPHA2 and EPHA2 phospho-Tyr⁷⁷² (p-Y772) in LM2 cells following tumor-endothelial co-culture. Dots represent independent biological experiments. log₂(H/M) ratios for total EPHA2 were calculated from the 3 most intense peptides within each experiment. *P*-value calculated by two-sided, unpaired t-test (raw values are presented in table S3). **(C)** Representative MS2 spectra showing fragment assignments that identify the EPHA2 Tyr⁷⁷² phospho-peptide alongside table of mass-to-charge (m/z) for a, b and y ion series.

Fig. 6. Phosphorylation of EPHA2-Tyr⁷⁷² is important for EPHA2-mediated inhibition of cancer cell transendothelial migration (TEM). **(A)** Working model for phospho-Tyr⁷⁷² regulation of EPHA2 function in tumor-endothelial interaction: upon tumor-endothelial cell contact, engagement of endothelial ephrin-A1 leads to EPHA2 phosphorylation at Tyr⁷⁷², receptor activation, and cell-cell repulsion, resulting in an inhibition of tumor-endothelial adhesion and TEM. Downregulation of phosphorylation at Tyr⁷⁷² overrides ligand-driven EPHA2 activation and signaling, thus enabling cell adhesion and cancer cell TEM. **(B)** TEM assay of

MDA-MB-468 and MDA-MB-453, stably expressing wild-type *EPHA2* (EPHA2-WT) or an *EPHA2*-Y772F mutant, containing a non-phosphorylatable phenylalanine at position 772. (C) In vitro kinase assay comparing activity of EPHA2-Y772F against wild-type and kinase dead K646M mutant EPHA2. (D) Representative western blot of transfection and EPHA2 immunoprecipitation (IP) efficiencies for kinase assay. (E) Representative western blots of EPHA2 phospho-Tyr⁷⁷² (p-Y772) in parental, LM2, BrM2a and BoM1 MDA-MB-231 subpopulations over the course of stimulation with ephrin-A1-Fc (eA1-Fc). (F) Densitometry analyses of EPHA2 p-Y772, quantified from western blots and normalized against respective GAPDH loading controls. Bars show mean $\pm$ SEM of changes in EPHA2 p-Y772, relative to the baseline of non-stimulated cells. All *P*-values were calculated by two-sided ratio paired t-test; n.s., not significantly different.