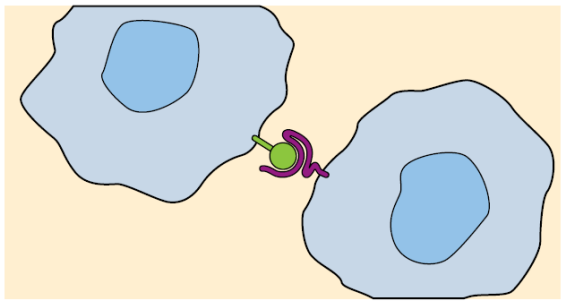
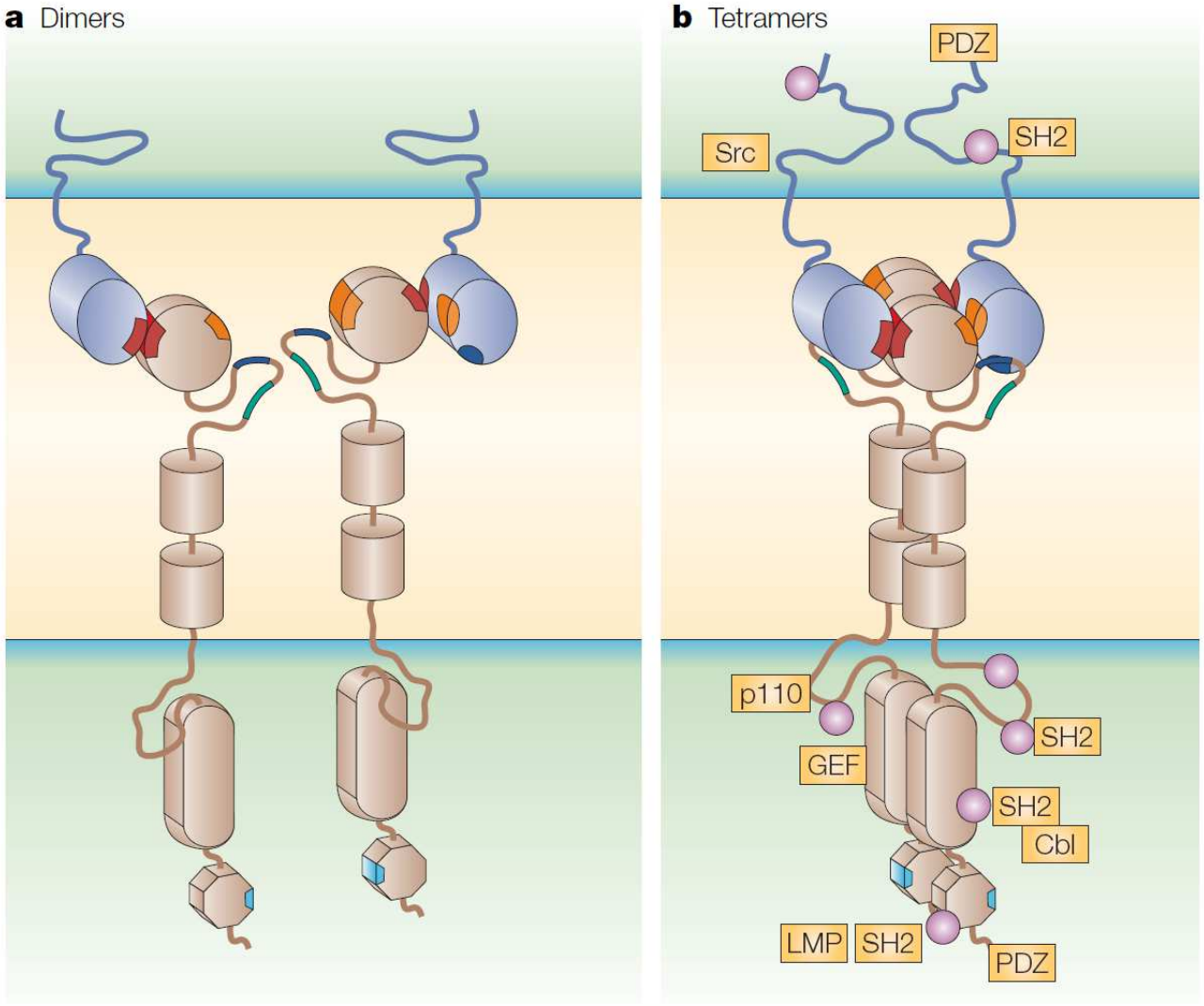
Comunicazione bidirezionale: come studiare in maniera quantitativa le proteine coinvolte nella segnalazione nelle due cellule?



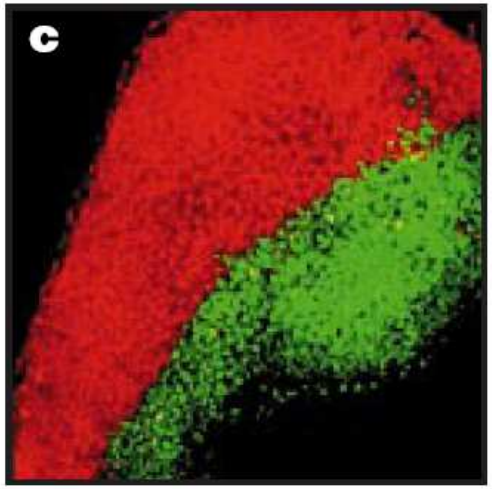


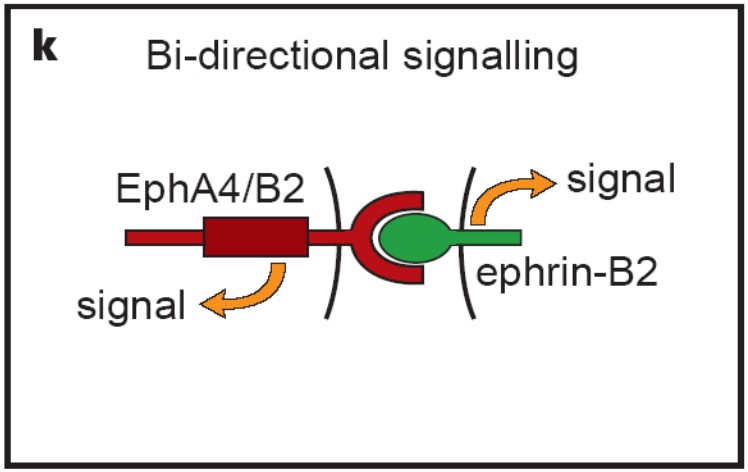
- la trasduzione del segnale di solito viene studiata stimolando le cellule bersaglio con un ligando solubile extracellulare, come ad esempio un fattore di crescita

- non è detto che questo approccio ripercorra il processo fisiologico della comunicazione intercellulare, in parte perché le proteine che fungono da ligandi per alcuni recettori sono esse stesse ancorate alla membrana della cellula

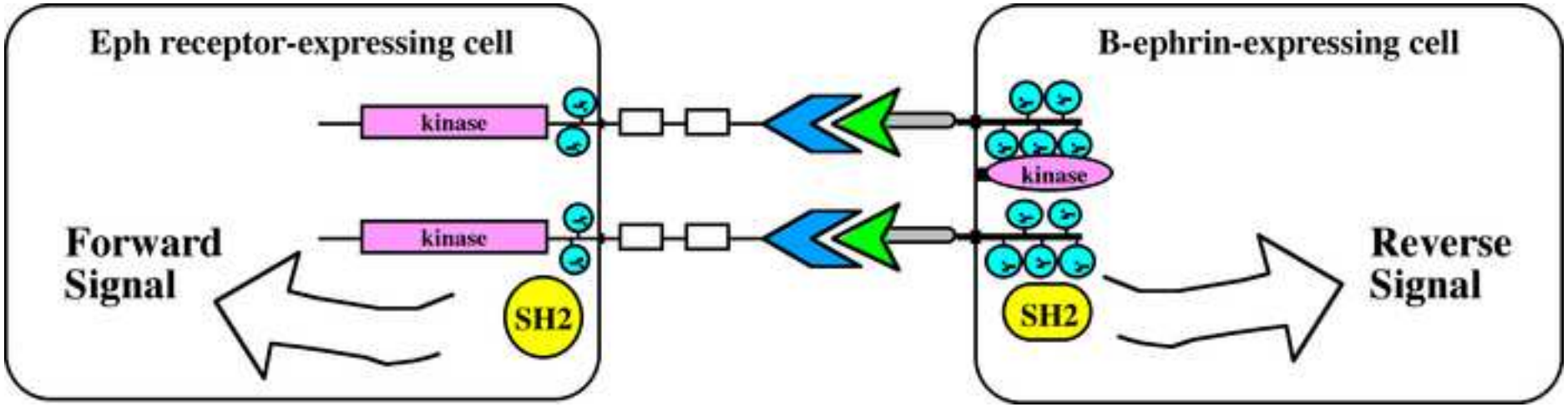
- la segnalazione iniziata dal contatto cellula-cellula di solito è un processo reciproco, nel quale due tipi cellulari si scambiano segnali distinti, che portano ad alterazioni mutualmente dipendenti nei loro rispettivi comportamenti

EphB2 /ephrin-B2

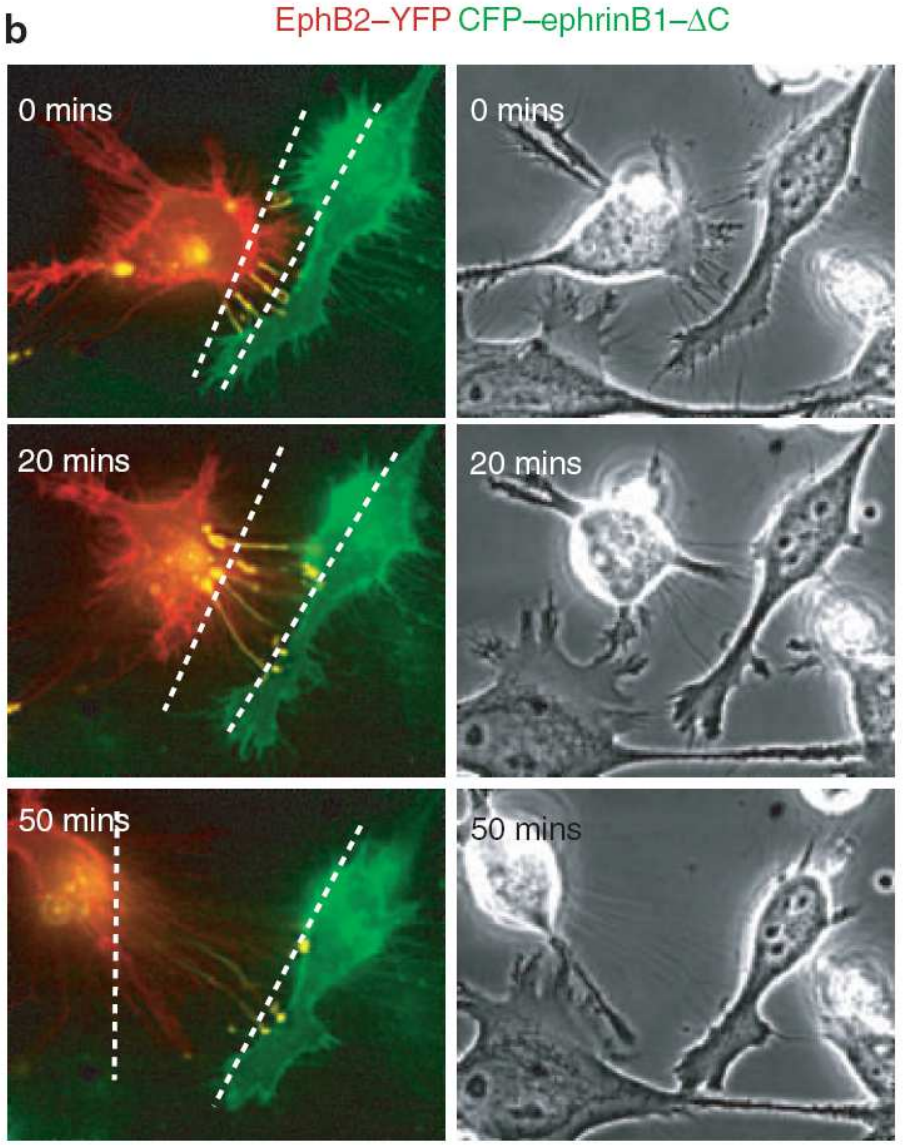




The Phenomenon of Bidirectional Signaling



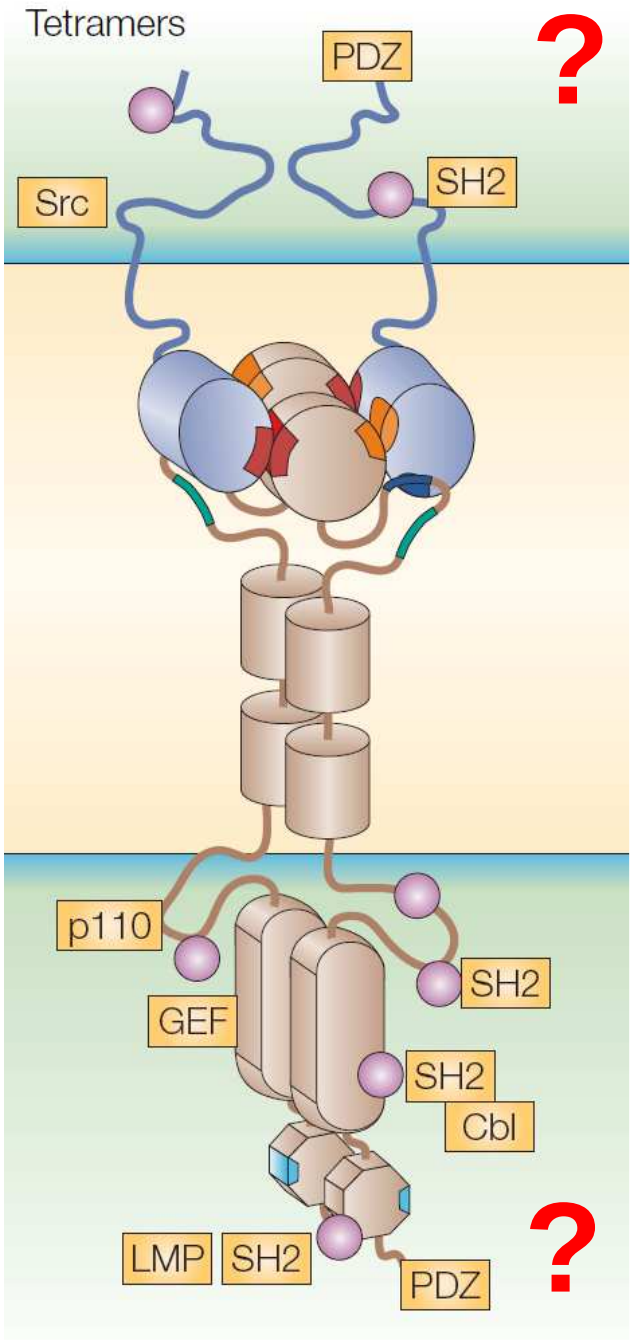
Interazione fra cellule adese (che esprimono il ligando transmembrana) e cellule adese (che esprimono il recettore transmembrana)



11 DECEMBER 2009 VOL 326 SCIENCE www.sciencemag.org

Cell-Specific Information Processing in Segregating Populations of Eph Receptor Ephrin–Expressing Cells

Claus Jørgensen,[1] Andrew Sherman,[1,2] Ginny I. Chen,[1,2] Adrian Pasculescu,[1] Alexei Poliakov,[3] Marilyn Hsiung,[1] Brett Larsen,[1] David G. Wilkinson,[3] Rune Linding,[4]* Tony Pawson[1,2]*



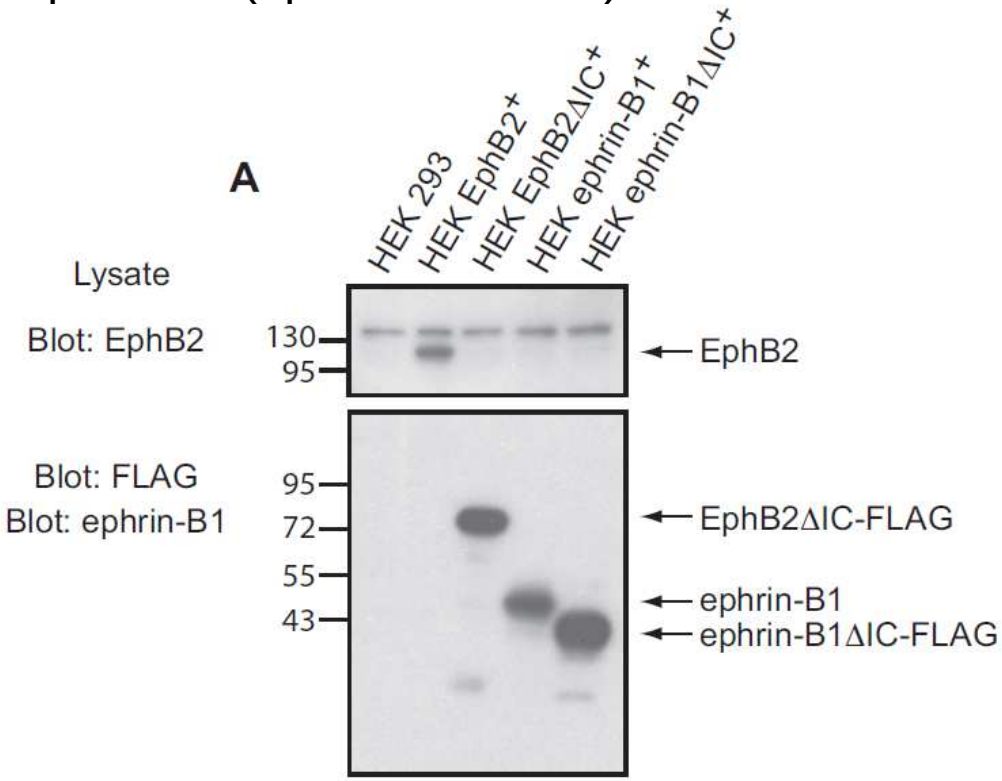
❁ direct interactions between transmembrane Eph receptor tyrosine kinases (EphRs) and their membrane bound ephrin ligands frequently lead to mutual **cell repulsion** and are important for **axon guidance** and **boundary formation** during tissue development

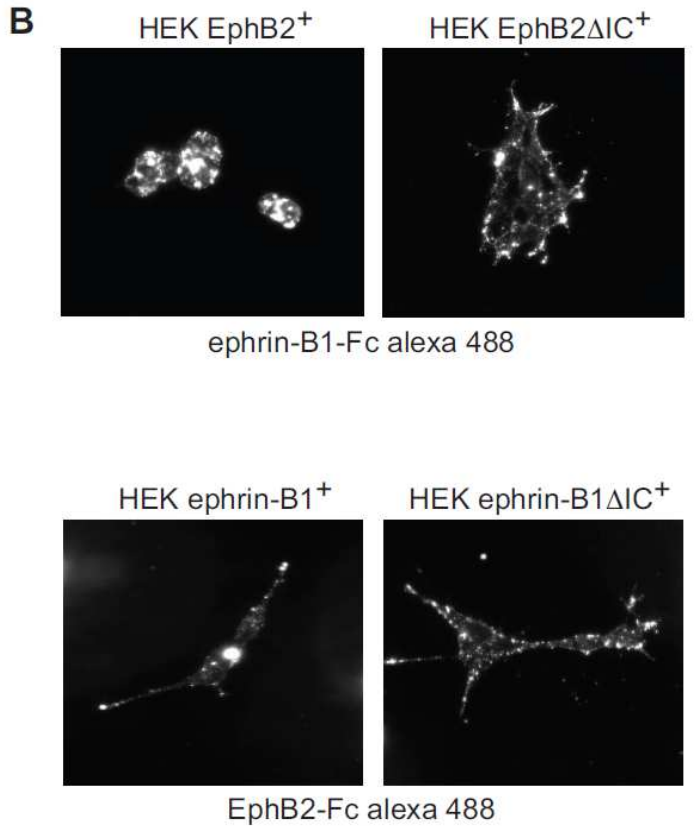
❁ clustering of B-type EphRs and ephrins at the surface of adjacent cells activates phosphotyrosine (pTyr) signaling in both the EphR- and ephrin-expressing cells, termed **forward** and **reverse** signaling, respectively

❁ systematic analysis of cell-specific networks in distinct populations of interacting cells is challenging primarily because the unique properties of each cell type are lost once co-cultured cells are processed for biochemical analysis, such as by immunoblotting

Quantitative analysis of Bidirectional Signaling (qBidS)

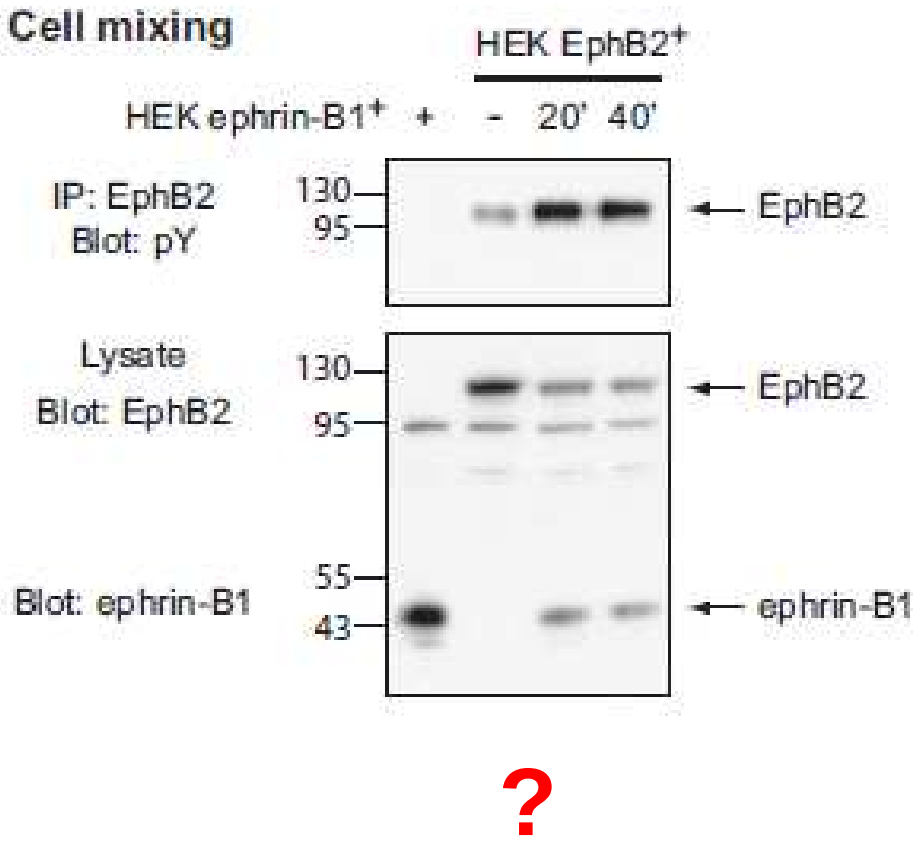
To study bidirectional EphR-ephrin signaling, they used the human embryonic kidney (HEK)293 cell line engineered to express either EphB2 (EphB2+ cells) or ephrin-B1 (ephrin-B1+ cells).





Relative surface levels of these proteins were tested using immunofluorescence (IF) and FACS. Ectodomain-Fc fusions of EphB2 or ephrin-B1 were labeled with Alexa-488 and used to mark surface exposed Eph receptor or ligand in live cells. Analysis by IF or FACS revealed similar surface levels of wild type or mutant forms of EphB2 and ephrin-B1 in all stable cell lines used.

To verify the ability of the EphB2+ and ephrin-B1+ cells to induce a pTyr response, signaling was initiated by cell mixing.
There is extensive tyrosine phosphorylation of both ephrin-B1 and EphB2, demonstrating a functional tyrosine kinase response.



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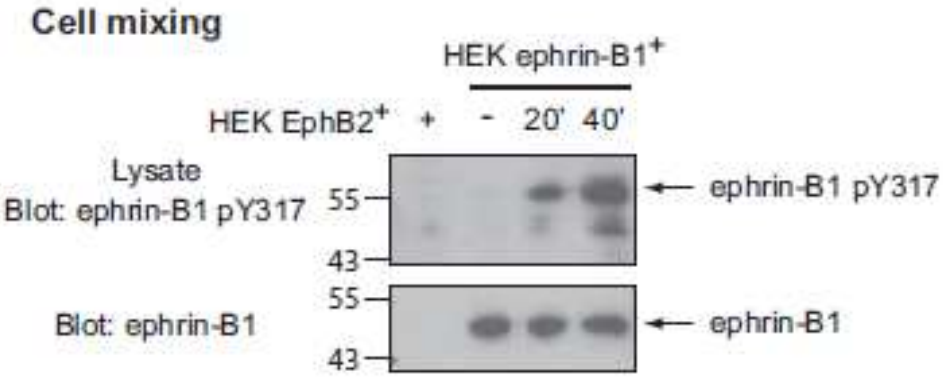
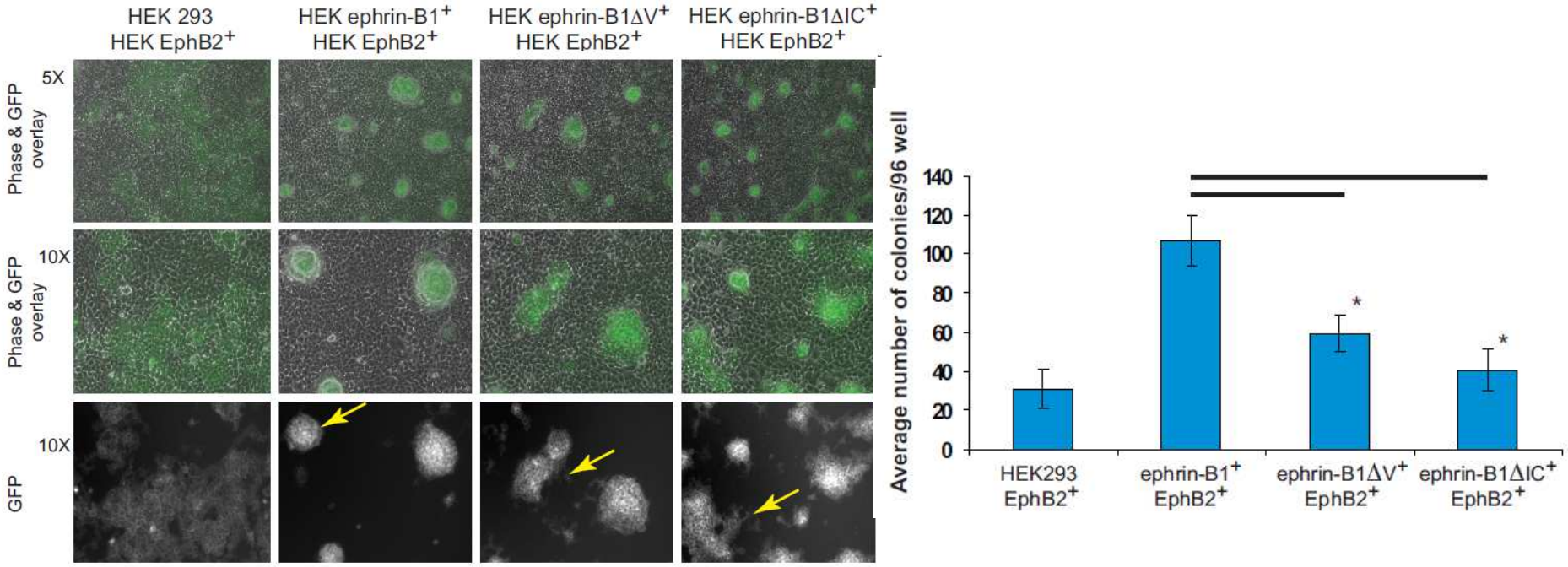
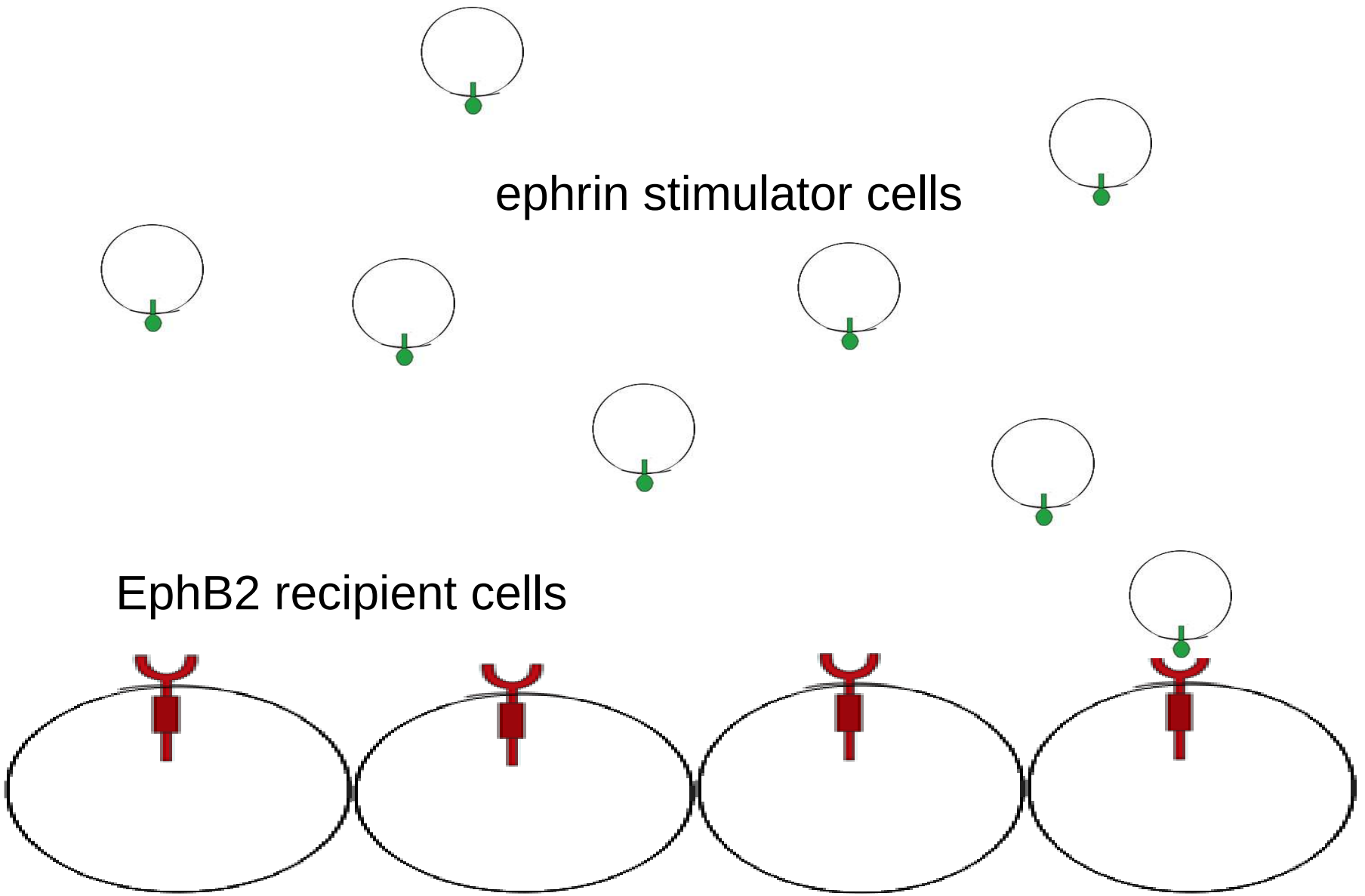


EphB2+ cells, which coexpress myristoylated GFP, were mixed with ephrin-B1+ cells. The ability of mixed populations of EphB2+ and ephrin-B1+ cells to sort and organize into distinct multi-cellular structures (colonies) was also confirmed, suggesting that all the relevant molecules required for this process are expressed within these cells.



Quantitative analysis of Bidirectional Signaling (qBidS) to better understand EphB2- and ephrin-B1-regulated cell sorting

Stimolazione di cellule adese (che esprimono il recettore transmembrana) con cellule in sospensione (che esprimono il ligando transmembrana)



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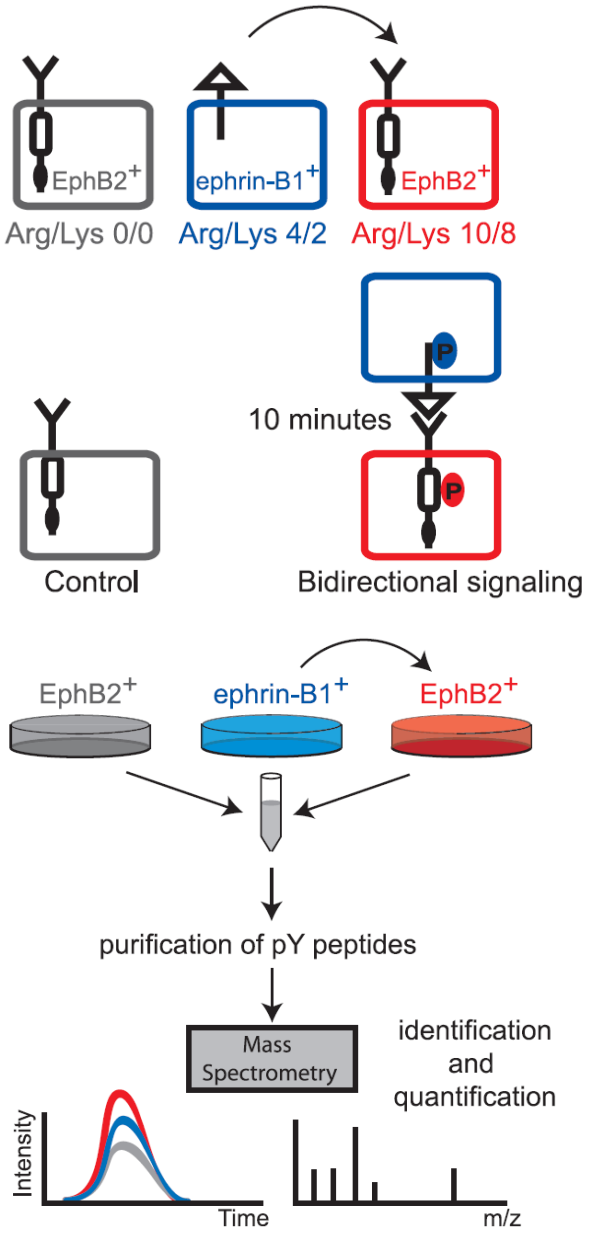


EphB2^{+} cells were labeled independently with
-**"light" ($C^{12}N^{14}$) arginine and lysine** or
-**"heavy" ($C^{13}N^{15}$) arginine and lysine**

ephrin-B1^{+} cells were labeled with
-**"medium" ($C^{12}N^{15}$) arginine and lysine**

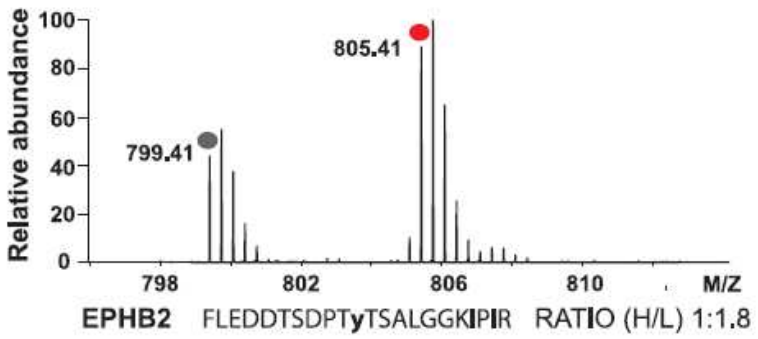
Bidirectional signaling was initiated, mixing **heavy labeled EphB2+ cells** and **medium labeled ephrin-B1+ cells**;

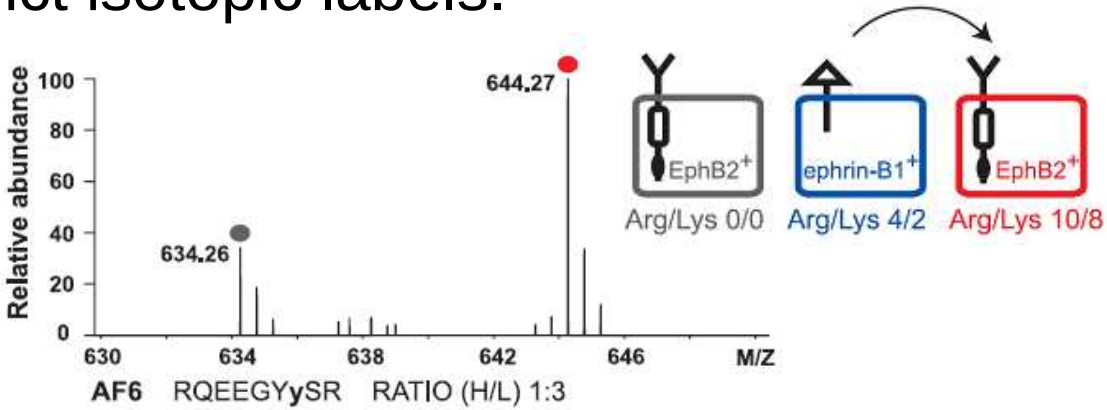
non-stimulated light-labeled EphB2+ cells served as a control.

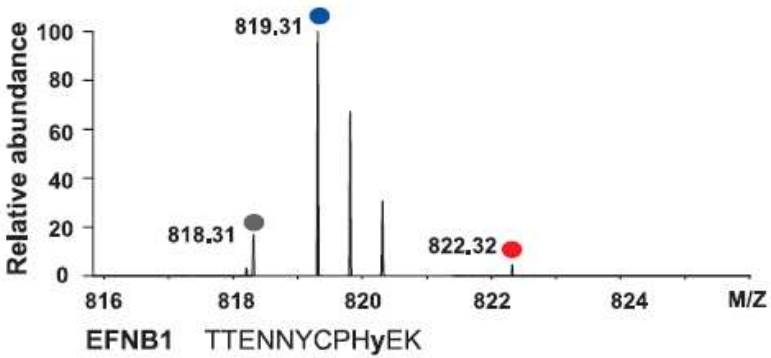
Mixed populations of cells were harvested after 10 min and combined with nonstimulated control cells.

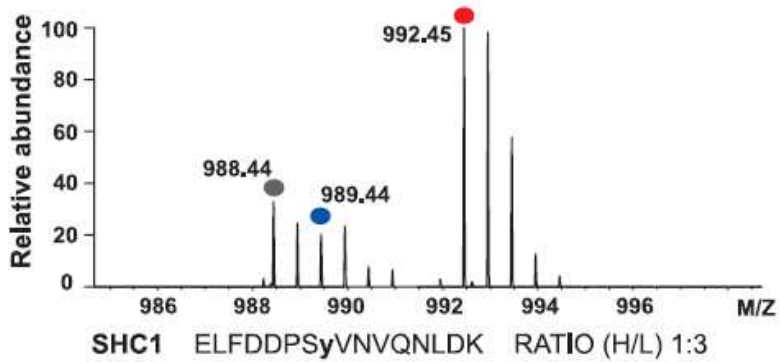
Cell lysates were digested with trypsin, and tyrosine-phosphorylated peptides were isolated and analyzed with liquid chromatography-mass spectrometry (LC-MS).

Peptides from mixed-cell populations were differentiated and quantified via their distinct isotopic labels.



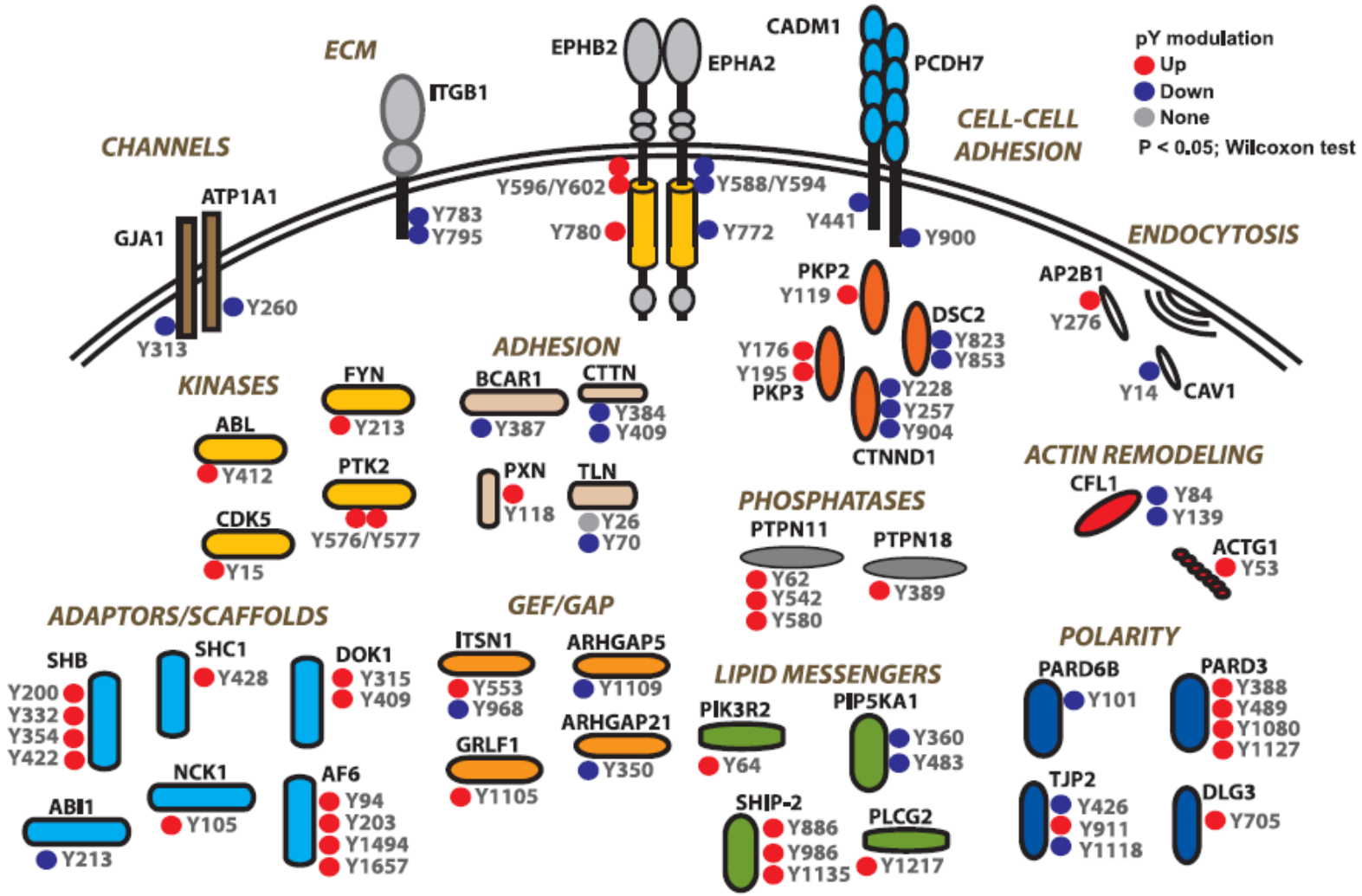






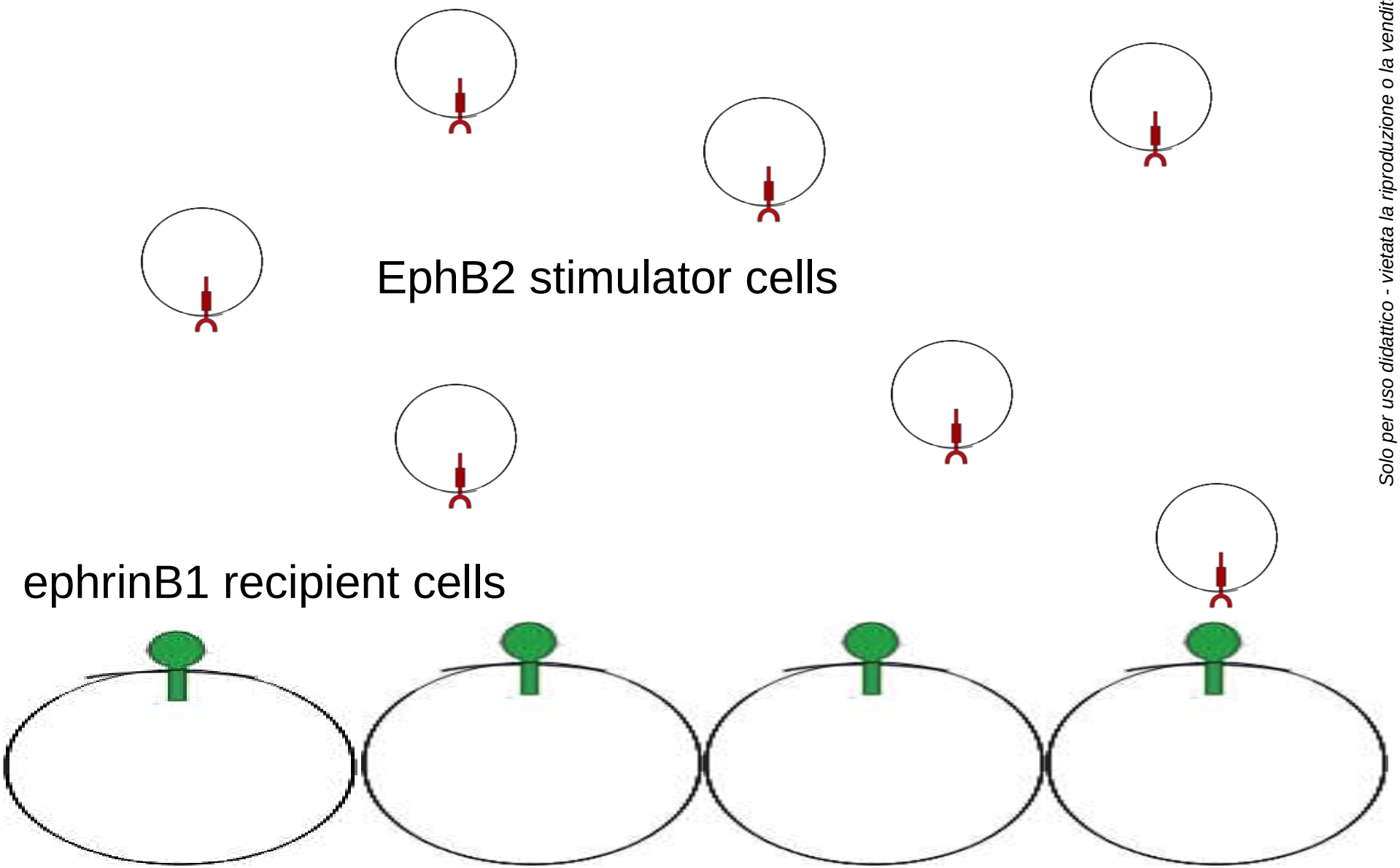
In **EphB2+ cells** mixed with ephrin-B1+ cells, phosphorylation of the activation loop of EPHB2 at Y780 was increased by 80%, as determined from the ratio of heavy- to light-labeled peptides. Tyrosine phosphorylation of ephrin-B1 (Y317) was only observed with a medium label, indicating that this peptide originates specifically from the **ephrin-B1+ cells**.
The previously described EphB2 targets AF6 (Y1657) and SHC1 (Y427) display threefold increased levels of phosphorylation in the **heavy-labeled EphB2+ cell** population.

Asymmetric regulation of tyrosine phosphorylation in EphB2+ cells.

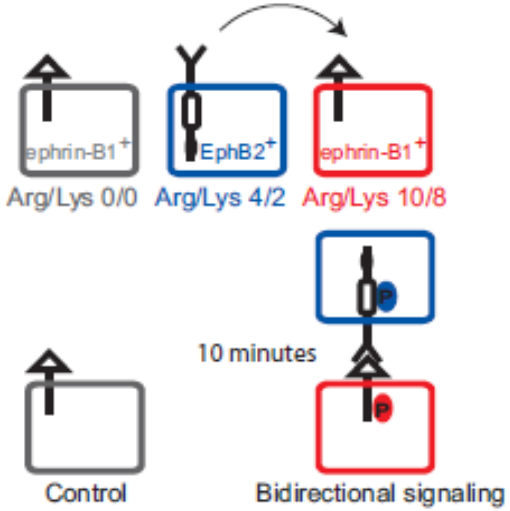


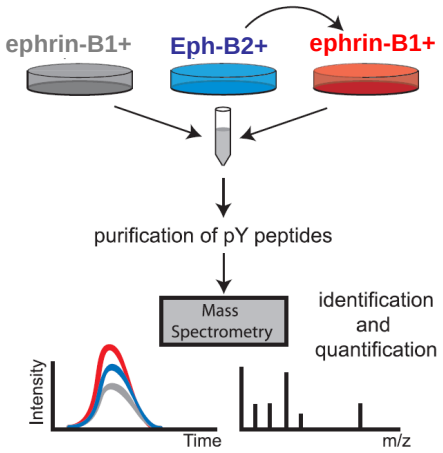
Overview of selected proteins in EphB2+ cells modulated by tyrosine phosphorylation after mixing with ephrin-B1+ cells. The modulation of pTyr sites is indicated as **significantly increased (red),** **not modulated (gray),** or **decreased (blue).** Molecules that are involved in a wide variety of cellular functions, such as lipid signaling (SHIP-2, PLCG2, and PIK3R2), polarity (DLG3 and PARD3), actin and myosin turnover (NCK1 and ACTG1), endocytosis and recycling (AP2B1 and CAV1), and adaptor proteins (SHB and SHC1), as well as kinases (ABL1 and CDK5) are modulated by tyrosine phosphorylation, indicating that phospho-regulation of numerous cellular processes may be important for cell sorting.

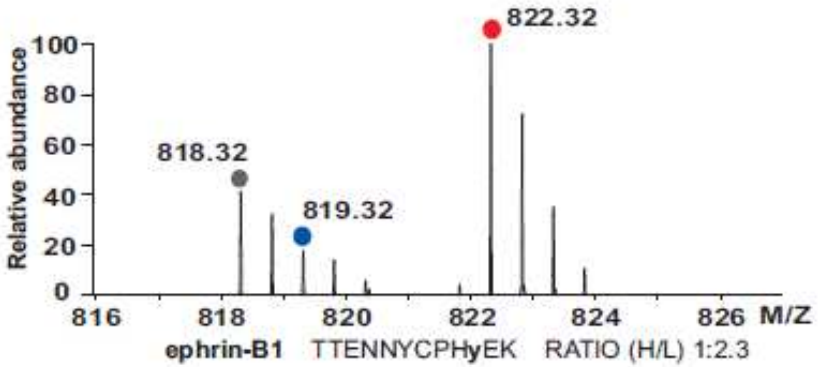
Stimolazione di cellule adese (che esprimono il ligando transmembrana)
con cellule in sospensione (che esprimono il recettore transmembrana)

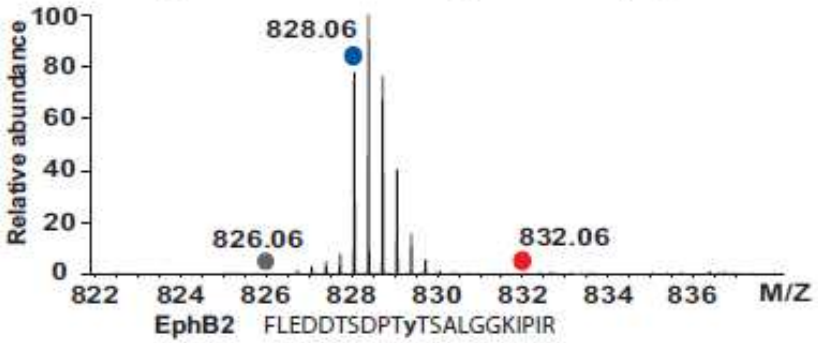


Quantitative analysis of Bidirectional Signaling (qBidS)









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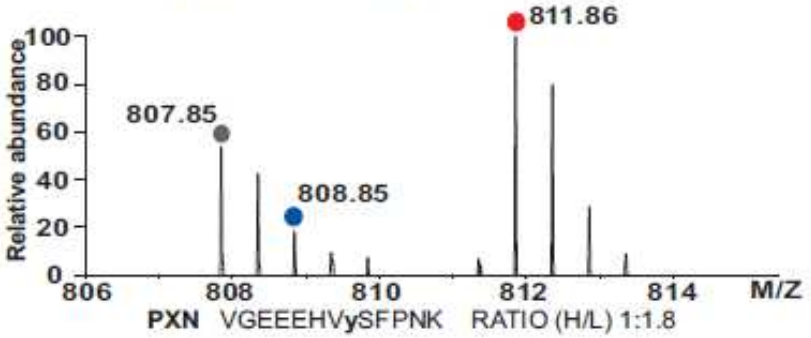


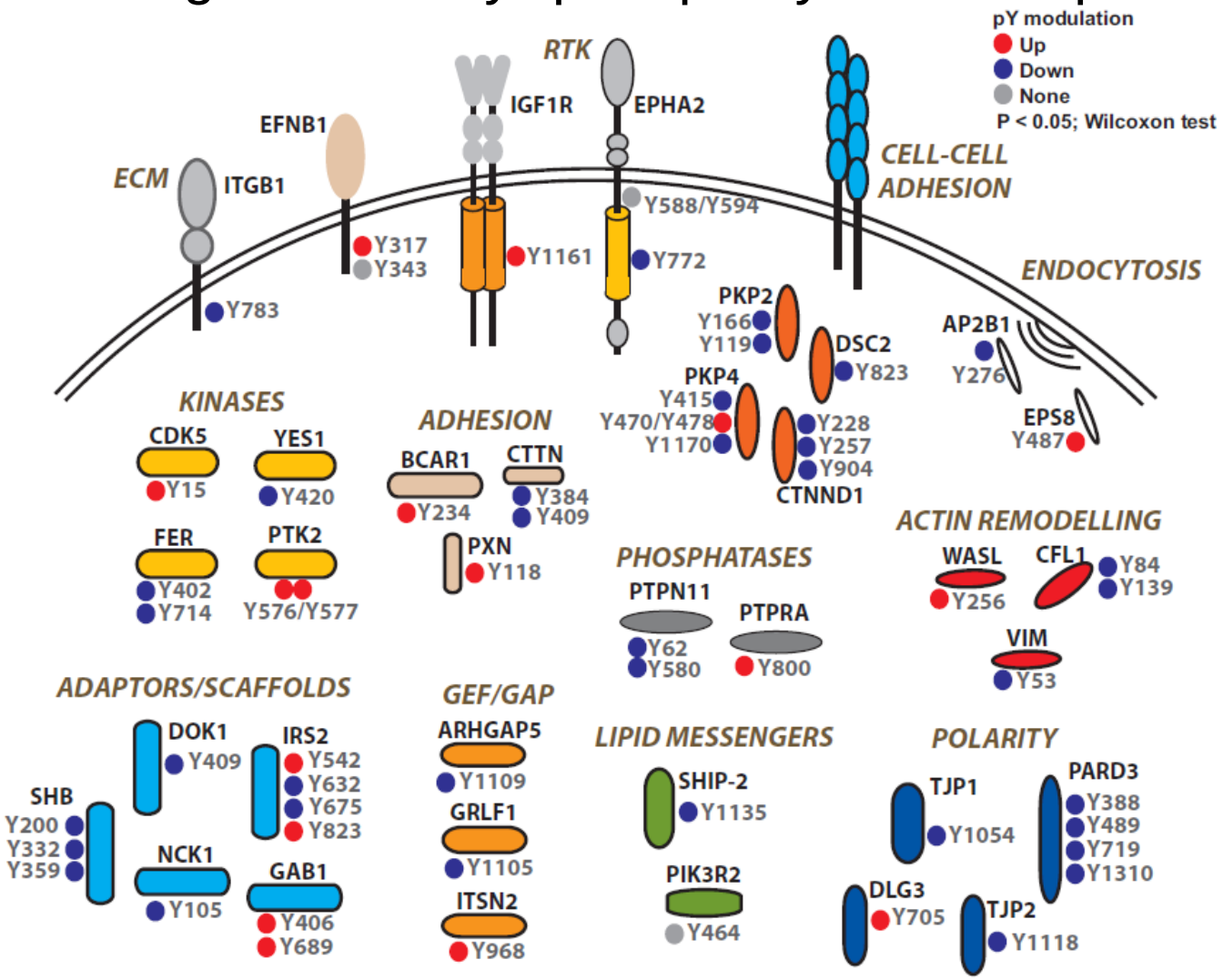
ephrin-B1^{+} cells were labeled independently with
-**"light" ($C^{12}N^{14}$) arginine and lysine** or
-**"heavy" ($C^{13}N^{15}$) arginine and lysine**

Eph-B2^{+} cells were labeled with
-**"medium" ($C^{12}N^{15}$) arginine and lysine**

Bidirectional signaling was initiated, mixing **heavy labeled ephrin-B1+ cells** and **medium labeled Eph-B2+ cells**;

non-stimulated light-labeled ephrin-B1+ cells served as a control.

Asymmetric regulation of tyr-phosphorylation in ephrin-B1+ cells



Overview of selected tyrosine phosphorylation sites regulated in ephrin-B1+ cells following mixing with EphB2+ cells. The positions of identified tyrosine phosphorylation sites are shown and are color-coded to display whether their phosphorylation was **significantly increased (red), decreased (blue)** or **not modulated (grey).** Proteins involved in a variety of cellular functions such as adhesion, actin remodeling, polarity and protein/lipid phosphorylation were modulated in ephrin-B1+ cells following contact with EphB2+ cells. Previously described downstream effectors of ephrin-B1 (PTK2, PXN) as well as novel participants (GAB1, ITSN2) were identified.

Bidirectional signaling between EphB2- and ephrin-B1–expressing cells is asymmetric.

Analysis of EphB2-ephrin-B1 bidirectional signaling identified:

- 557 distinct tyrosine phosphorylation sites in EphB2$^+$ cells of which 276 were significantly regulated in 185 proteins.

- 353 distinct tyrosine phosphorylation sites in ephrin-B1$^+$ of which 166 were significantly regulated in 119 proteins.

→ Analysis of the global changes in tyrosine phosphorylation induced by contact between EphB2$^+$ and ephrinB1$^+$ cells identified a total of

- 442 tyrosine phosphorylation sites on 304 target proteins

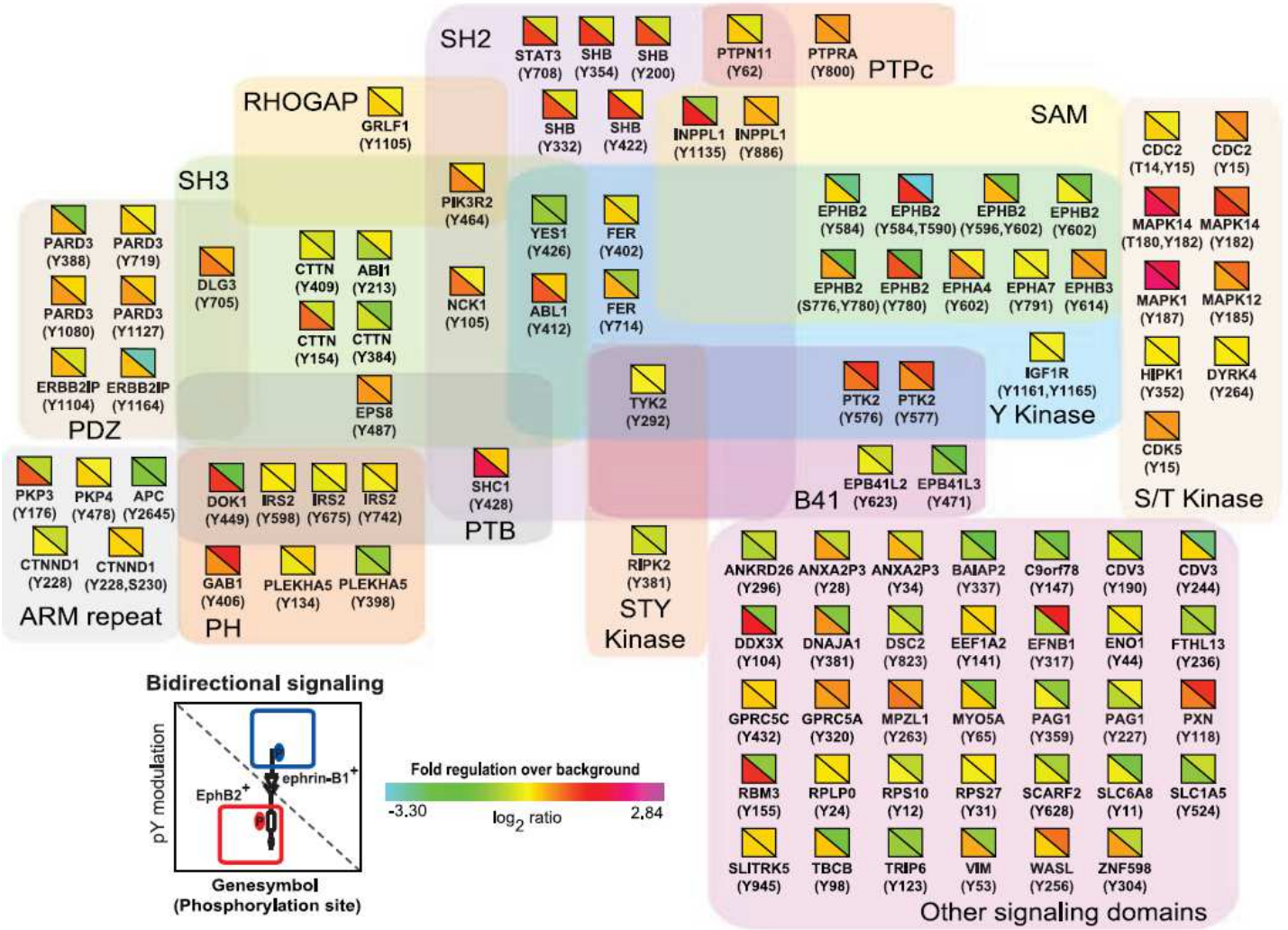
To quantitatively assess cell-specific signaling events, they identified identical peptides that were tyrosine-phosphorylated in both EphB2^{+} and ephrin-B1^{+} cells and compared their dynamic profiles between the two cell types.

They compared the cell-specific modulation of the 100 common tyrosine phosphorylation sites, which they grouped according to the signaling domains present on the phosphorylated proteins.

Of these sites, 71% displayed asymmetric modulation between the cell lines.

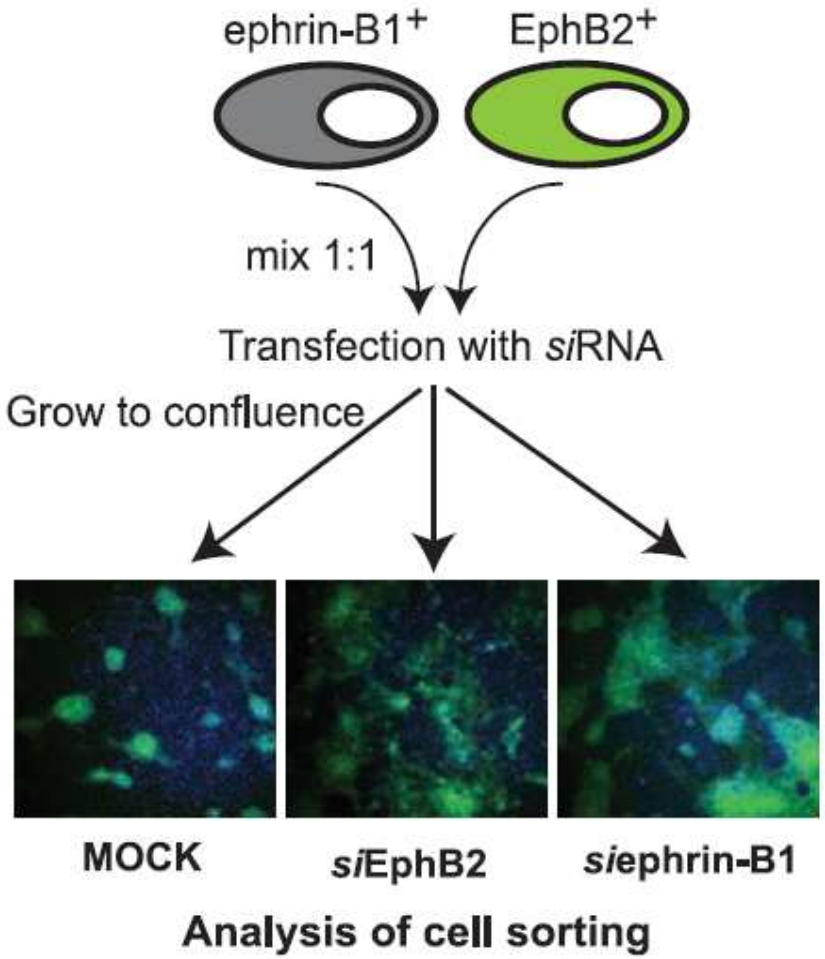
→ Signaling networks induced by contact between EphB2^{+} and ephrin-B1^{+} cells show both cell specific and shared modes of regulation.

Comparison of contact-initiated pTyr signaling between ephrin-B1^{+} and EphB2^{+} cells



Identical pTyr sites are shown grouped according to the domain composition of their host proteins. Cell-specific modulation of pTyr sites is indicated by slashed boxes, each triangle representing specific regulation within EphB2+ (bottom triangles) or ephrin-B1+ (top triangles) cells, respectively. The fold regulation of each pTyr site is shown with a color range. In total, we identified 100 identical tyrosine-phosphorylated sites in the two cell types, of which 71 displayed asymmetric modulation between EphB2+ and ephrin-B1+ cells.

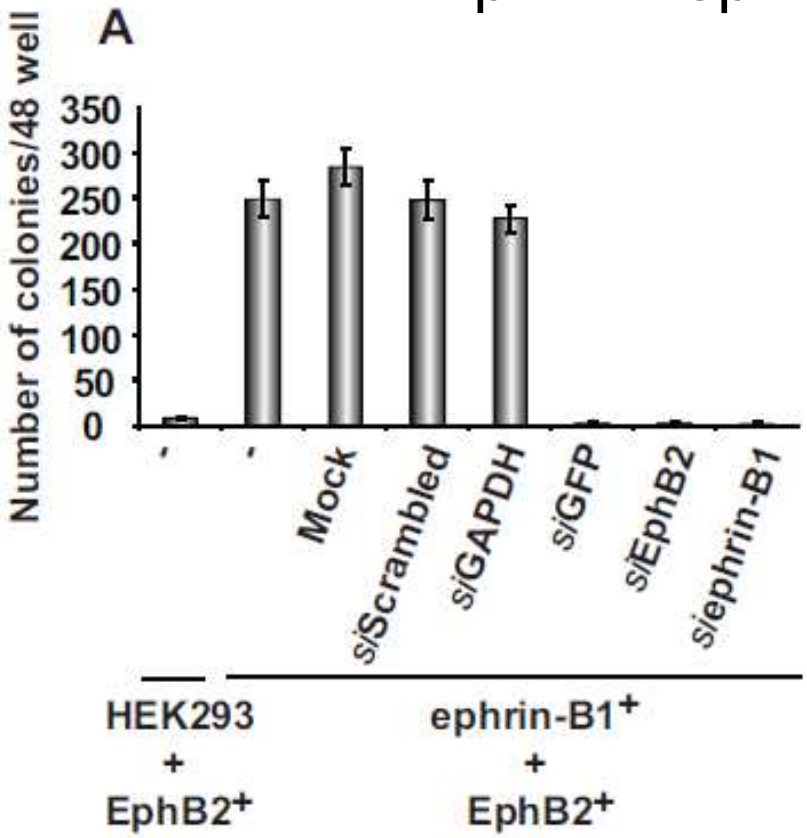
Functional analysis of EphB2- and ephrin-B1-regulated cell sorting through siRNA screening .

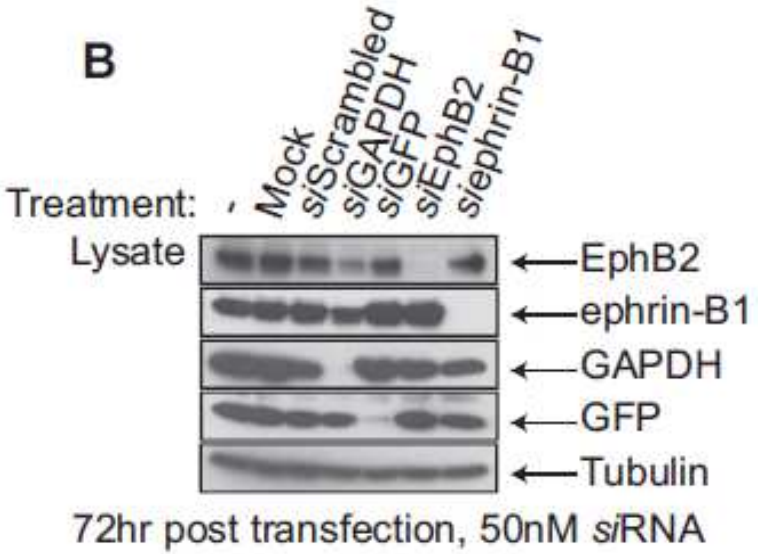


EphB2+ cells, which coexpress myristoylated GFP, were mixed with ephrin-B1+ cells, transfected with siRNA pools, and grown to 100% density. The number of GFP-positive EphB2+ colonies was used to determine the effect of siRNAs on cell sorting. Disruption of EphB2 or ephrin-B1 expression by means of siRNA inhibits colony formation

Functional analysis of EphB2- and ephrin-B1-regulated cell sorting through siRNA screening

Characterization of siRNA-dependent inhibition of EphB2+-ephrin-B1+ cell sorting.

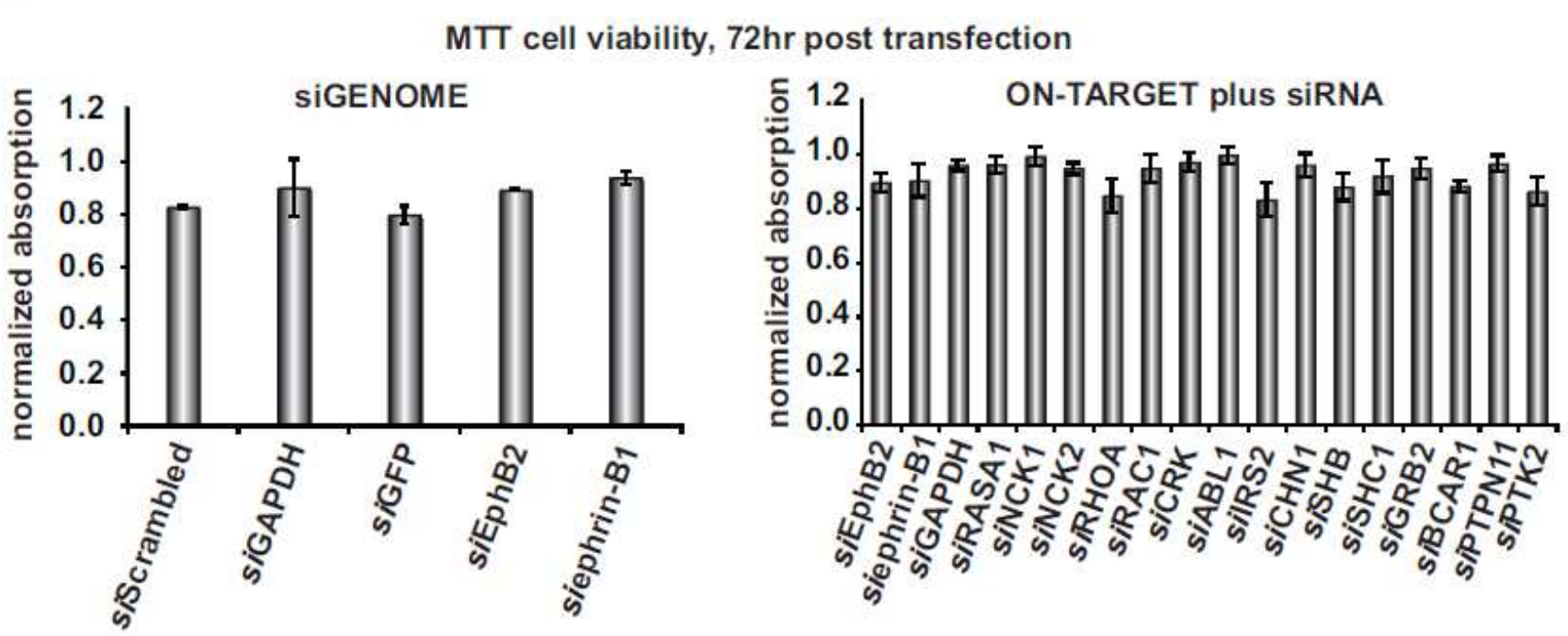




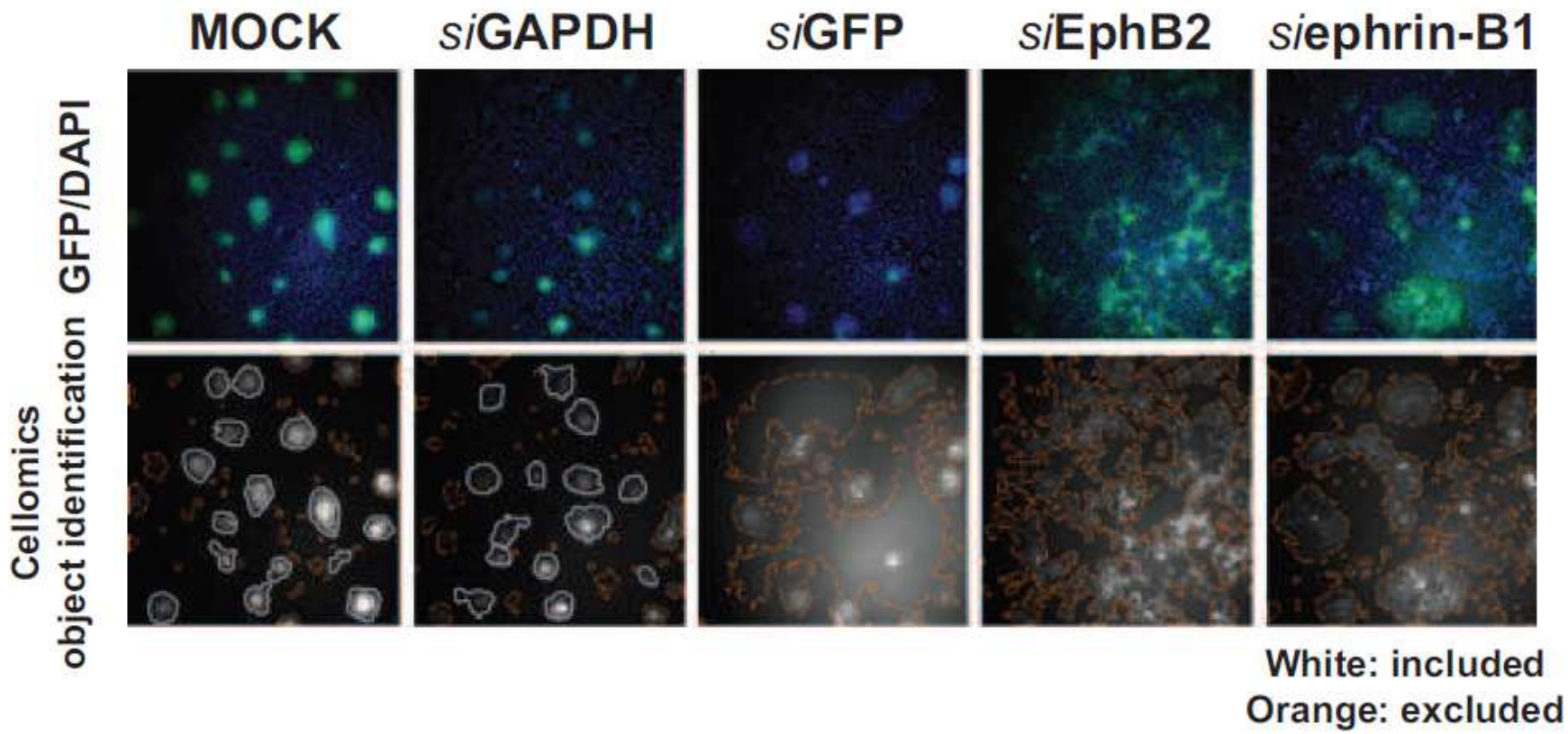
(**A**) siRNAs to EphB2 or ephrin-B1 decreased the number of colonies formed in mixed populations of ephrin-B1+ and EphB2+ cells. Treatment of mixed cell populations with siRNA to GAPDH, non-targeting control siRNA or mock transfection did not affect the number of EphB2+ colonies formed. siRNA to GFP decreased the number of colonies that could be detected.

(**B**) Efficiency of siRNA induced knockdown assessed by immunoblotting. Mixed populations of EphB2+ and ephrin-B1+ cells were transfected with siRNAs to GAPDH, GFP, EphB2, ephrin-B1, and a non-targeting siRNA or were mock-transfected; cell populations were lysed after 72 hours and immunoblotted with antibodies to the indicated proteins.

A custom library of **2172** siRNA pools directed against targets with selected signaling domains, including kinases, phosphatases, and pTyr recognition modules to screen for proteins involved in cell sorting was used.

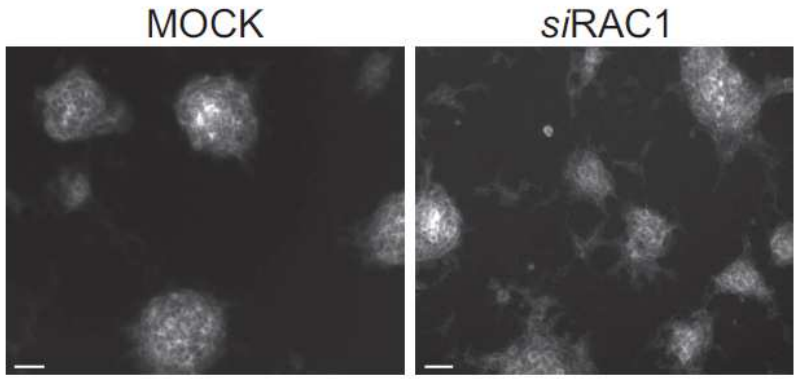


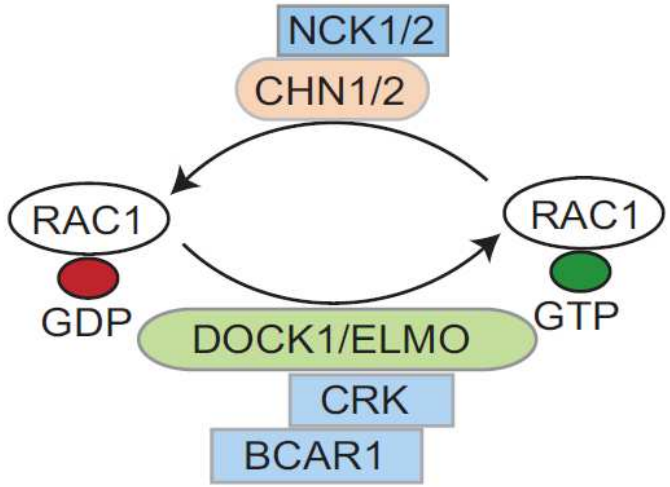
Control or siRNAs affecting cell sorting does not alter cell viability or proliferation. Co-cultured EphB2+-ephrin-B1+ cells were transfected with either siGENOME R siRNA (40nM final concentration, left panel) or ON-TARGET*plus*TM siRNA (100nM final concentration, right panel) and grown for 72 hrs. Cell viability were tested by MTT assay according to manufactures instruction (Roche) and normalized to mock transfected control cells. Neither control siRNA or siRNA identified to perturb cell sorting affected cell proliferation or apoptosis.



Automated analysis of EphB2-GFP cell-sorting by the cellomics platform. Hoechst staining of nuclei was used to identify the focal plane after which nine images were acquired for GFP. A threshold of four standard deviations above background was set for automated detection of GFP positive colonies (outlined in white), while GFP staining below this threshold was excluded (outlined in orange).

siRNAs to RAC1 and known regulators of RAC1 activity interfered with cell sorting





Functional analysis of EphB2- and ephrin-B1-regulated cell sorting through siRNA screening

2172 siRNA

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They analyzed the number of resulting EphB2+ GFP positive colonies by means of automated microscopy and identified **589** siRNAs that significantly affected colony formation (the mock transfection averaged (90 colonies ± 15 within each biological replicate; n = 4 biological replicates)

2172 siRNA

589 affect colony formation

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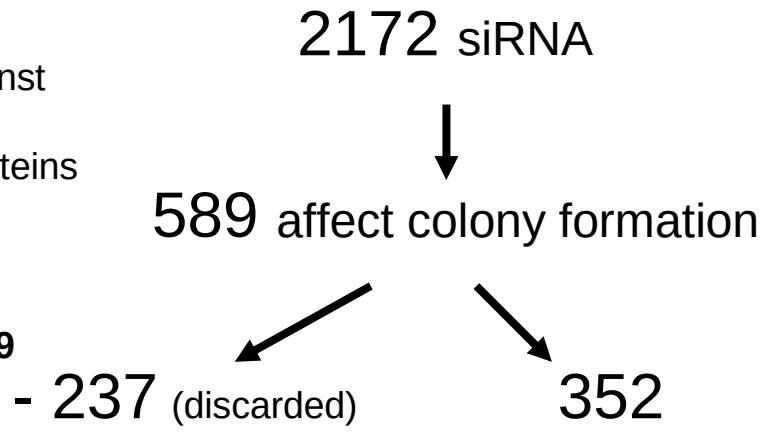
All affected cultures were then visually inspected, and **237** siRNAs that affect growth and adhesion were discarded.



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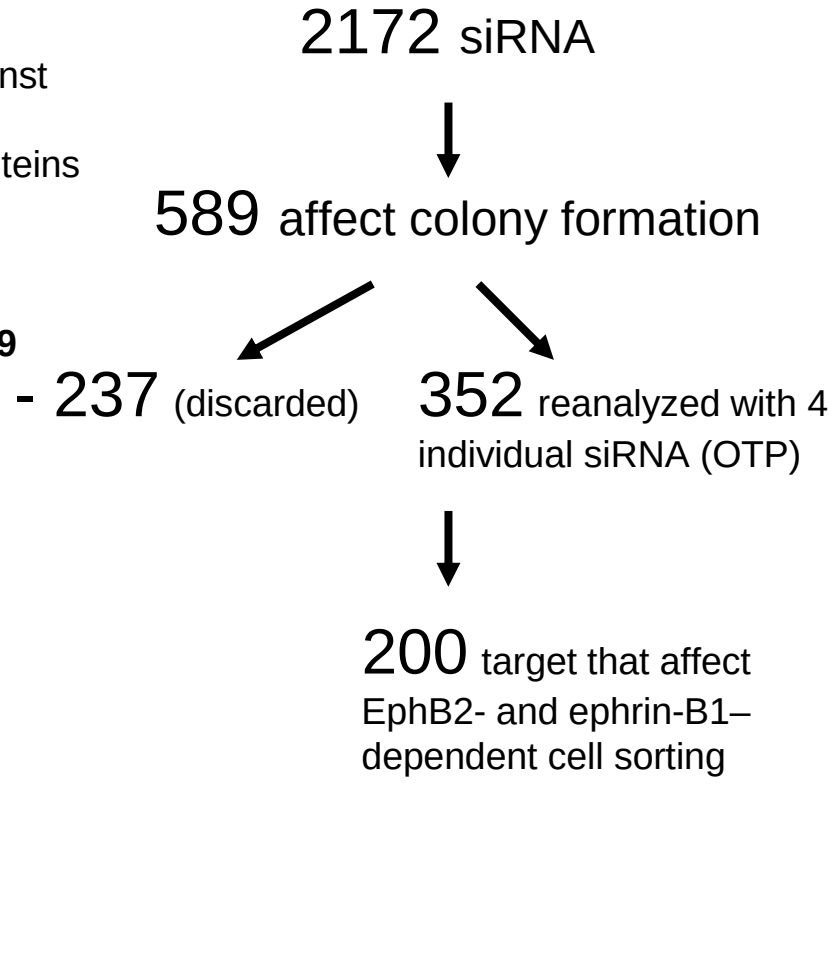
Off-target effects are inherent to siRNA technology so they reanalyzed the remaining **352** targets with **4** individual siRNAs [ON-TARGETplus (OTP)] reagents that were distinct from those used in the initial screen and applied them to each target.
Cocultures were also visually confirmed.



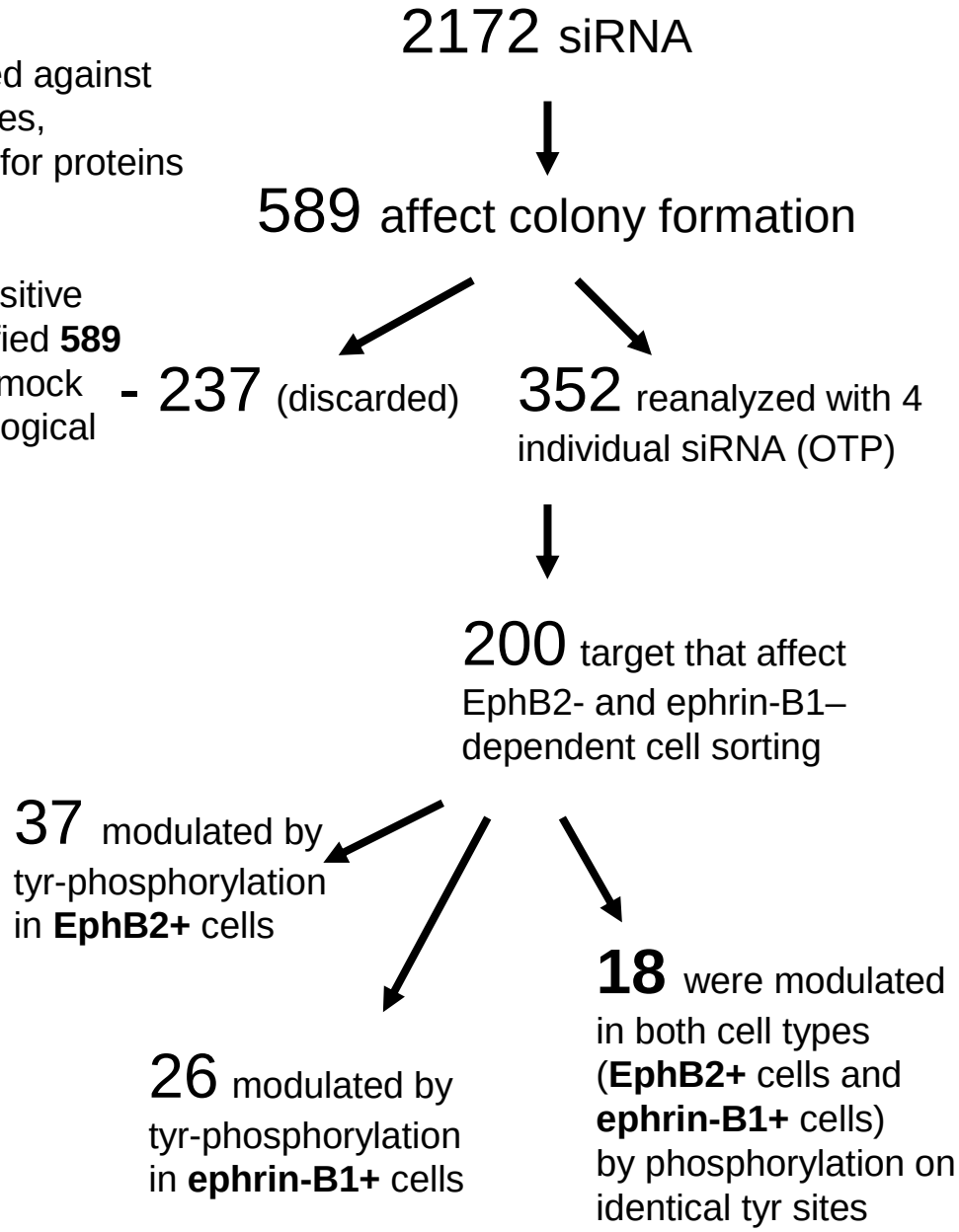
Functional analysis of EphB2- and ephrin-B1-regulated cell sorting through siRNA screening

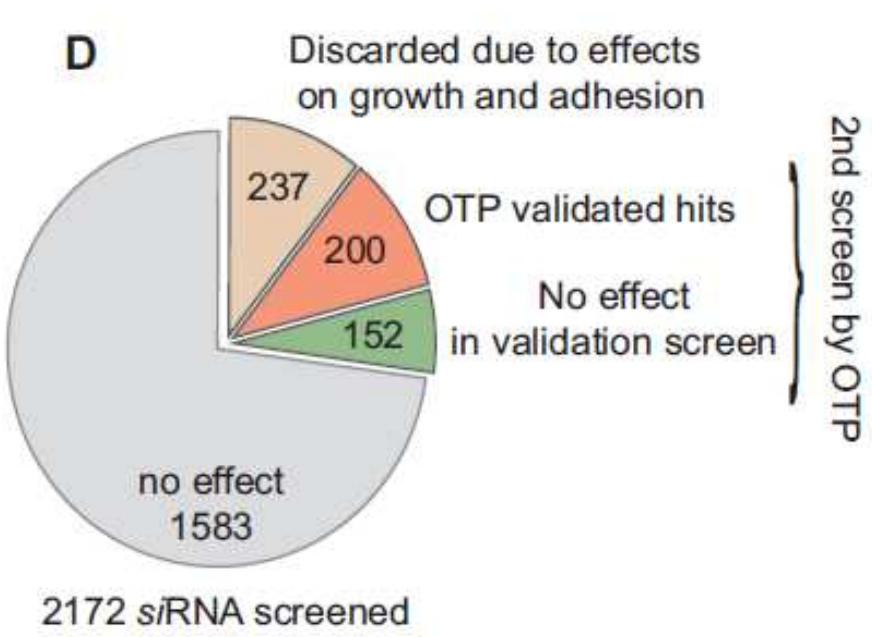
They used a custom library of **2172** siRNA pools directed against targets with selected signaling domains, including kinases, phosphatases, and pTyr recognition modules to screen for proteins involved in cell sorting

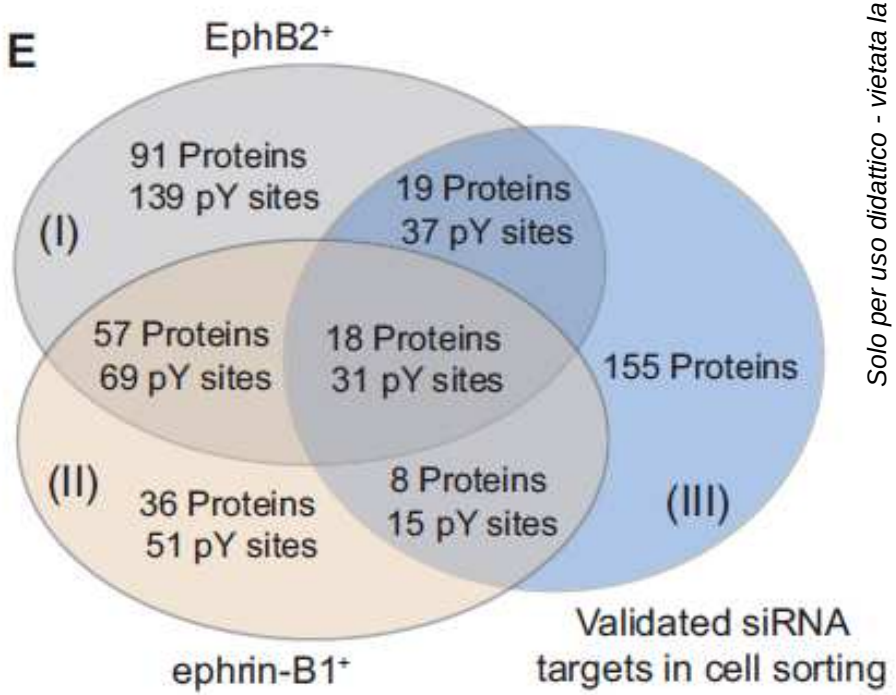
They analyzed the number of resulting EphB2+ GFP positive colonies by means of automated microscopy and identified **589** siRNAs that significantly affected colony formation (the mock transfection averaged (90 colonies ± 15 within each biological replicate; n = 4 biological replicates)

All affected cultures were then visually inspected, and **237** siRNAs that affect growth and adhesion were discarded.
Off-target effects are inherent to siRNA technology so they reanalyzed the remaining **352** targets with **4** individual siRNAs [ON-TARGETplus (OTP)] reagents that were distinct from those used in the initial screen and applied them to each target.
Cocultures were also visually confirmed.

They compared the **200** targets identified in the siRNA screen with those containing pTyr sites modulated by the mixing of EphB2+ and ephrin-B1+ cells.

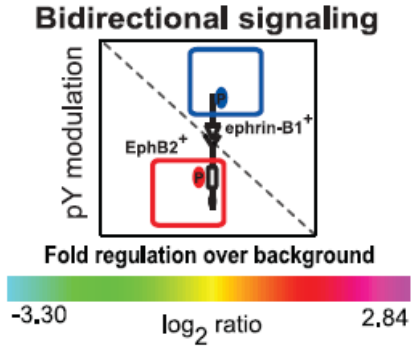






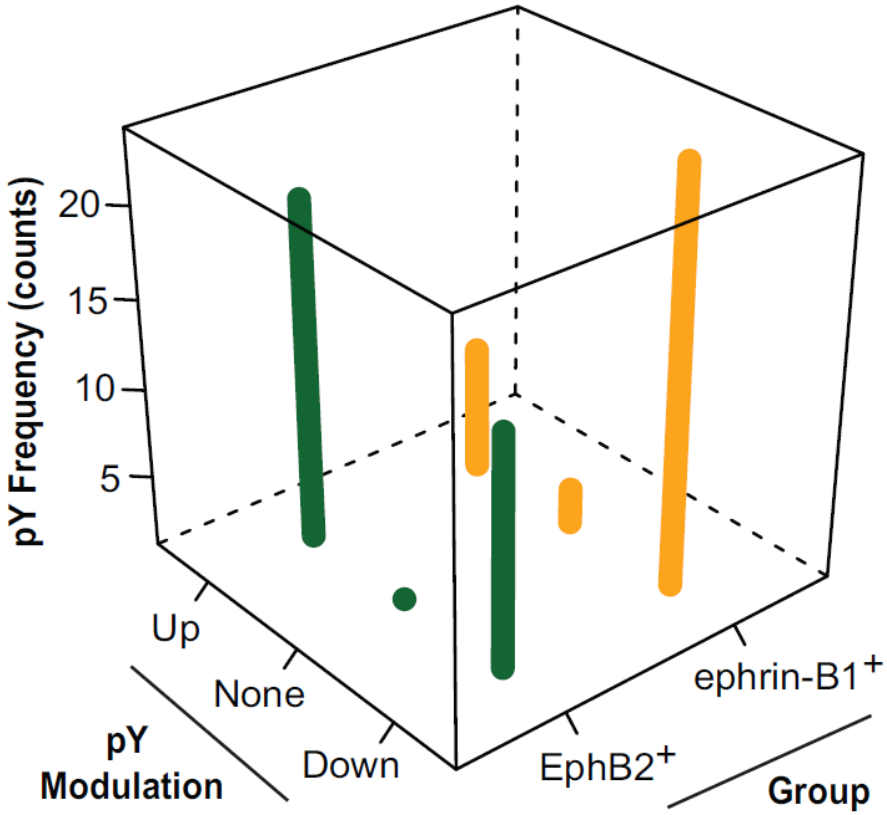
Proteins identified through siRNA screening as functionally important for cell sorting tend to be asymmetrically phosphorylated

#*si*RNA	Genesymbol	pY site	Cellular function	Modulation
	EFNB1	Y317	ephrin ligand	
	IRS2	Y598/Y675/Y742	adaptor	
	PTK2	Y576/Y577	focal adhesion kinase	
	PXN	Y118	adhesion molecule	
	WASL	Y256	actin dynamics	
	APC	Y2645	tumor suppressor/polarity	
	INPPL1	Y886/Y1135	lipid phosphatase	
	PAG1	Y277/Y359	Src regulation	
	STAT3	Y708	transcription factor	
	ABL1	Y412	non-receptor tyrosine kinase	
	DLG3	Y705	polarity	
	EPHB2	Y584/Y602/Y780	Eph receptor tyrosine kinase	
	PARD3	Y388/Y719/Y1080/Y1127	polarity	
	PKP4	Y478	cell-cell adhesion	
	PTPN11	Y62	protein phosphatase	
	SHB	Y200/Y332/Y354/Y422	adaptor molecule	
	SHC1	Y428	adaptor molecule	
	YES1	Y426	non-receptor tyrosine kinase	



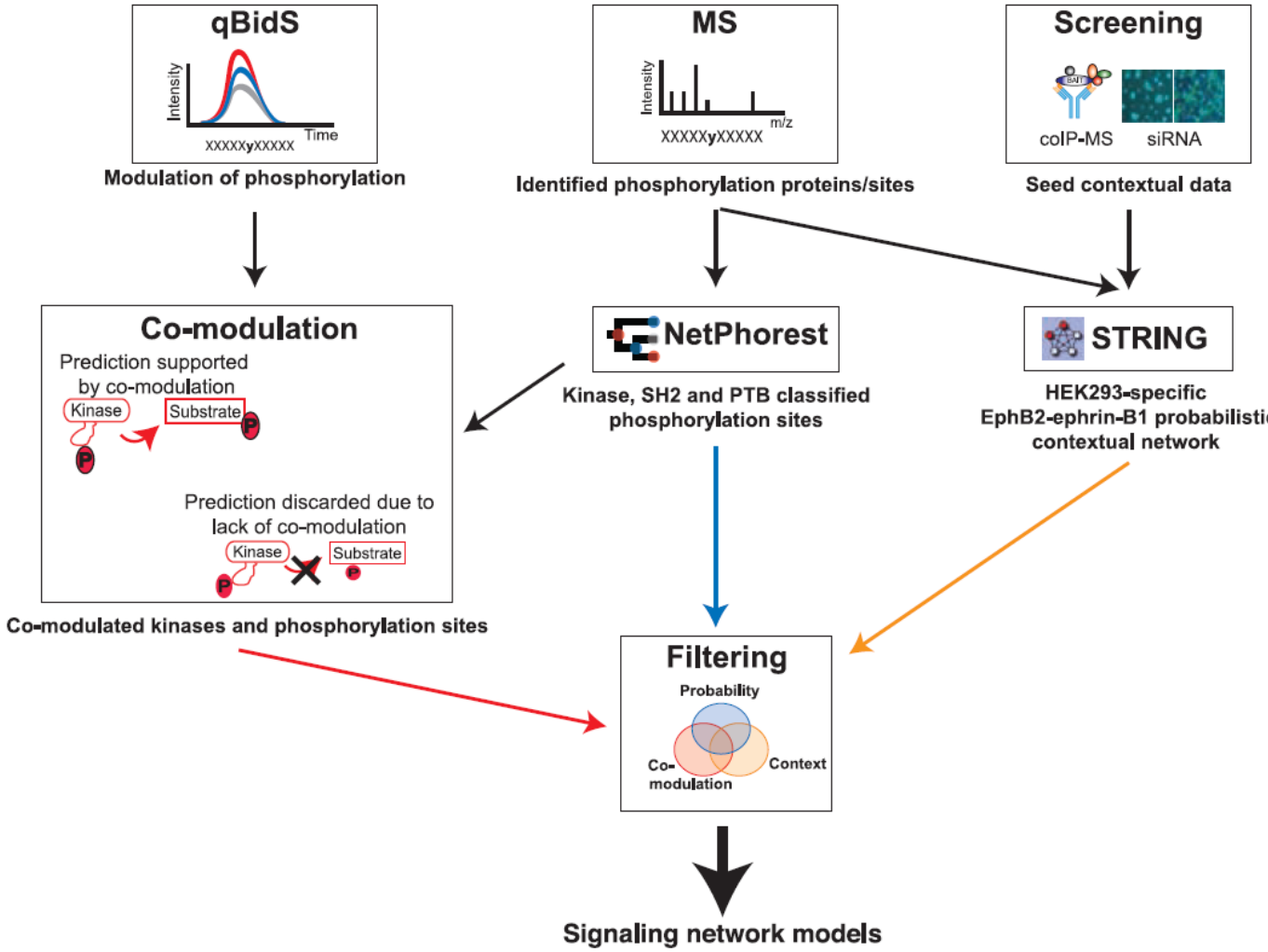
The modulation of pTyr sites residing in proteins important for cell sorting that were identified in both EphB2+ and ephrin-B1+ cells was compared.The number of OTP duplexes recapitulating a loss of cell sorting is shown to the left in red, followed by the gene name, the identified pTyr sites, and the cellular function of the protein. The cell-specific modulation of each pTyr site is shown by color code.

Proteins identified as functionally important for cell sorting by siRNA screening tend to be asymmetrically phosphorylated.



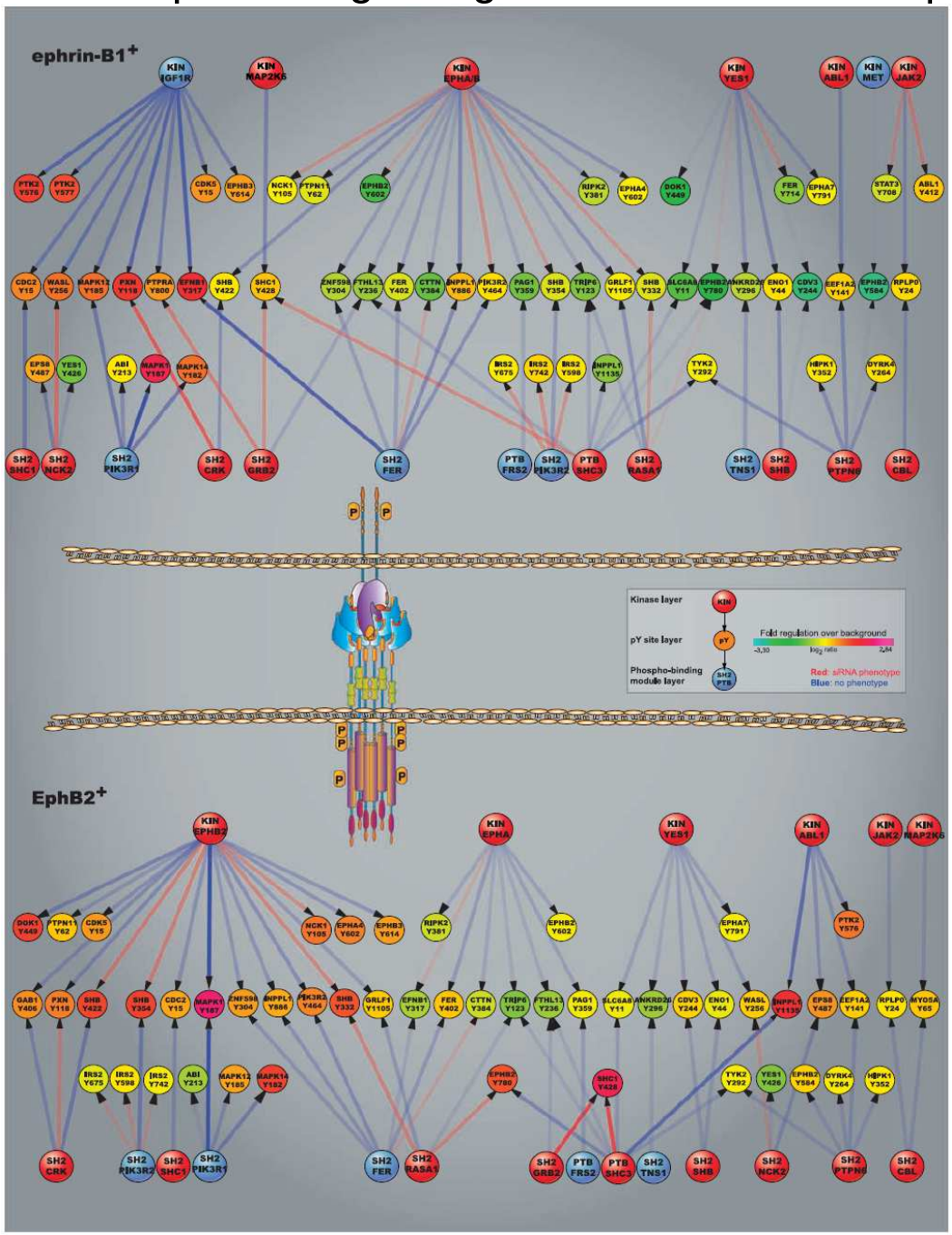
The modulation of pTyr sites residing in proteins important for cell sorting that were identified in both EphB2+ and ephrin- B1+ cells was compared. The specific modulation of each pTyr site (up, none, down) is depicted for either EphB2+ or ephrin-B1+ cells as a 3D boxplot, where the height of the bars indicates the number of pTyr sites. Modulation of pTyr sites of these proteins is significantly different between EphB2+ and ephrin-B1+ cells (P = 0.008; Fishers exact test).

Computational data integration and network modeling

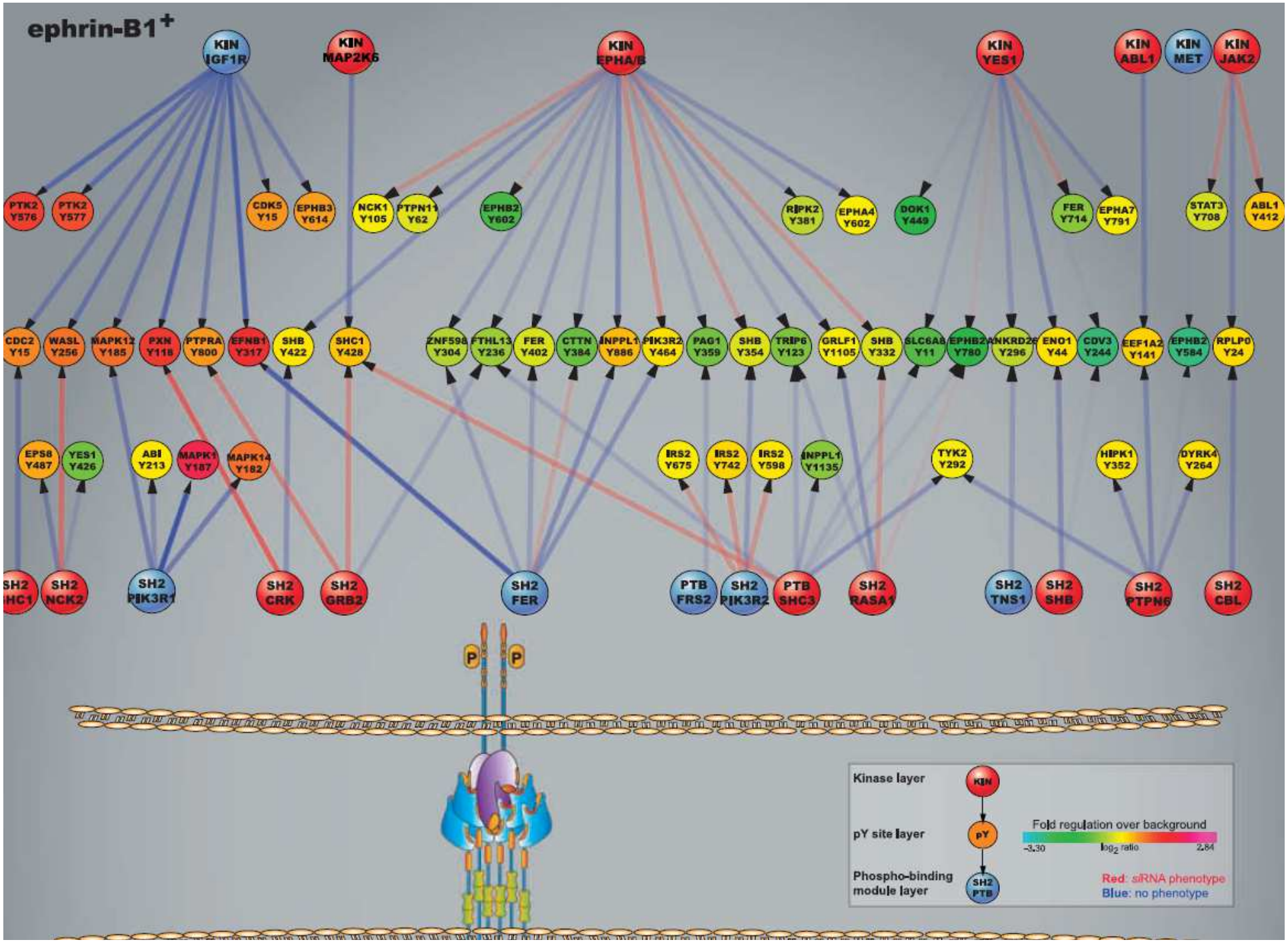


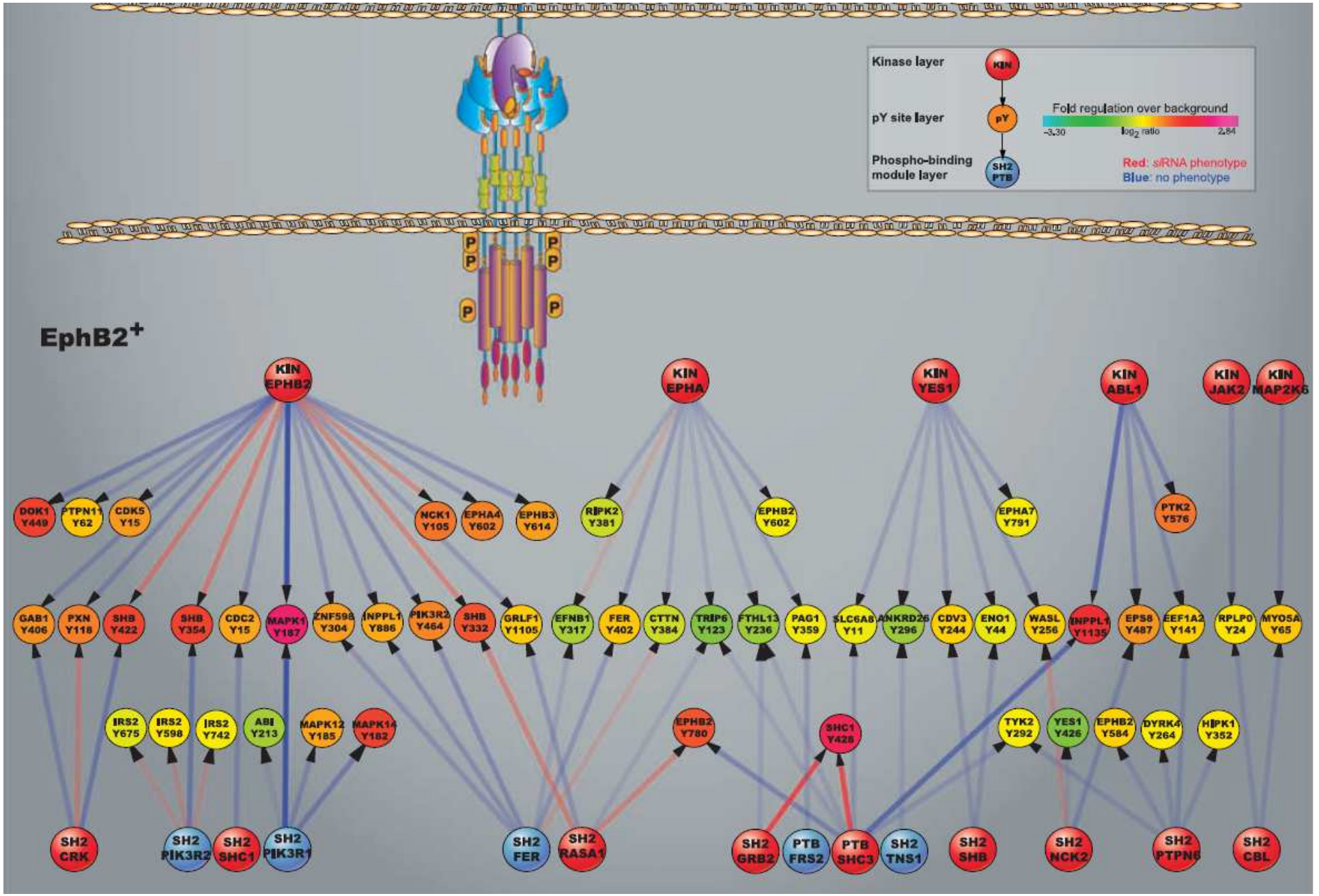
All observed phosphorylation sites were first processed by the NetPhorest algorithm so as to predict kinase-substrate relationships and SH2 and PTB domain interactions. These predictions were subjected to several subsequent filtering schemes:
1- tyrosine phosphorylation sites and the activation loop phosphorylation of their predicted kinases are required to be comodulated (**red path**).
2- predictions were filtered on the basis of the probability score from Netphorest (**blue path**).
3- proteins identified through qBidS or siRNA screening or by coprecipitation were used as input to the STRING resource to generate a systems-specific protein-protein interaction network, permitting contextual filtering (**orange path**) similar to the NetworKIN algorithm.

Cell-specific signaling network models in EphB2- and ephrin-B1- expressing cells



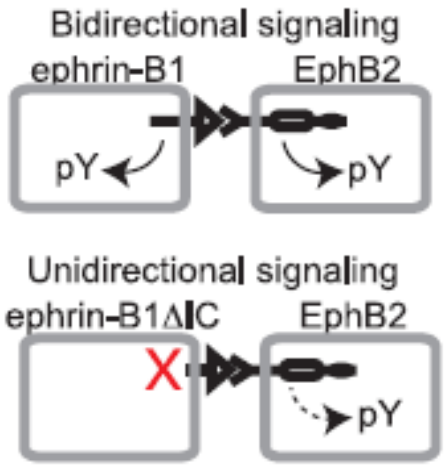
Cell-specific information flow in EphB2+ and ephrin-B1+ cells is shown in the form of modular protein networks with kinases (KIN), pTyr sites, and phospho-binding modules (SH2 and PTB) organized in layers. The color of the pTyr sites represents their cell-specific modulation, according to the indicated color code, whereas the color of each kinase and phospho-binding node represents whether through siRNA it was identified as a cell-sorting target (red) or not (blue). The arrows represent the strength of the information flow within the network and the intensity is proportional to the modulation of the pTyr site involved in the specific edge. Arrows color-coded red represent an interaction supported by the contextual protein association network. Different kinases between the EphB2+ and ephrin-B1+ cells appear to be responsible for the change in information flow, and it appears that SH2- and PTB-binding proteins are used to a larger degree in EphB2+ cells than in ephrin-B1+ cells.





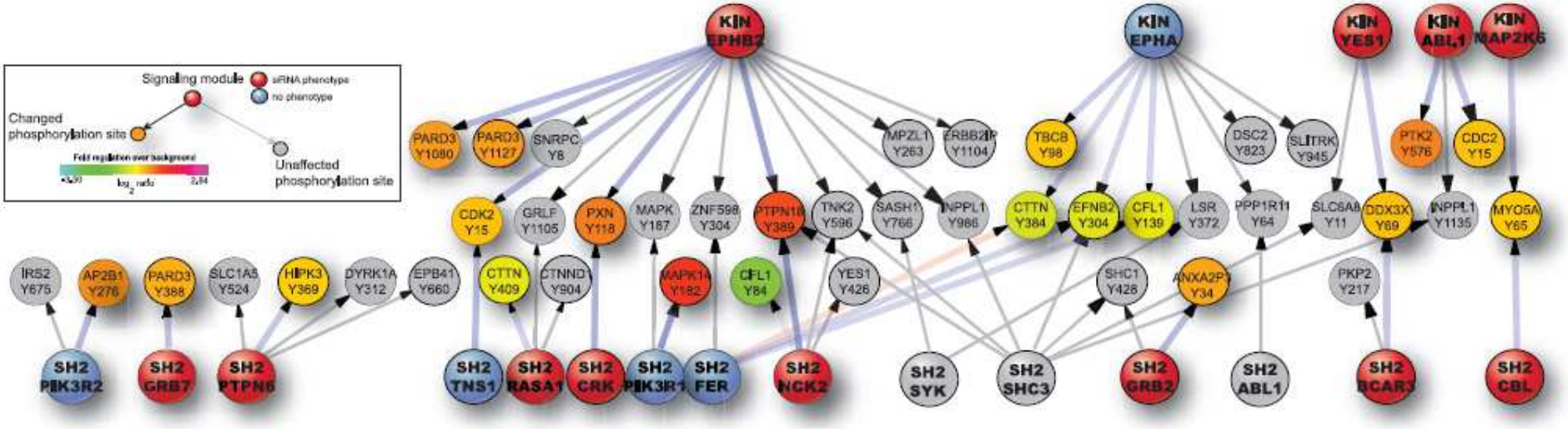
Changes in network structure and utilization caused by ephrin mutations

pTyr signaling in EphB2+ cells mixed with ephrin-B1+ cells is compared by means of qBidS with the pTyr dynamics in EphB2+ cells mixed with ephrin-B1ΔIC+ cells.

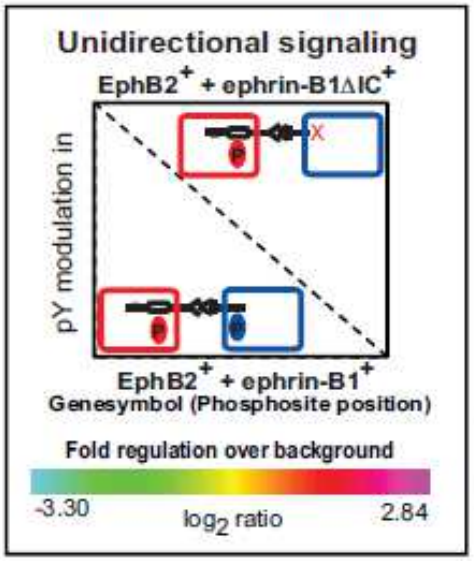


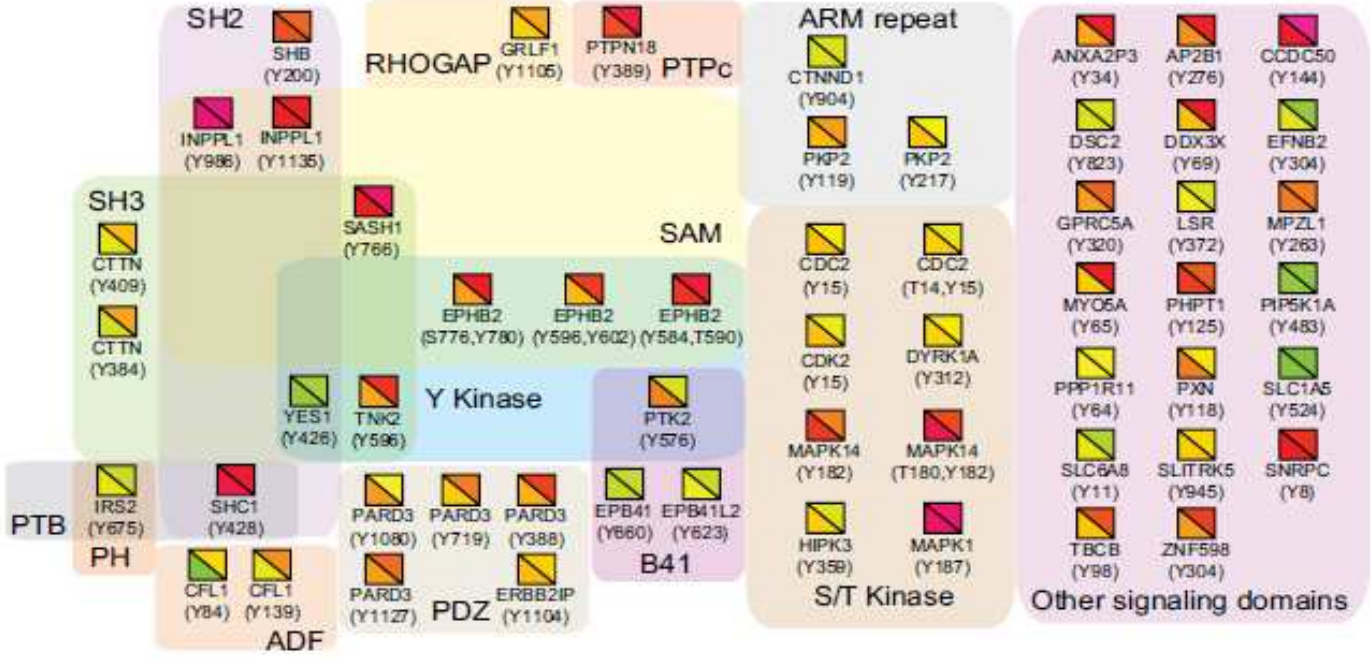
A differential network model of signaling flow in EphB2+ cells mixed with ephrin-B1+ or ephrin-B1 ΔIC+ cells. Information flow in EphB2+ cells is shown as a modular network with kinases (KIN), pTyr sites, and phospho-binding modules (SH2 and PTB) shown for each pTyr site. The network is shown for EphB2+ cells mixed with ephrin-B1+ cells, and differential utilization of the network in EphB2+ cells mixed with ephrin-B1 ΔIC+ cells is highlighted with color-coding, whereas similar network structures and utilization is represented in gray.

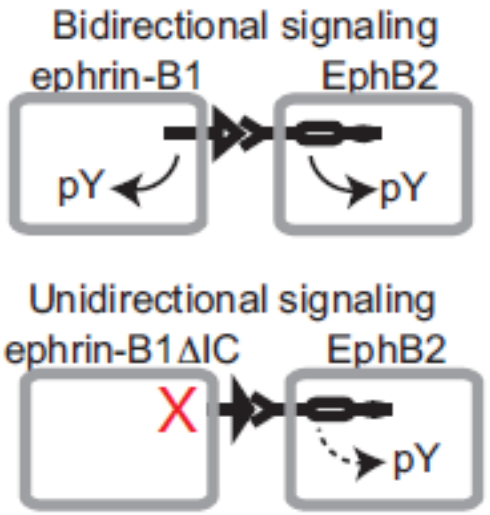
→ The network model highlights that ephrin-B1+–initiated signaling in EphB2+ cells utilizes different network paths in EphB2+ cells, depending on the presence or absence of the intracellular region of ephrin-B1.



Changes in pTyr modulation by ephrin-B1 intracellular region.







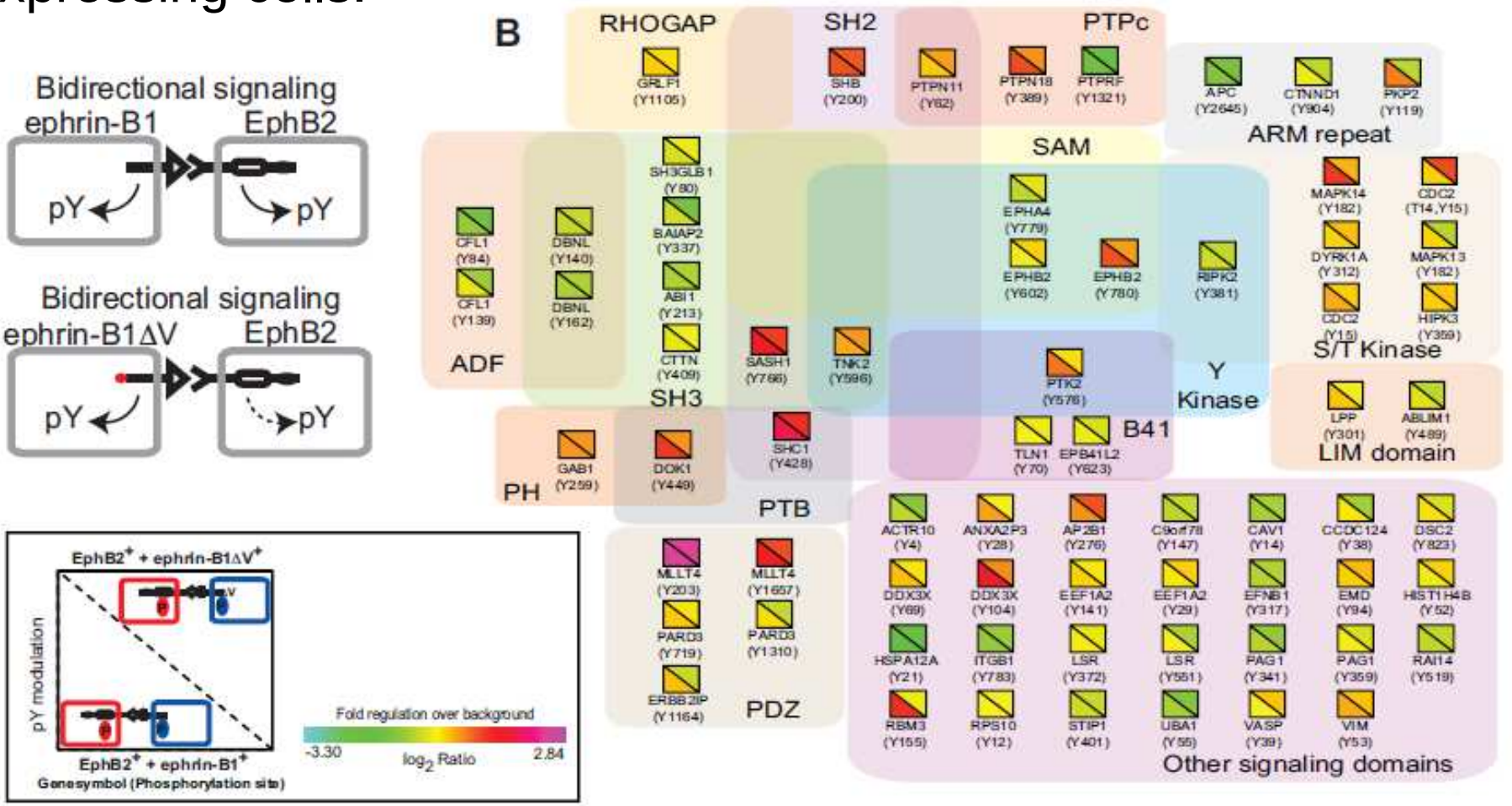
Outline of the experimental design

Phosphotyrosine signaling in EphB2+ cells mixed with ephrin-B1+ cells is compared to the pTyr dynamics in EphB2+ cells mixed with ephrin-B1IΔC+ cells, using qBidS.
Contact-initiated pTyr signaling is compared by mixing of EphB2+ with ephrin-B1+ or ephrin-B1IΔC+ cells. Specific modulation of individual pTyr peptides from EphB2+ cells is shown by boxes, with the color-coded triangles representing their modulation in EphB2+ cells mixed with ephrin-B1+ (lower triangle) or ephrin-B1IΔC+ cells (upper triangle).

These data suggest that the intracellular region of ephrin-B1 affects signal processing not only within ephrin-B1+ cells but ALSO in neighboring cells that express EphB2, revealing a non–cell-autonomous mode of regulation in EphR-ephrin signaling.

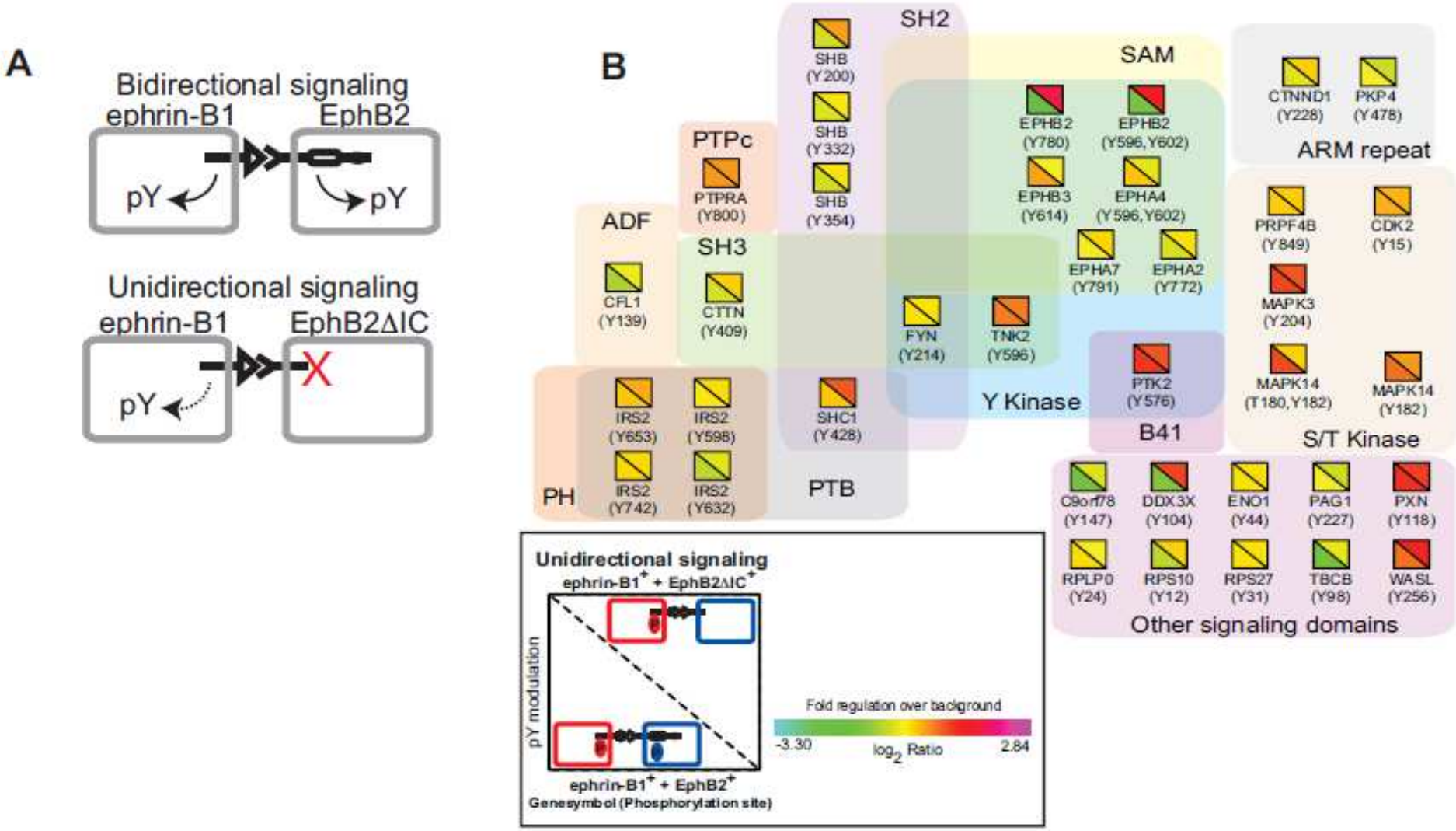
The same applies to the C-terminal valine of ephrin-B1 required for PDZ domain–binding of ephrin-B1, which also influenced signaling in EphB2+ cells

Phosphotyrosine signaling in EphB2+ cells is modified by the presence or absence of the PDZ binding motif in ephrin-B1 expressing cells.



pTyr signaling is differently modulated in EphB2+ cells depending on whether they are mixed with ephrin-B1ΔIC+ or ephrin-B1ΔV+ cells, suggesting that additional components besides PDZ domain–containing proteins may be required to orchestrate the signaling effects of ephrin-B1 on EphB2.

Phosphotyrosine signaling in ephrin-B1+ cells is modified by the intracellular region of EphB2.



Signaling response to soluble ephrin ligand is different from cell-contact initiated signaling

- Soluble fusion proteins containing the extracellular regions of ephrins are commonly used to activate EphR signaling, so they compared the responses of EphB2+ cells to stimulation either by an artificial clustered ectodomain ephrin-B1-Fc fusion or by mixing with ephrin-B1+ cells.

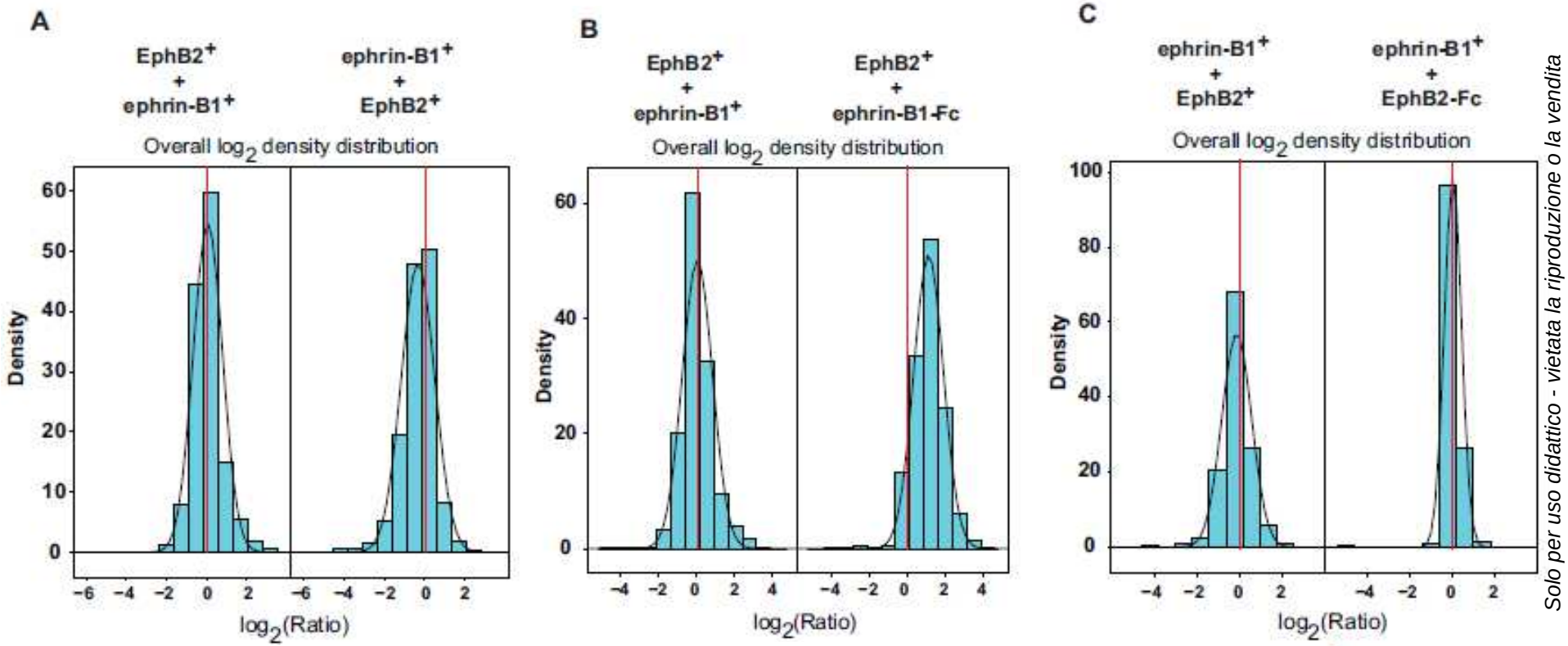
- The two types of stimulation resulted in **significantly different pTyr modulation** of identical sites.

- Soluble ephrin-B1 ligand led to increased phosphorylation in EphB2+ cells when compared to the same pTyr sites in EphB2+ cells induced by mixing with ephrin-B1+ cells.

- However, whereas increased phosphorylation of PXN (Y118) and PTK2 (Y576, Y577) was observed in EphB2+ cells during contact-initiated signaling, the phosphorylation of these sites was decreased when soluble ephrin-B1 was used for stimulation.

- This suggests that the observed differences were not only due to a general increased activity of EphB2 when artificial soluble ligand was used.

Modulation of identical phosphorylated tyrosine residues in EphB2+ or ephrin-B1+ cells is dependent on the nature of the stimulus



The diagrams represents the number of pTyr sites (y-axis/density) compared to the log2 ratio of their modulation (x-axis). Identical pTyr sites identified using the qBidS approach are represented according to their cell-specific modulation.

(**A**) Distribution of pTyr sites in EphB2+ cells mixed with ephrin-B1+ cells; the panel on the right shows the distribution of the same pTyr sites in ephrin-B1+ cells mixed with EphB2+ cells.

(**B**) Distribution of phosphorylated tyrosine residues modulated in EphB2+ cells stimulated either by ephrin-B1+ cells (left panel) or by clustered artificial soluble ephrin-B1-Fc ligand (right panel) are represented according to their stimulus-dependent modulation.

(**C**) Distribution of phosphorylated tyrosine residues modulated in ephrin-B1+ cells stimulated either by EphB2+ cells (left panel) or clustered artificial soluble EphB2-Fc ligand (right panel) are represented according to their stimulus-dependent modulation.

Conclusions

- information processing between EphB2+ and ephrin-B1+ cells is asymmetric

- there are both structural and dynamic differences in the networks mediating the molecular information flow induced by bidirectional signaling, as compared with unidirectional signaling induced either by C-terminally truncated cell surface ligands, or soluble proteins

- The systems-level approaches described here are of general utility in studying the effects of cell-cell interactions and network utilization in both normal and pathologic processes.