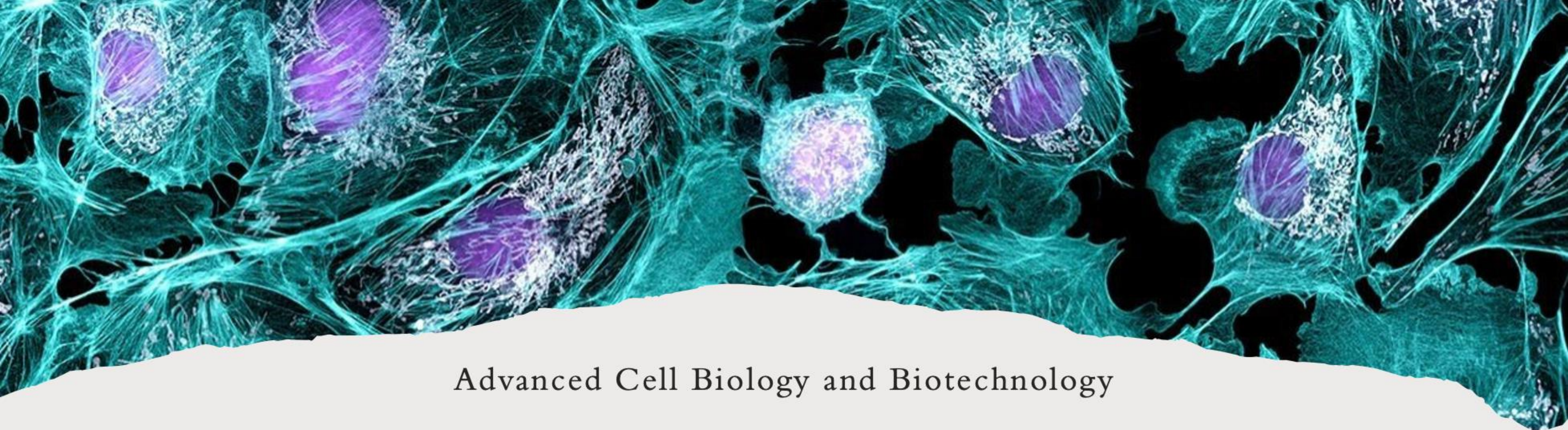


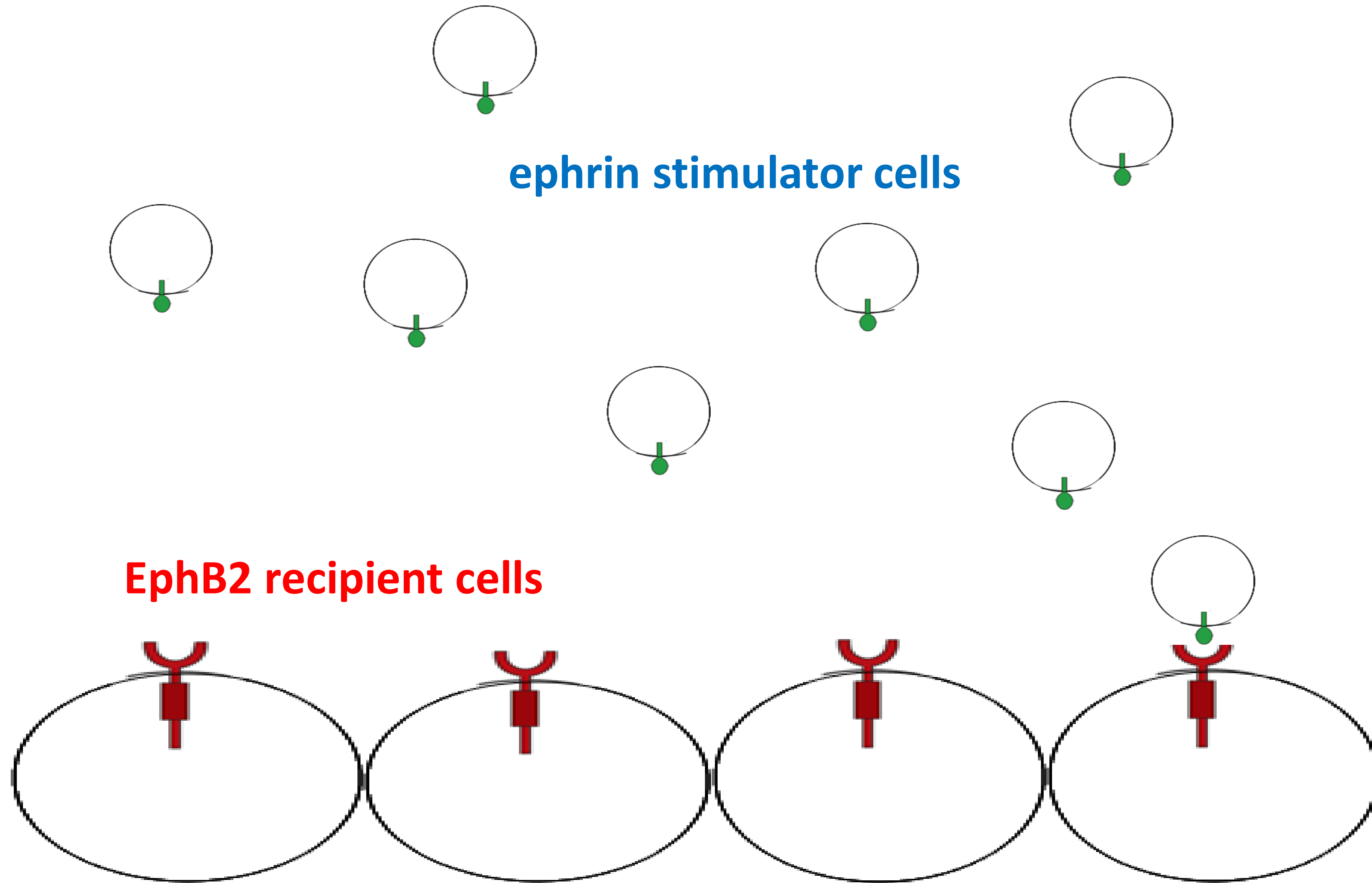
Advanced Cell Biology and Biotechnology

ACBB 2021/22

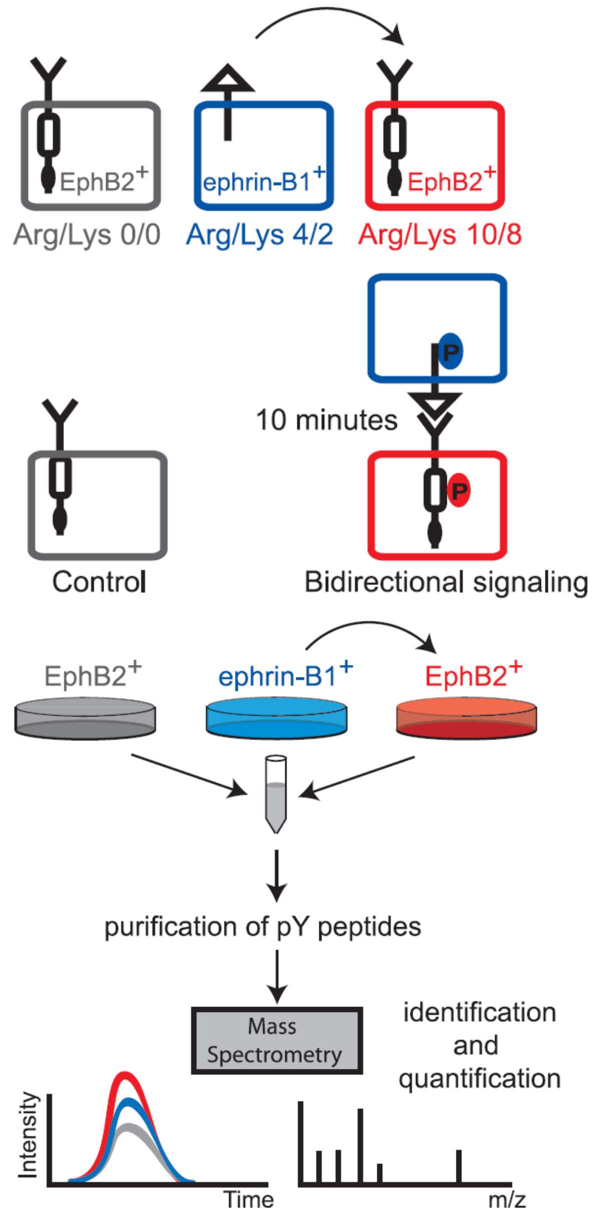
…the lecture of December 17th is about to begin…

- methods to study cell-cell communication:
  - chemotaxis & chemokinesis
  - attraction & repulsion
  - substrate preference
  - bidirectional signalling

Stimulation of adherent recipient cells (expressing transmembrane receptor) with cells in suspension (expressing transmembrane ligand)



Quantitative analysis of Bidirectional Signaling (qBidS)



$EphB2^+$ 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**

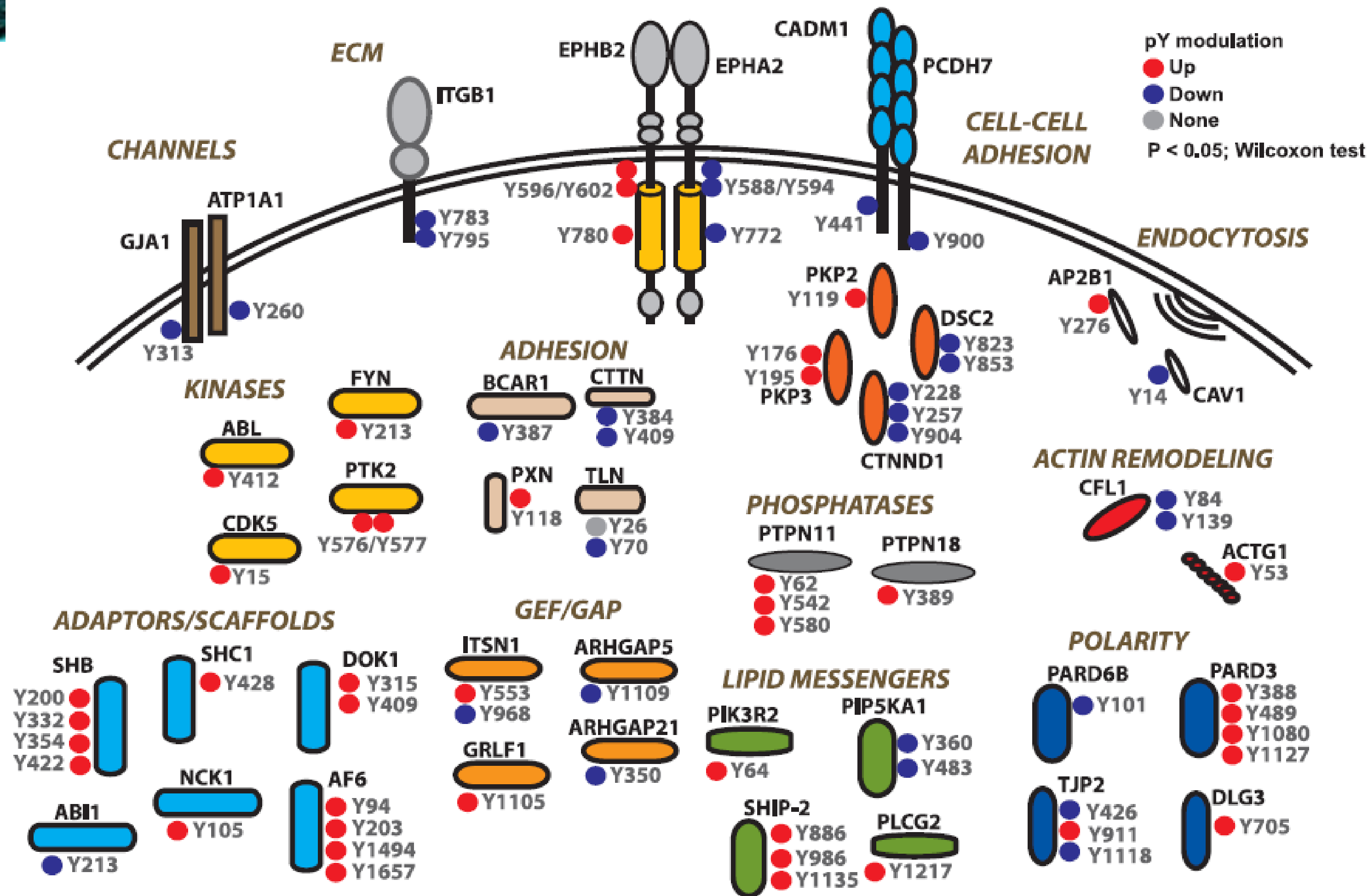
Bidirectional signaling was initiated, mixing **heavy labeled EphB2+ cells** and **medium labeled ephrin-B1+ cells**

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.

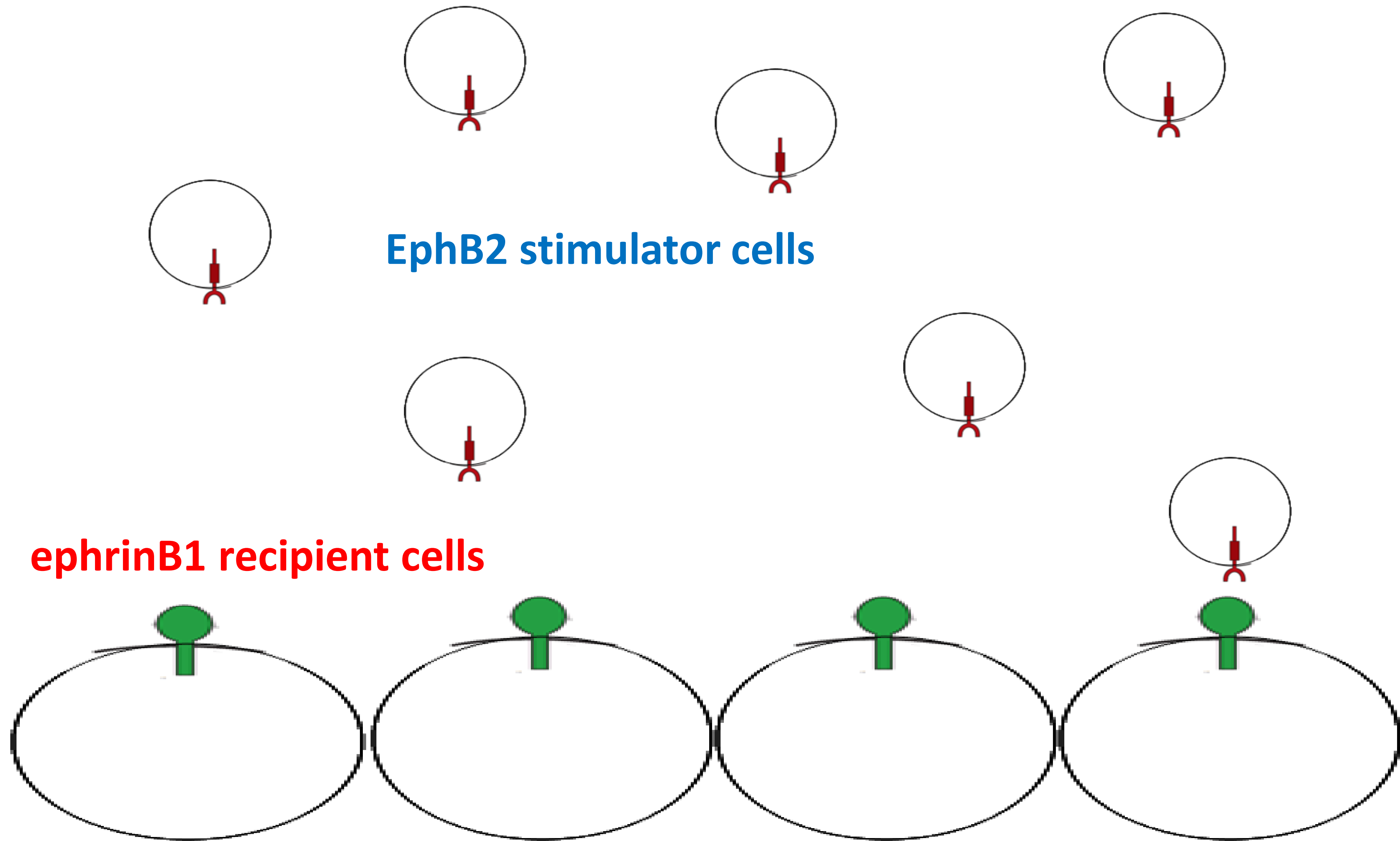
Cell lysates were digested with trypsin, and tyrosine-phosphorylated peptides were isolated and analyzed with liquid chromatography-mass spectrometry (LC-MS).

Overview of selected proteins in EphB2+ cells modulated by tyrosine phosphorylation after mixing with ephrin-B1+ cells

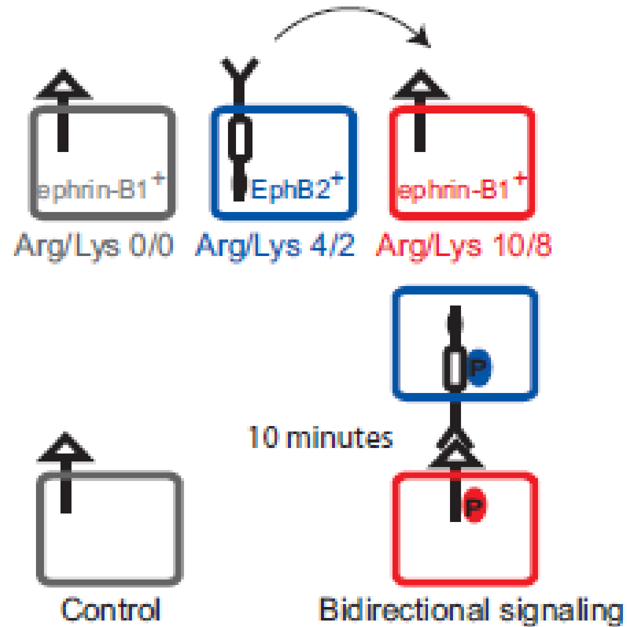


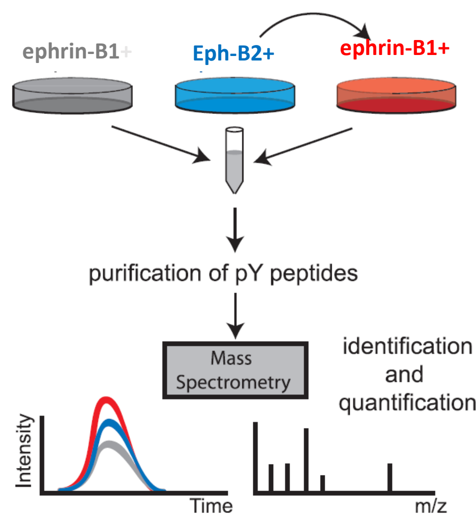
The modulation of pTyr sites is indicated as **significantly increased (red)**, **not modulated (gray)**, or **decreased (blue).** Molecules that are involved in a wide variety of cellular functions are modulated by tyrosine phosphorylation, indicating that phospho-regulation of numerous cellular processes may be important for cell sorting.

Stimulation of adherent recipient cells (expressing transmembrane ligand) with cells in suspension (expressing transmembrane receptor)



Quantitative analysis of Bidirectional Signaling (qBidS)





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**

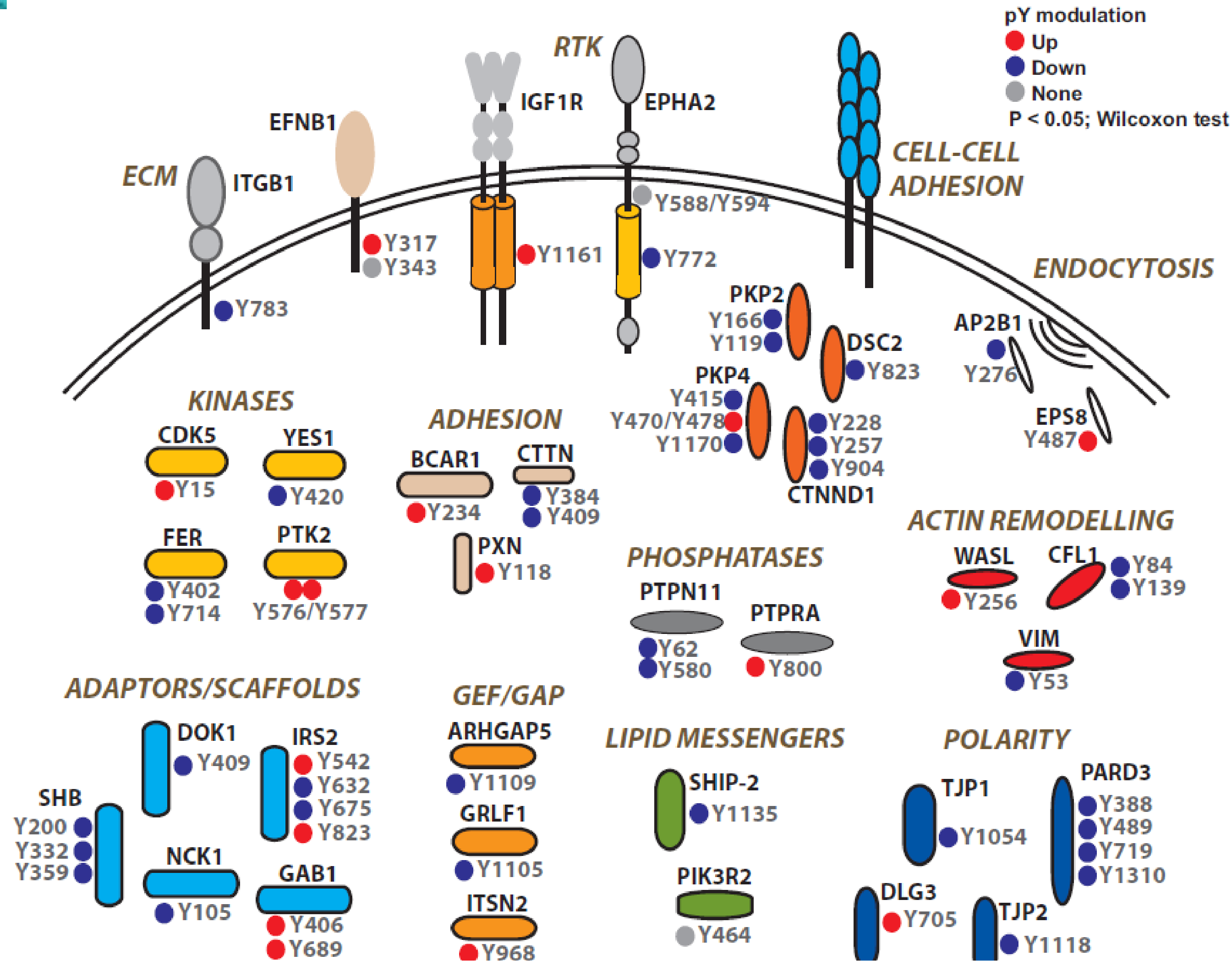
Bidirectional signaling was initiated, mixing
heavy labeled ephrin-B1+ cells and
medium labeled Eph-B2+ cells;

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

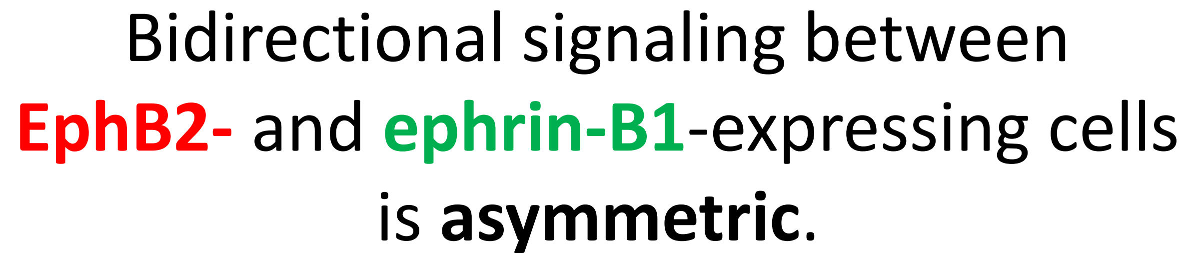
Mixed populations of cells were harvested after 10 min and combined with nonstimulated control cells.

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

Overview of selected proteins in ephrin-B1+ cells modulated by tyrosine phosphorylation after 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 were modulated in ephrin-B1+ cells following contact with EphB2+ cells.



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

Comparison of 100 common tyrosine phosphorylation sites

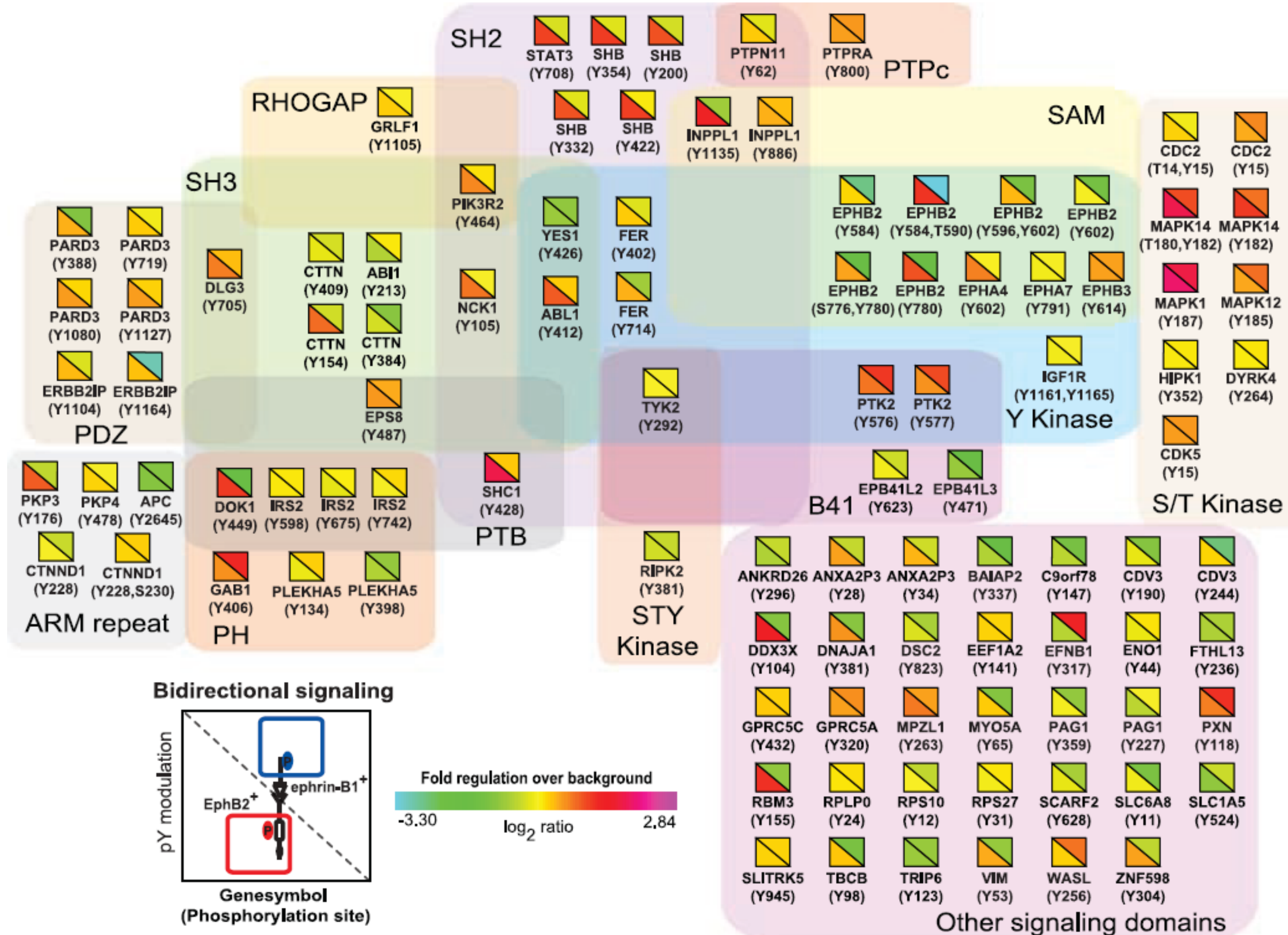
To quantitatively assess cell-specific signaling events, they identified **100** identical peptides that were tyrosine-phosphorylated in both **EphB2$^+$** and **ephrin-B1$^+$** cells and compared their dynamic profiles between the two cell types.

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.

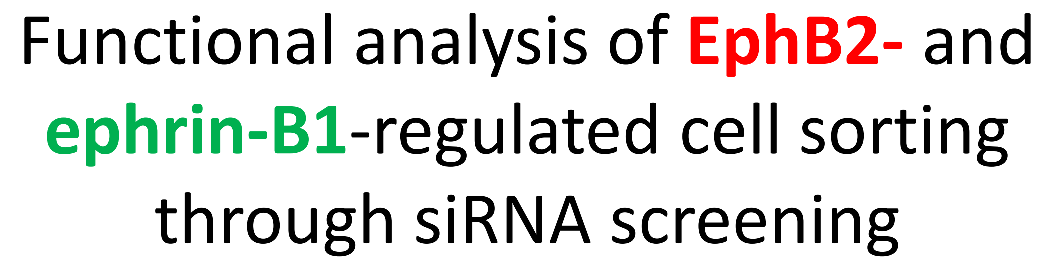
Of these sites, **71%** displayed **asymmetric modulation** between the cell lines.

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

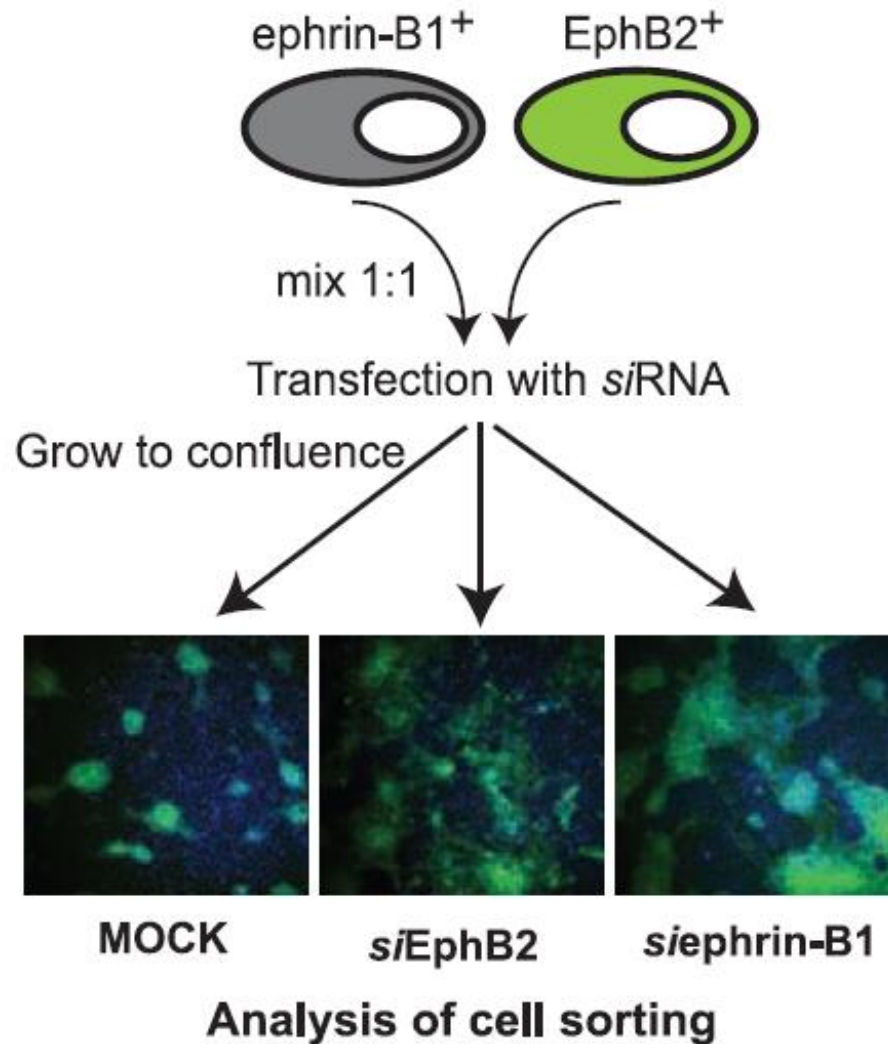
Comparison of contact-initiated pTyr signaling between ephrin-B1$^+$ and EphB2$^+$ cells



100 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 (**71** pTyr displayed asymmetric modulation).

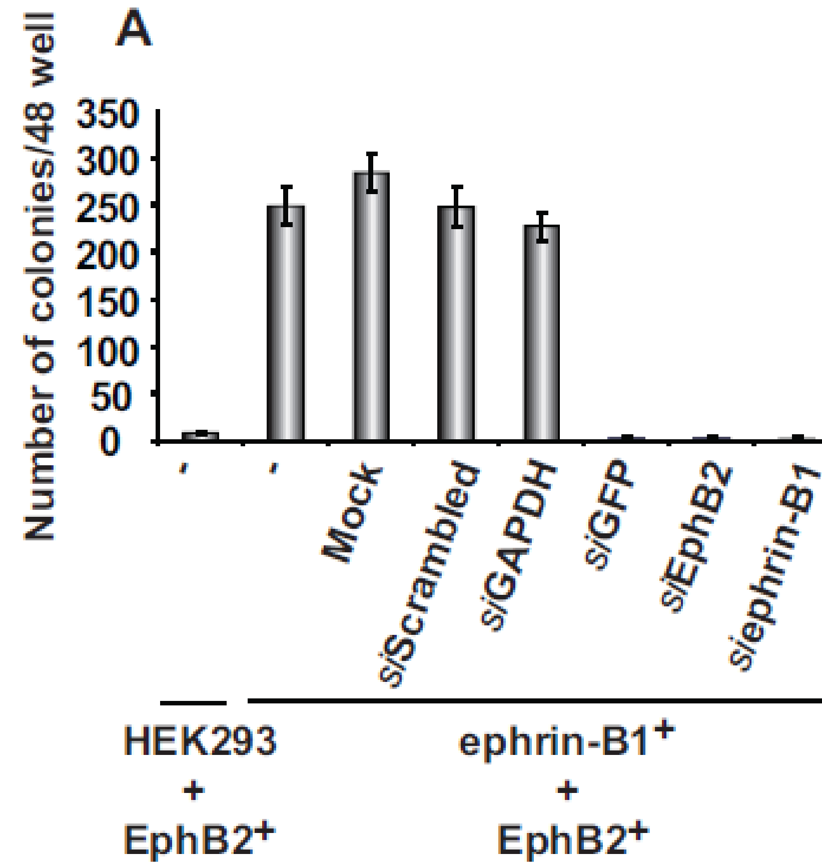


Functional analysis of EphB2- and ephrin-B1-regulated cell sorting through siRNA screening



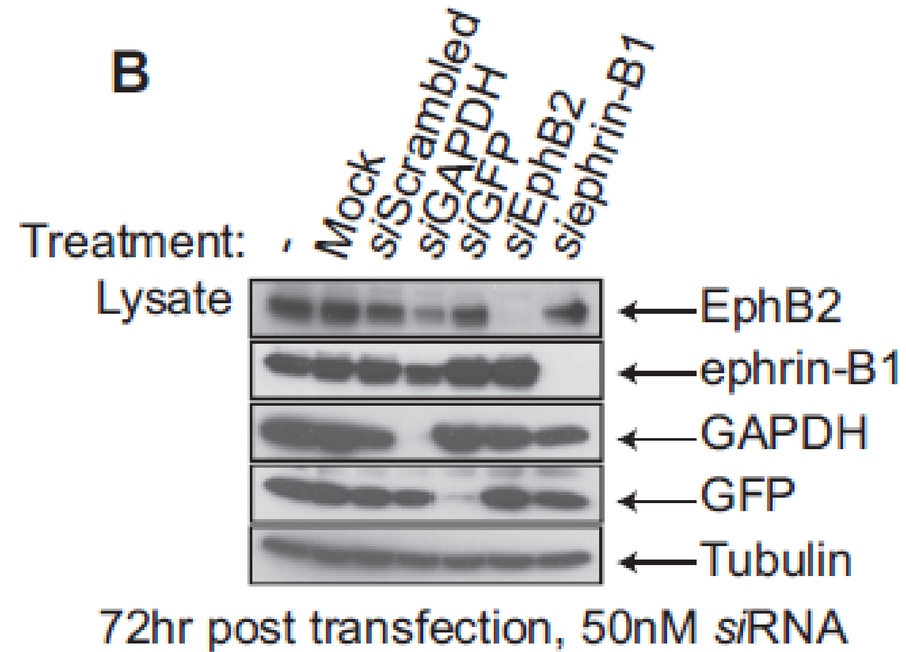
EphB2⁺ cells, which coexpress myristoylated GFP, were mixed with **ephrin-B1⁺** cells, transfected with siRNA pools, and grown to 100% density. The number of GFP-positive **EphB2⁺** colonies was used to determine the effect of siRNAs on cell sorting. Disruption of EphB2 or ephrin-B1 expression by means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

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



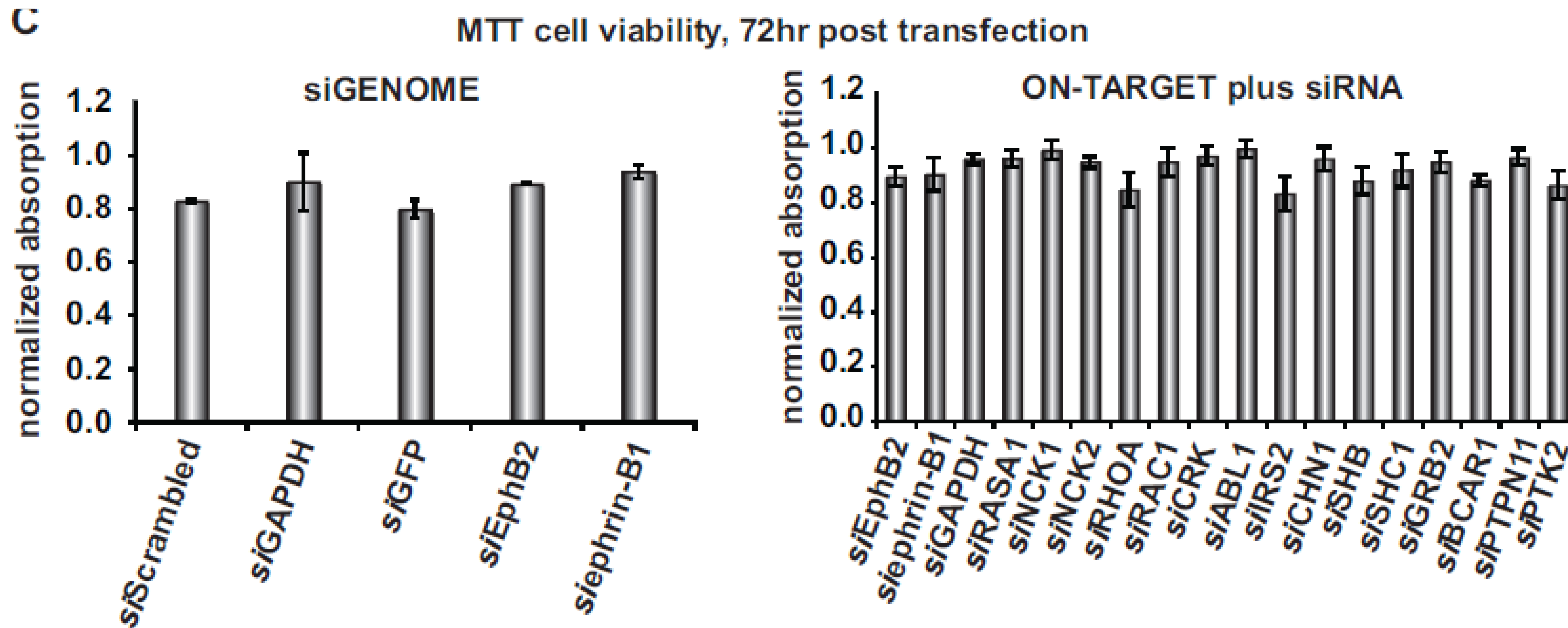
(**A**) siRNAs to EphB2 or ephrin-B1 decreased the number of colonies formed in mixed populations of ephrin-B1+ and EphB2+ cells. Treatment of mixed cell populations with siRNA to GAPDH, non-targeting control siRNA or mock transfection did not affect the number of EphB2+ colonies formed. siRNA to GFP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

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



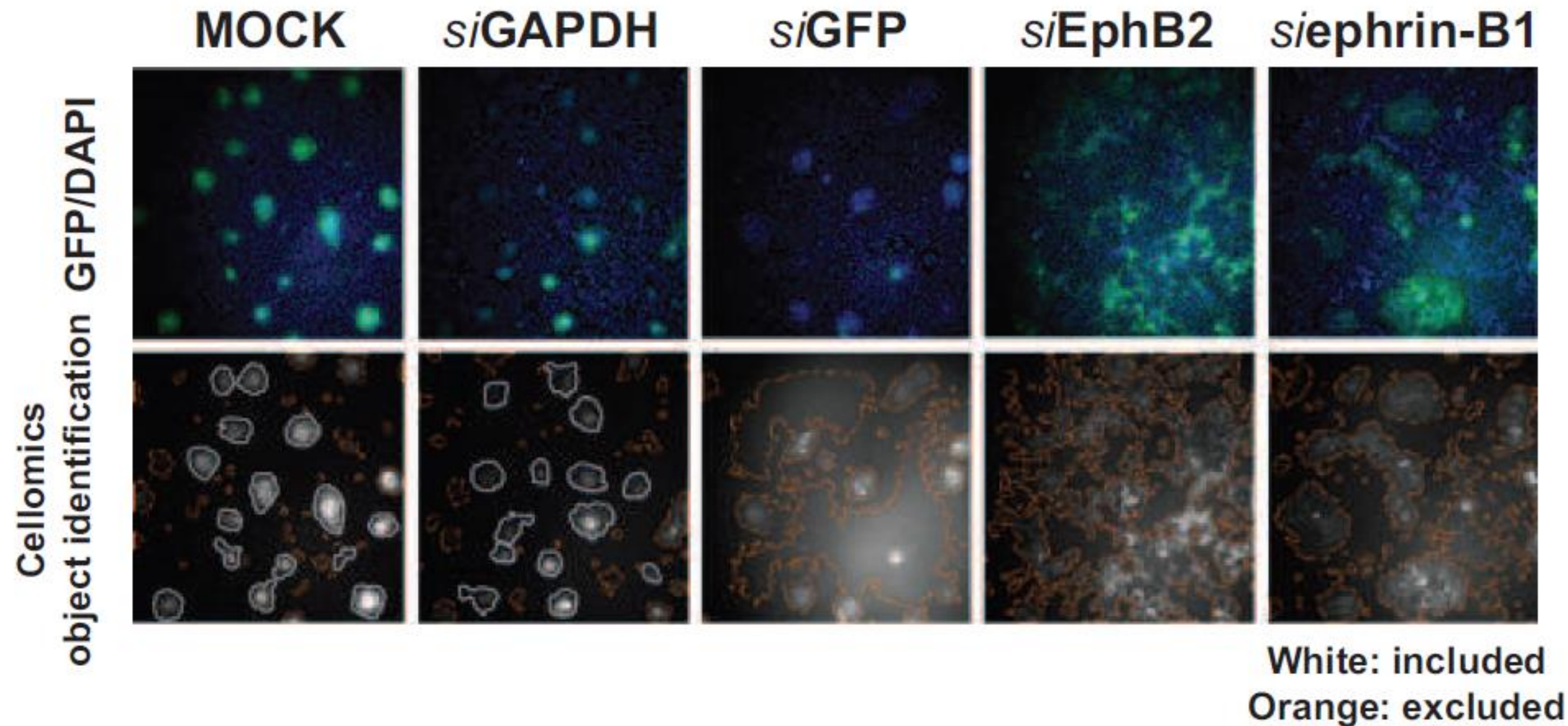
(**B**) Efficiency of siRNA induced knockdown assessed by immunoblotting. Mixed populations of EphB2+ and ephrin-B1+ cells were transfected with siRNAs to GAPDH, GFP, EphB2, ephrin-B1, and a non-targeting siRNA or were mock-transfected; cell populations were lysed after 72 hours and immunoblotted with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

A custom library of **2172** siRNA pools directed against kinases, phosphatases, and pTyr recognition modules to screen for proteins involved in cell sorting



Control or siRNAs affecting cell sorting does not alter cell viability or proliferation.
Co-cultured **EphB2$^+$-ephrin-B1$^+$** cells were transfected with either siGENOME siRNA or ON-TARGET*plus*TM siRNA and grown for 72 hrs.
Cell viability was tested by MTT assay and normalized to mock transfected control cells. Neither control siRNA or siRNA identified to perturb cell sorting affected cell proliferation or apoptosis.

Cellomics platform



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).

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

2172 siRNA

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)

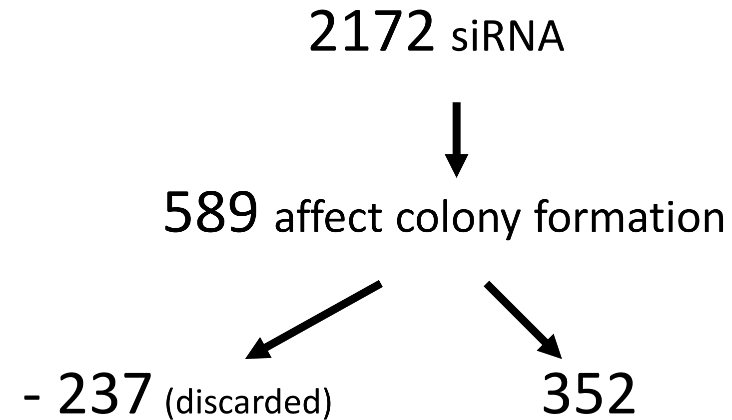
2172 siRNA

589 affect colony formation

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)



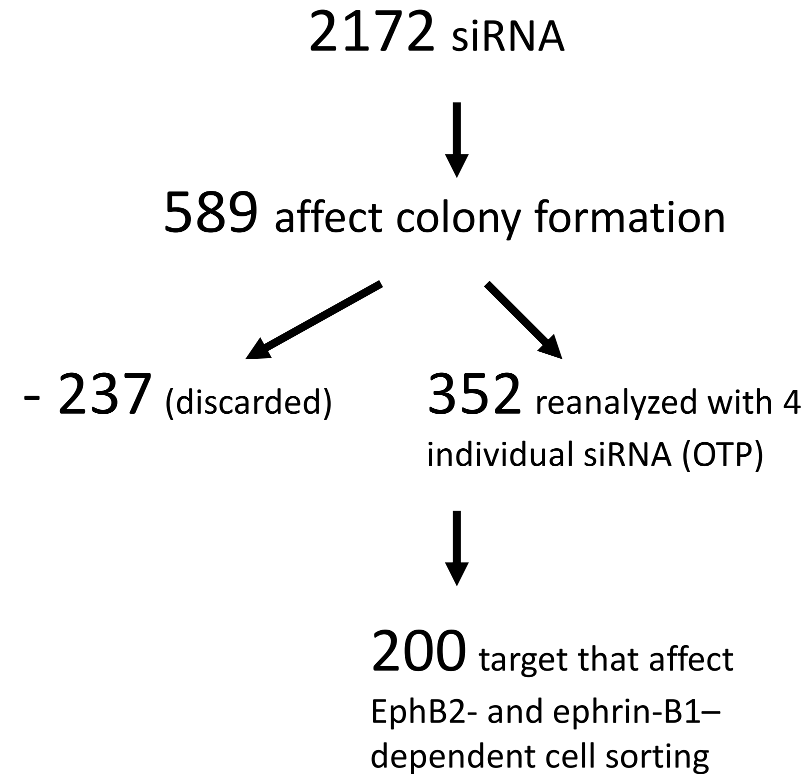
All affected cultures were then visually inspected, and **237** siRNAs that affect growth and adhesion were discarded.

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 obtained **200** targets.



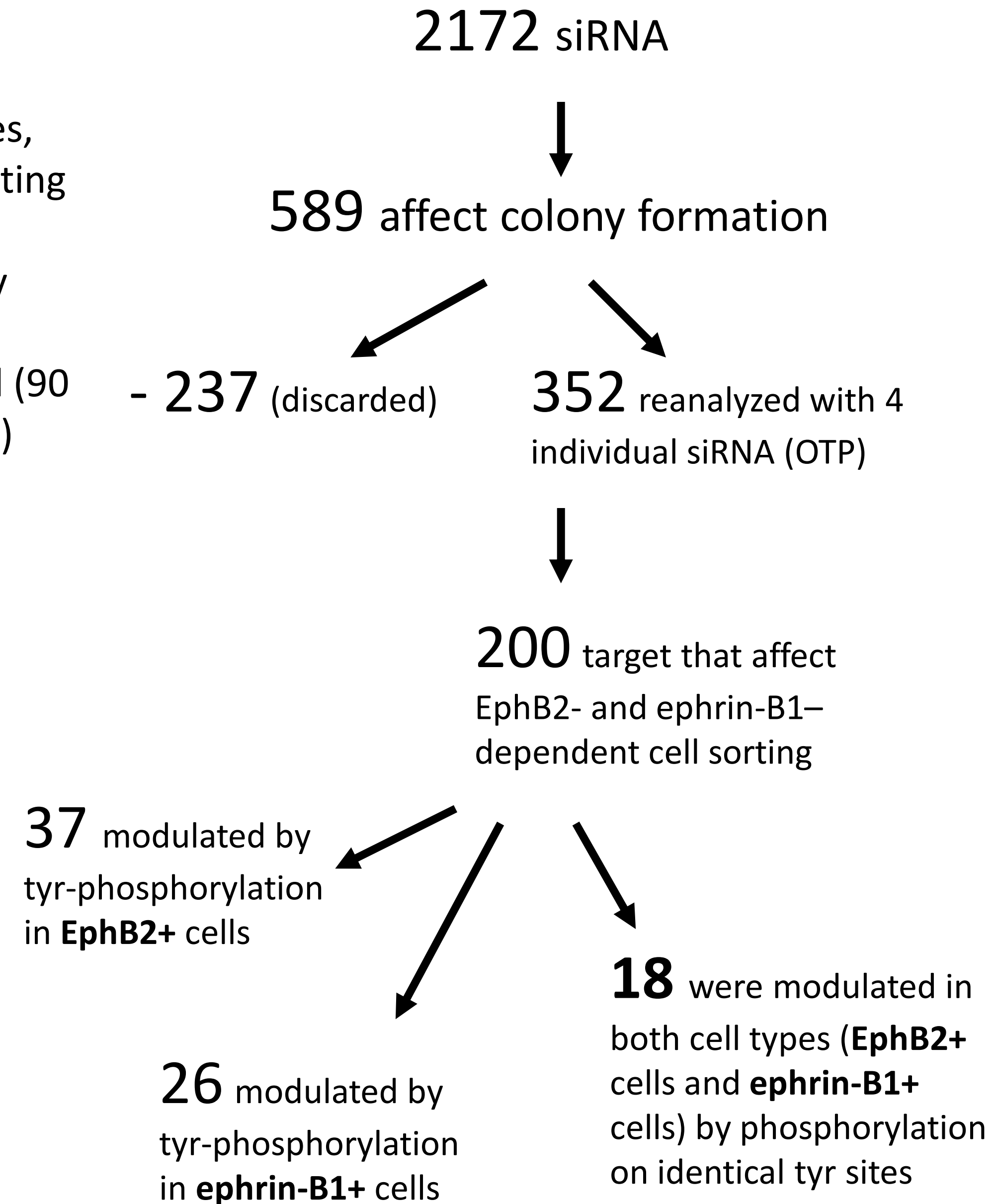
Functional analysis of EphB2- and ephrin-B1-regulated cell sorting through siRNA screening

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)

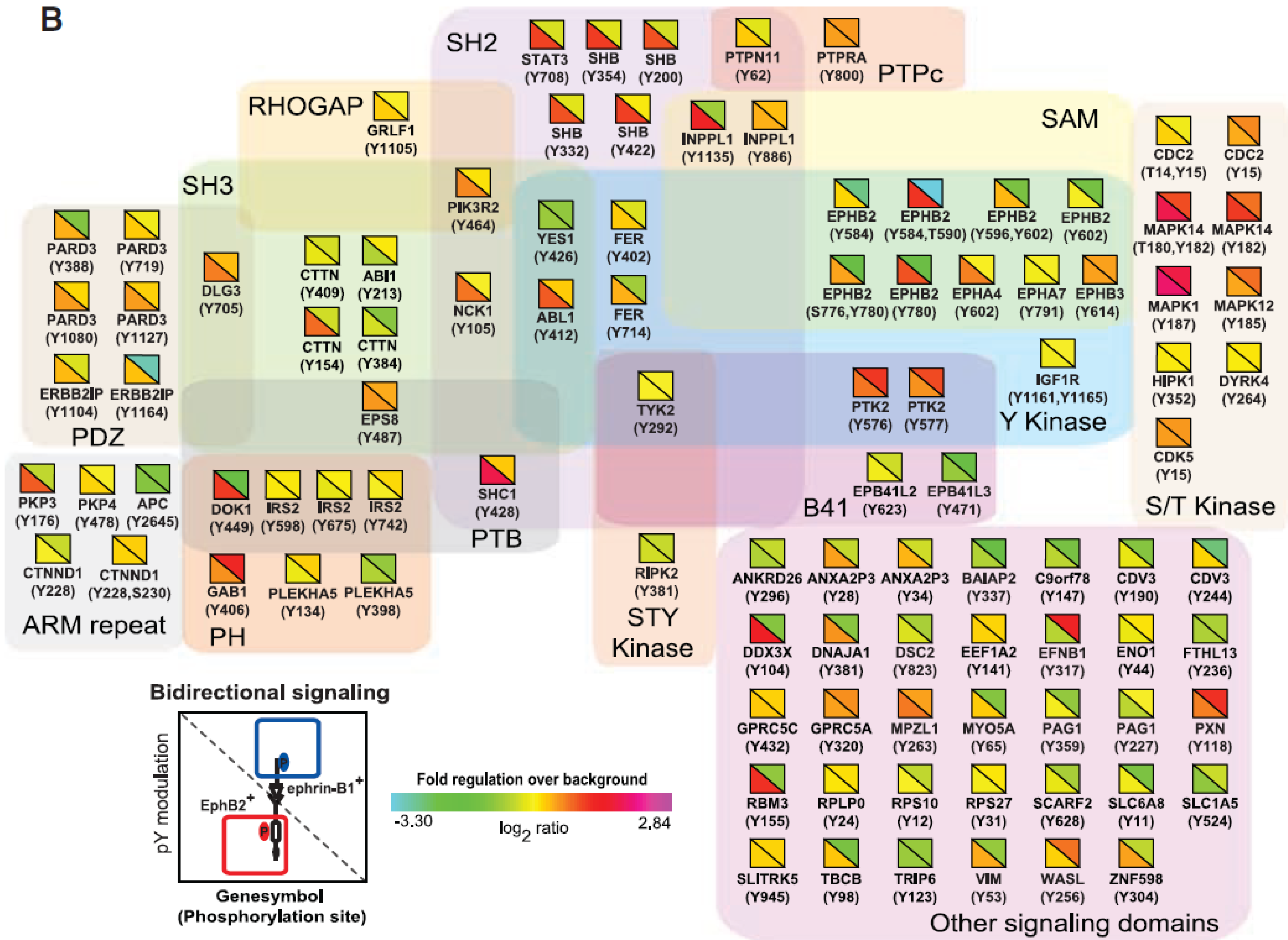
All affected cultures were then visually inspected, and **237** siRNAs that affect growth and adhesion were discarded.
Off-target effects are inherent to siRNA technology so they reanalyzed the remaining **352** targets with **4** individual siRNAs [ON-TARGETplus (OTP)] reagents that were distinct from 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 screening** with the proteins containing **pTyr sites modulated by the mixing of EphB2+ and ephrin-B1+ cells**

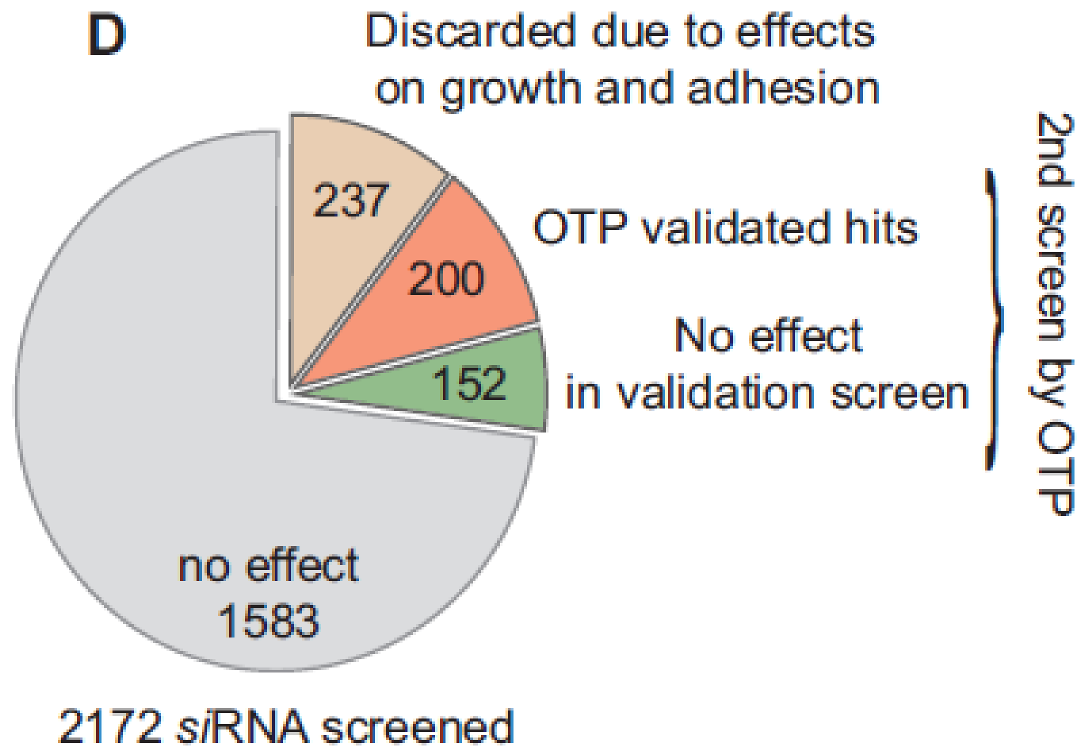


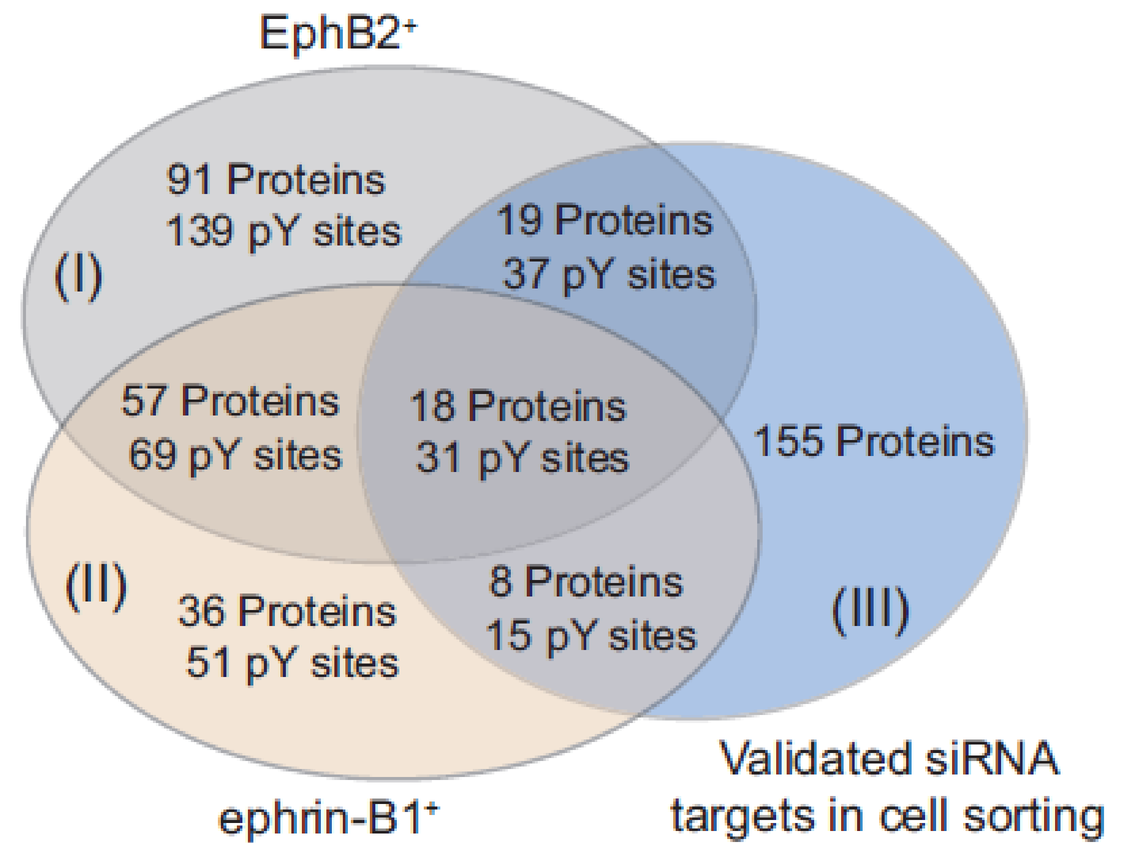
100 phosphorylation sites "modulated by the mixing of

ephrin-B1+ and EphB2+ cells



100 phosphorylation sites modulated by the mixing of ephrin-B1^{+} and EphB2^{+} cells

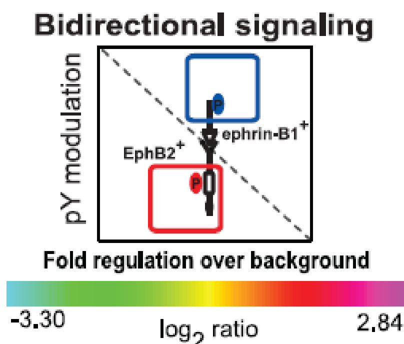




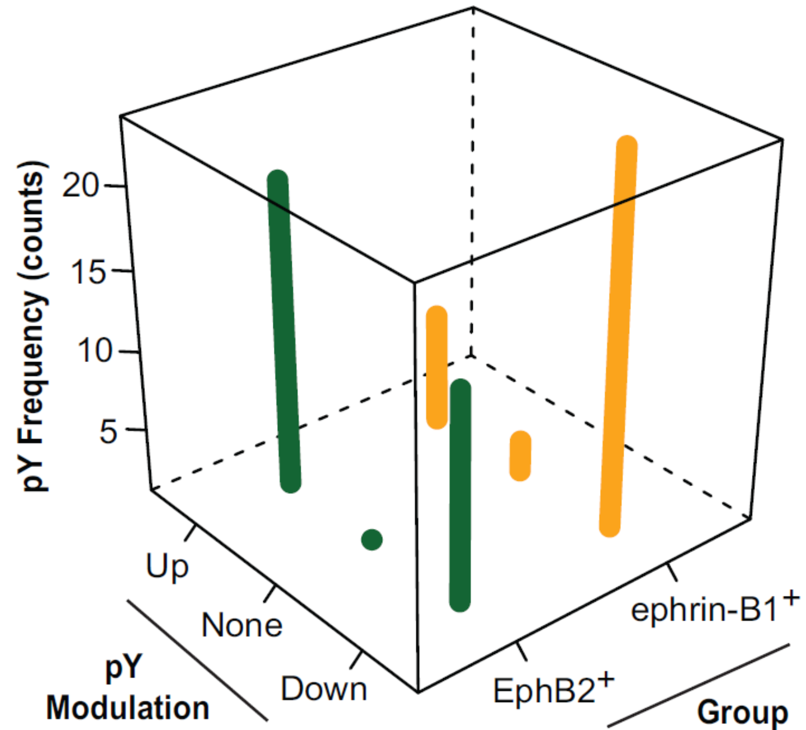
Proteins identified through siRNA screening as functionally important for cell sorting tend to be asymmetrically phosphorylated

#siRNA	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.



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.

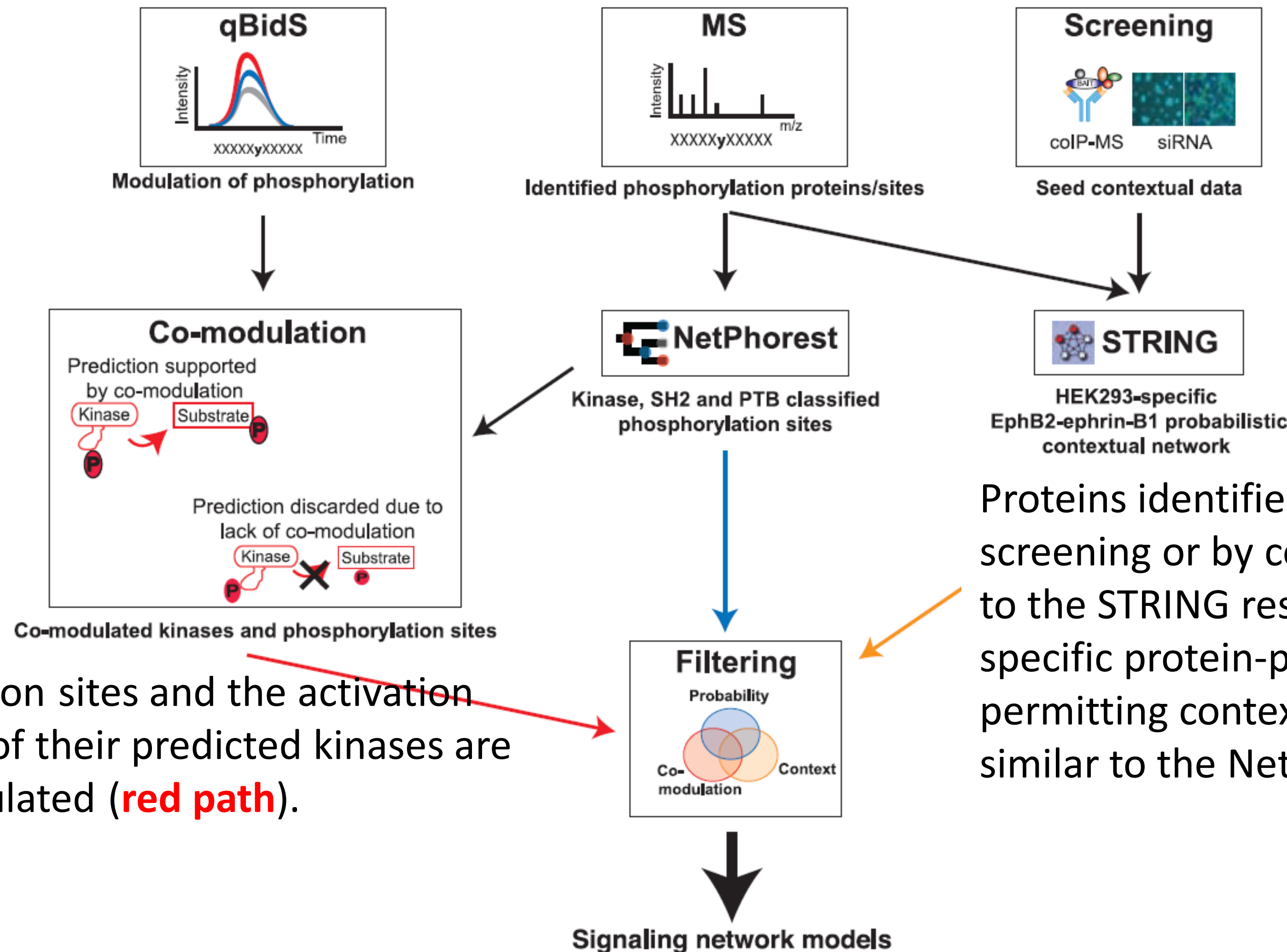
The specific modulation of each pTyr site is depicted for either EphB2+ or ephrin-B1+ cells, where the height of the bars indicates the number of pTyr sites.

Modulation of pTyr sites of these proteins is significantly different between EphB2+ and ephrin-B1+ cells.

Computational data integration and network modeling

All observed phosphorylation sites were processed by the NetPhorest algorithm to predict kinase-substrate relationships and SH2 and PTB domain interactions.

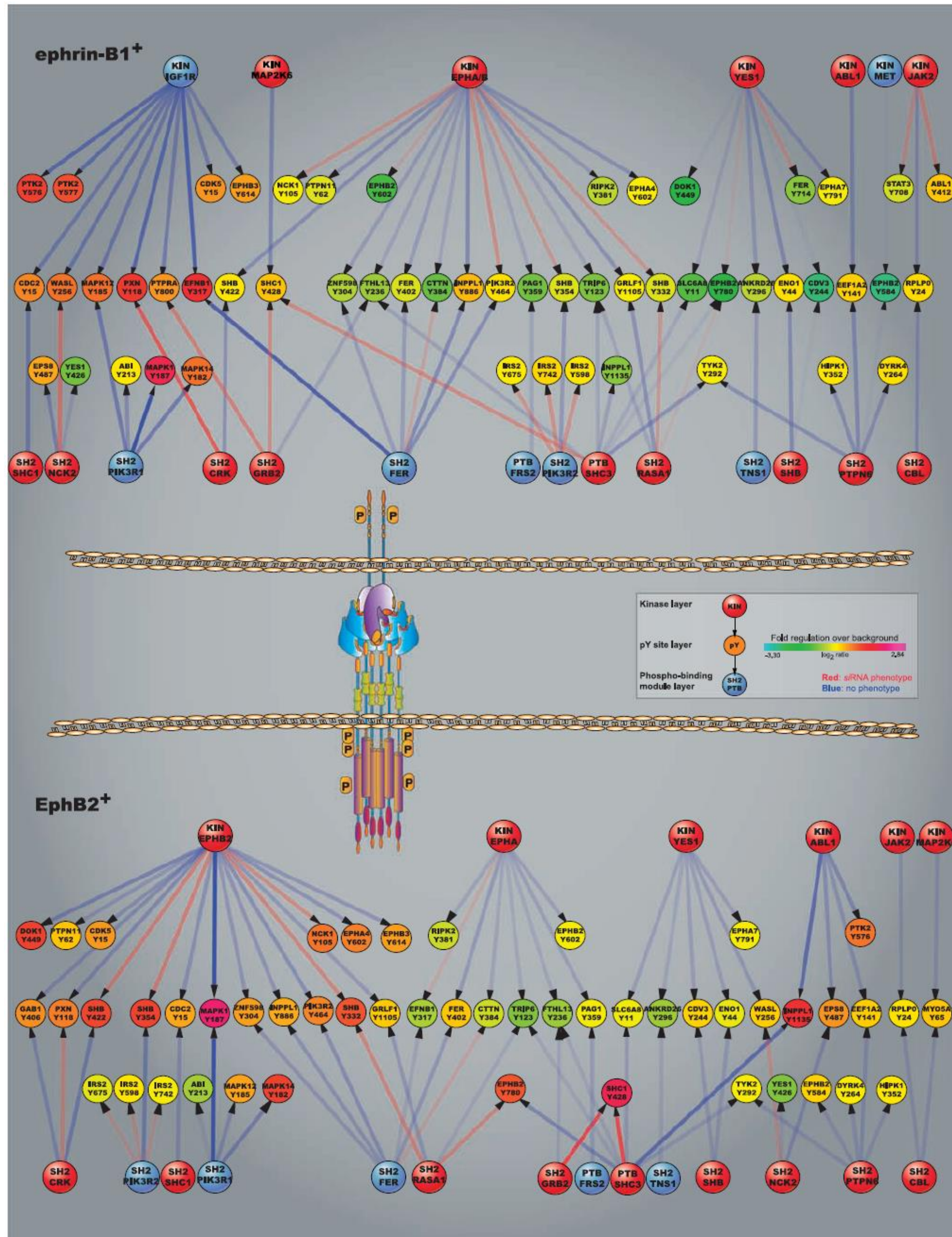
Predictions were filtered on the basis of the probability score from Netphorest (**blue path**).



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.

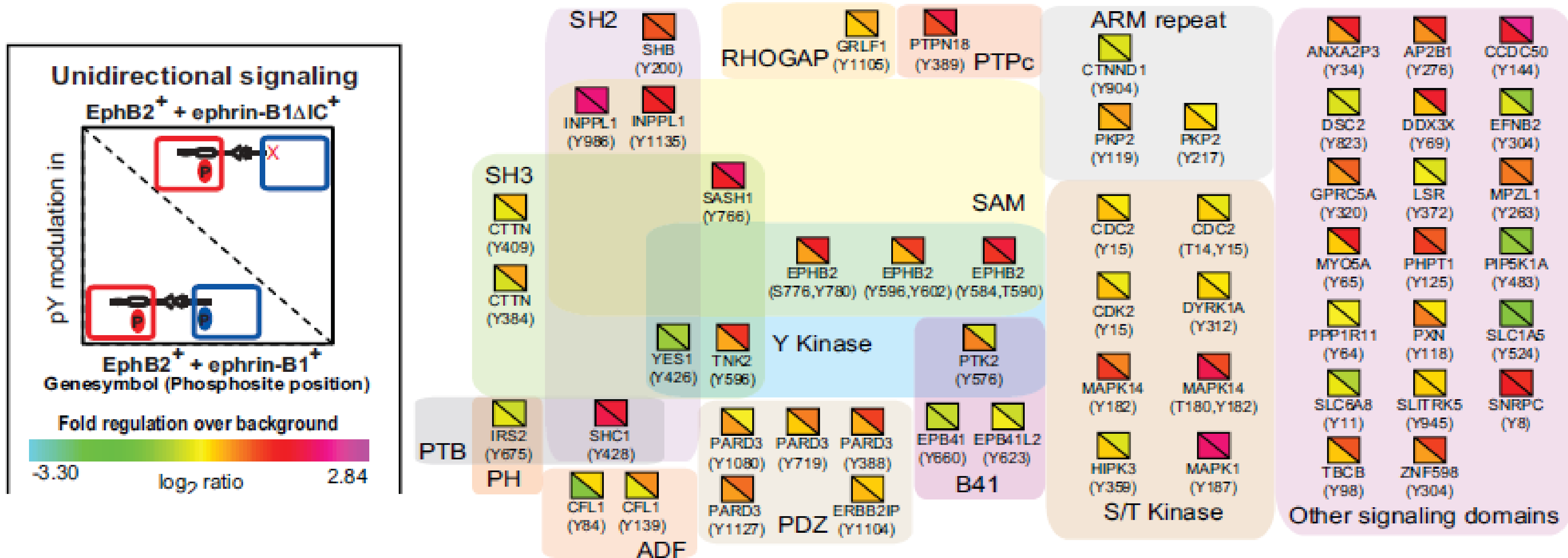
Tyrosine phosphorylation sites and the activation loop phosphorylation of their predicted kinases are required to be comodulated (**red path**).

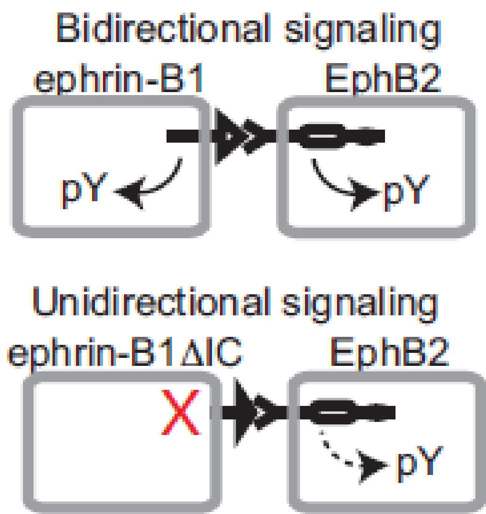
Cell-specific signaling network models in EphB2- and ephrin-B1- expressing cells



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 pTyr modulation by ephrin-B1 intracellular region.

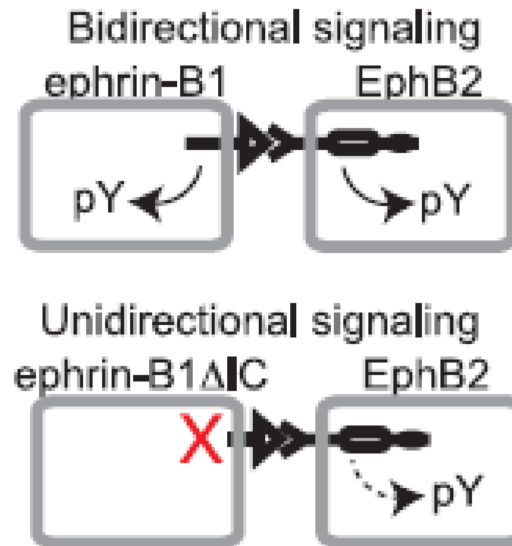




Phosphotyrosine signaling in **EphB2+** cells mixed with **ephrin-B1+** cells is compared to the pTyr dynamics in **EphB2+** cells mixed with **ephrin-B1ΔIC+** cells, using qBidS.

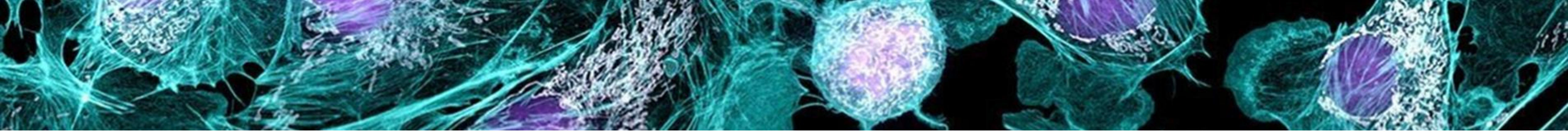
Contact-initiated pTyr signaling is compared by mixing of EphB2+ with ephrin-B1+ or ephrin-B1ΔIC+ cells. Specific modulation of individual pTyr peptides from EphB2+ cells is shown by boxes, with the color-coded triangles representing their modulation in EphB2+ cells mixed with ephrin-B1+ (lower triangle) or ephrin-B1 ΔIC+ cells (upper triangle).

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.

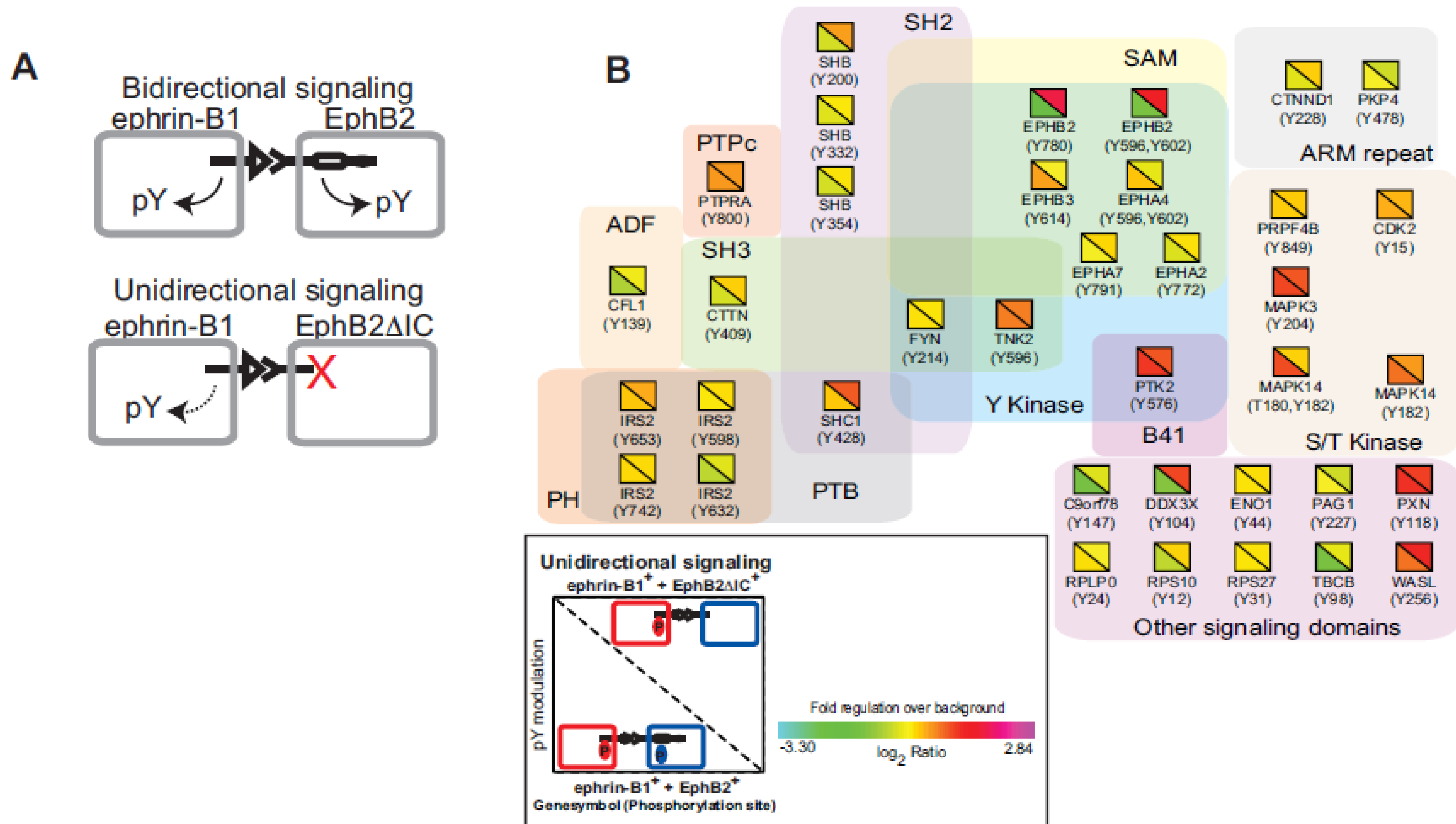
→ ephrin-B1 initiated signaling in EphB2+ cells utilizes different network paths in EphB2+ cells, depending on the presence or absence of the intracellular region of ephrin-B1.



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.

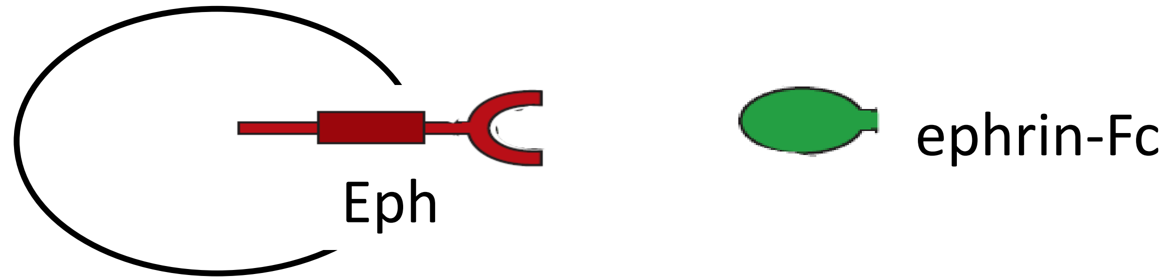
The same applies to the C-terminal valine of ephrin-B1 required for PDZ domain–binding of ephrin-B1, which also influenced signaling in EphB2^{+} cells

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

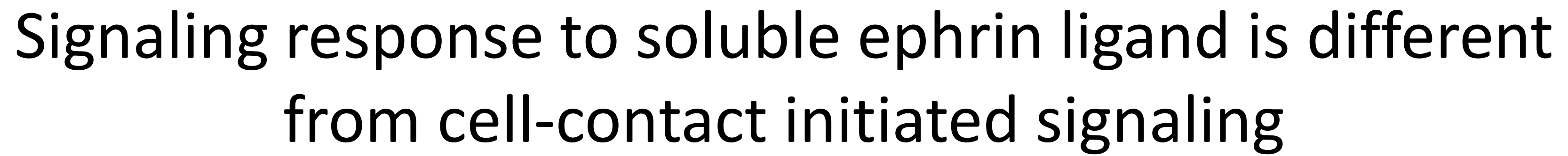


signal transduction is often studied by stimulating the target cells with a recombinant soluble ligand or with a recombinant soluble receptor

Stimulation of cells expressing the receptor with soluble ligand

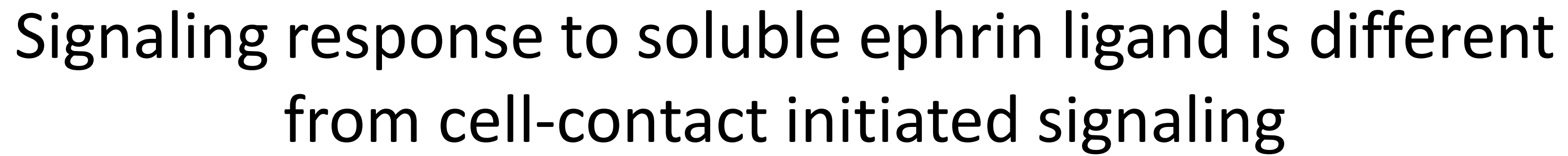


Stimulation of cells expressing the ligand with soluble receptor



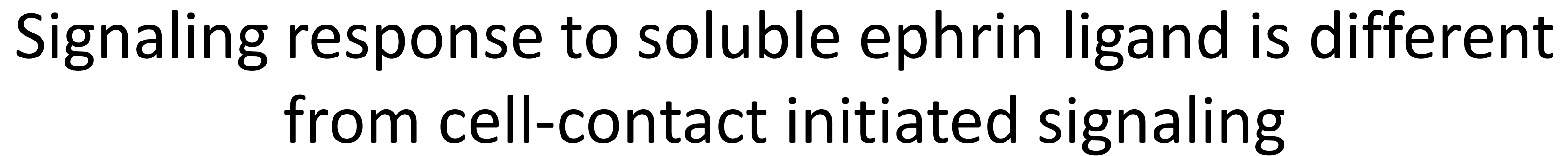
Signaling response to soluble ephrin ligand is different from cell-contact initiated signaling

- they compared the responses of **EphB2^{+} cells** to stimulation either with **ephrin-B1-Fc** or by mixing with **ephrin-B1+ cells**.



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

- they compared the responses of **EphB2^{+} cells** to stimulation either with **ephrin-B1-Fc** 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**.



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

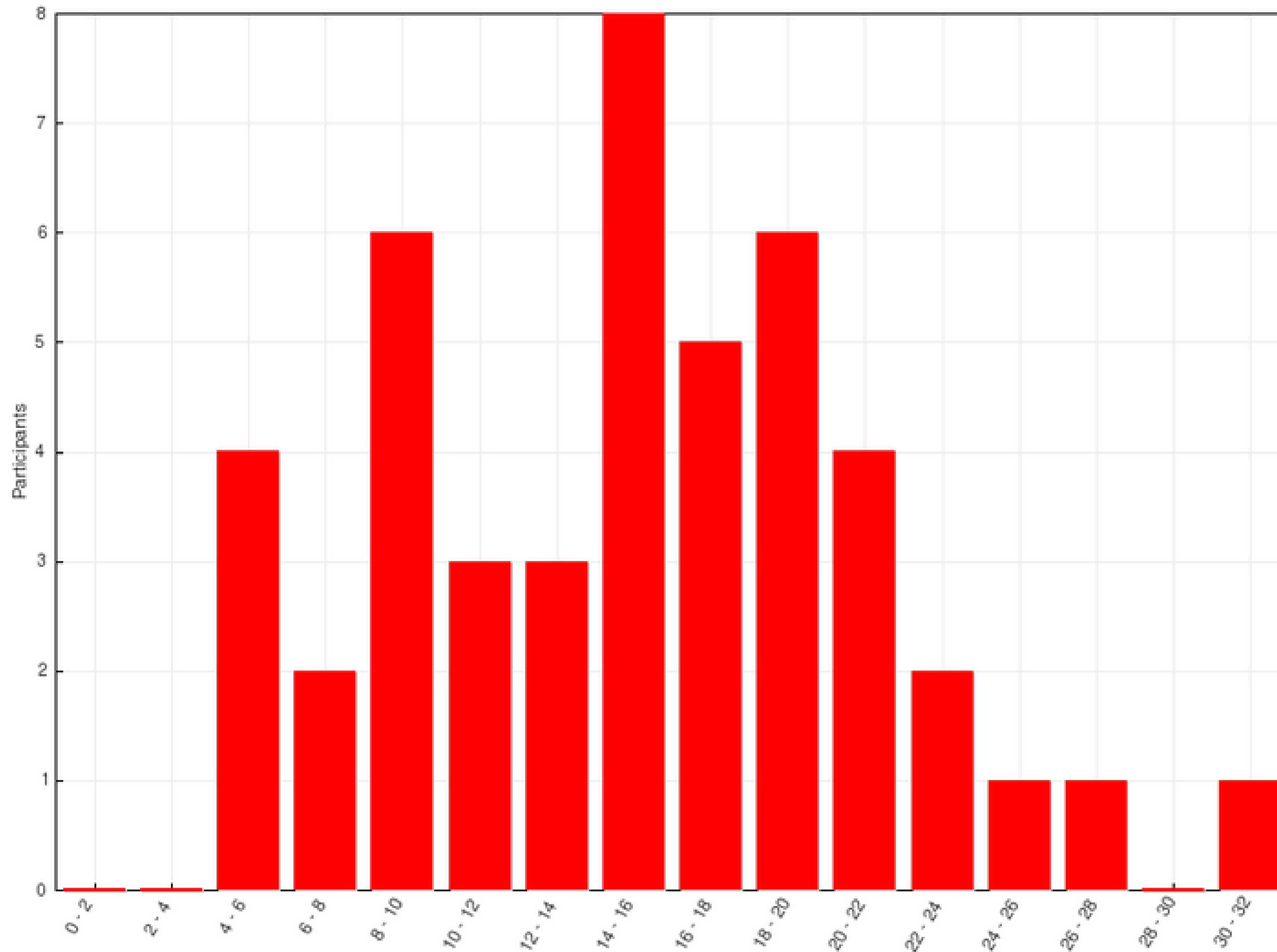
- they compared the responses of **EphB2$^+$ cells** to stimulation either with **ephrin-B1-Fc** or by mixing with **ephrin-B1+ cells**.
- The two types of stimulation resulted in **significantly different pTyr modulation** of identical sites.
- **Soluble ephrin-B1 ligand** led to **increased** phosphorylation 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, **decreased** phosphorylation was observed 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.**

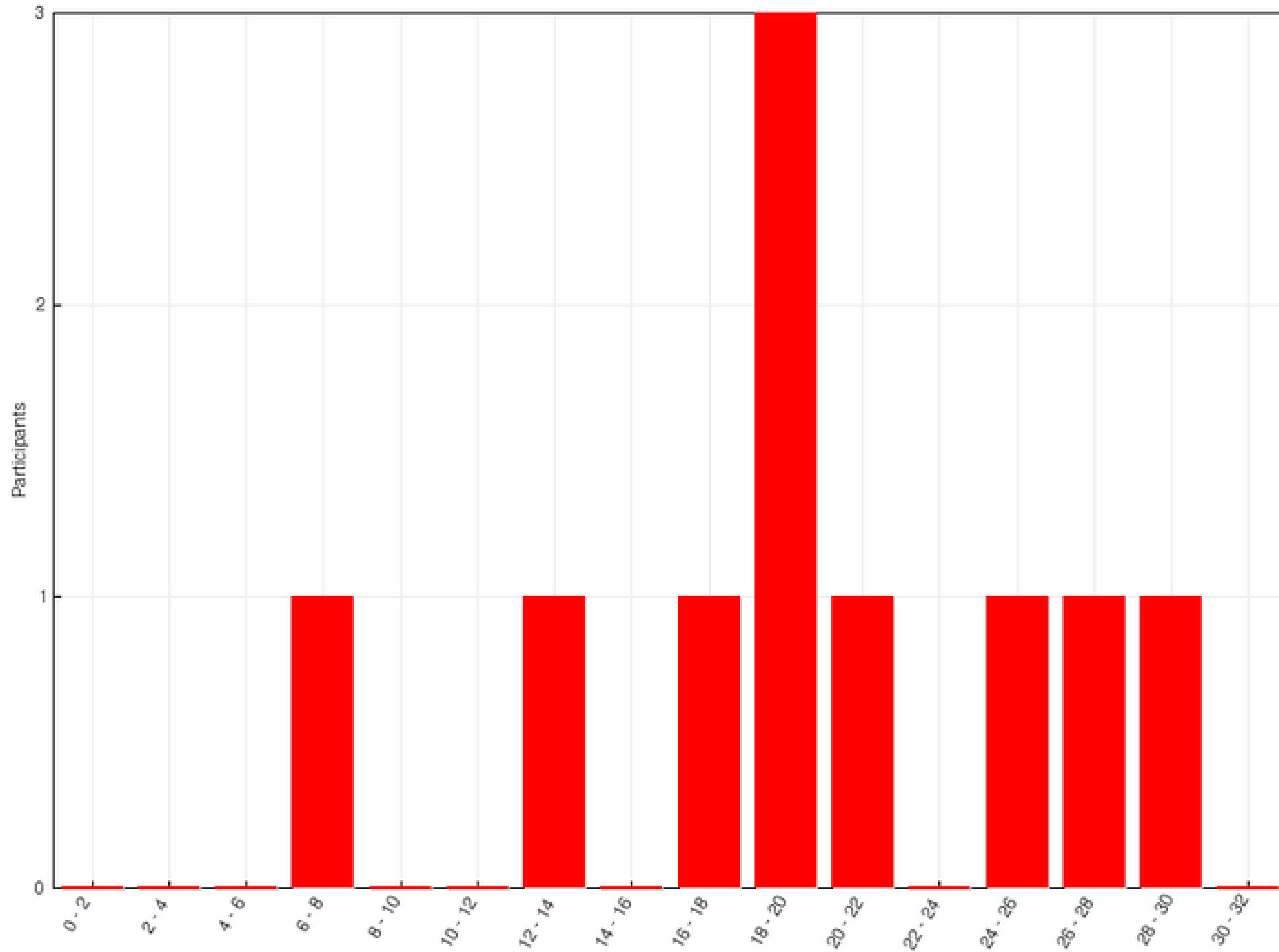
❅ information processing between **EphB2^{+}** and **ephrin-B1^{+}** cells is asymmetric

❅ 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 system-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.



46 students
Average 15/30



14 students
Average 15/30