Structurally encoded intraclass differences in EphA clusters drive distinct cell responses

Elena Seiradake1, Andreas Schaupp2, Daniel del Toro Ruiz2, Rainer Kaufmann1,3, Nikolaos Mitakidis1, Karl Harlos1, A Radu Aricescu1, Rüdiger Klein2 & E Yvonne Jones1

Functional outcomes of ephrin binding to Eph receptors (Ephs) range from cell repulsion to adhesion. Here we used cell collapse and stripe assays, showing contrasting effects of human ephrinA5 binding to EphA2 and EphA4. Despite equivalent ligand binding affinities, EphA4 triggered greater cell collapse, whereas EphA2-expressing cells adhered better to ephrinA5-coated surfaces. Chimeric receptors showed that the ectodomain is a major determinant of cell response. We report crystal structures of EphA4 ectodomain alone and in complexes with ephrinB3 and ephrinA5. These revealed closed clusters with a dimeric or circular arrangement in the crystal lattice, contrasting with extended arrays previously observed for EphA2 ectodomain. Localization microscopy showed that ligand-stimulated EphA4 induces smaller clusters than does EphA2. Mutant Ephs link these characteristics to interactions observed in the crystal lattices, suggesting a mechanism by which distinctive ectodomain surfaces determine clustering, and thereby signaling, properties.

The 14 erythropoietin-producing hepatocellular receptors (Ephs) comprise the largest family of receptor tyrosine kinases in humans. Eph receptor signaling can exert localized effects on cytoskeletal dynamics, thereby directing repulsive or migratory responses. Family members are expressed in many tissues during morphogenesis and have essential roles in cell-cell communication to guide cell positioning, segregation and migration in tissue homeostasis as well as in development1–3. Conversely, Ephs are widely expressed in cancer cells and in tumor blood vessels; they are implicated in tumor progression and metastatic spread, with examples of both increased and decreased levels of expression linked to malignancy4–6.

Eph receptors bind protein ligands, the Eph receptor-interacting proteins (ephrins), presented on the surface of an opposing cell (that is, a trans mode of ligand-receptor binding requiring direct cell-cell contact). All eight members of the human Eph family are membrane tethered, either by a glycosylphosphatidylinositol anchor in the ephrinA proteins or by a transmembrane helix and short cytoplasmic region in the ephrinB proteins. The Eph family is also subdivided into two classes—EphAs and EphBs—groupings that are in part determined by sequence similarity but that also reflect the conserved architecture of an N-terminal receptor-binding domain (RBD) and a sterile-alpha-motif (SAM) domain that can carry a C-terminal PDZ-binding motif. Likewise, the ephrins are characterized by the β-barrel fold of eight β-strands (designated A–K), and the Eph LBD consists of a β-sandwich ‘jelly roll’ fold of 12 β-strands (A–M). Structural studies of complexes between the Eph LBD and the ephrin RBD have revealed a conserved 1:1 interaction interface generic to all ligand-receptor combinations10. This high-affinity binding mode is, in essence, insertion of a single long loop from the ephrin RBD (loop G–H) into a substantial cavity on the surface of the Eph LBD. The detailed architecture of this RBD-LBD interface determines the specificity and binding affinity of ephrin-Eph interactions10. However, the 1:1 ligand-receptor binding mode does not, in isolation, provide a molecular mechanism for Eph receptor kinase autophosphorylation and signaling. Eph signaling requires receptor clustering11.

The first crystal structure for an Eph–ephrin complex, namely EphB2 LBD in complex with ephrinB2 RBD, highlighted a tetrameric arrangement involving a second low-affinity Eph–ephrin interaction surface. The region of the Eph LBD contributing to this interaction was designated the HI loop12. Functional data13 provided support for the biological significance of the tetrameric arrangement; however, crystallographic studies for a series of other Eph LBD–ephrin RBD complexes only revealed interfaces mediating the 1:1 high-affinity binding mode10. Subsequent structural and functional studies on
the full EphA2 ectodomain identified two in-cis Eph-Eph dimerization surfaces, which are equally essential for EphA2 clustering at cell-cell contacts.  These are a polar region on the LBD that includes the HI loop plus adjacent surfaces and a hydrophobic region on the sushido domain. Together, these interactions promote the assembly of extended EphA2 arrays that can form in the presence or absence of ephrin. 

The functionality of the ephrin-Eph system is complex. Depending on receptor and ligand availability, as well as cell-specific factors, Eph signaling mediates responses ranging from cell repulsion to adhesion. This variation in cellular response complicates dissection of fundamental molecular mechanisms in ephrin-Eph signaling. Eph ectodomain binding to the RBD of class A or B ephrin ligands on neighboring cells classically triggers cell-cell repulsion through activation of the Eph intracellular kinase domain. In the nervous system, repulsive EphA4-eprhin interaction is required for the formation of precise neuronal connections in the brain and spinal cord and for motor and sensory innervation of peripheral targets. Although EphA2 protein is found in axons, its overall expression in the brain is low. It is expressed in many other tissues including the eye, where EphA2-ephrinA5 interaction is implicated in the adhesive packing of fiber cells through cross-talk with cadherin and catenin protein families. EphA2 is also associated with invasive cell behavior and is upregulated in many cancers, in which it increases cancer malignancy and poor clinical prognosis.

EphA2 binds only class A ephrins, whereas EphA4 is unusually promiscuous, binding ephrinAs and Bs. Both Ephs bind ephrinA5. To investigate the potential role of Eph receptor clustering characteristics in cellular response, we carried out a comparative structural and functional analysis of the EphA2-ephrinA5 and EphA4-ephrinA5 systems.

RESULTS

Eph-dependent differences in cellular response to ephrinA5

Although many factors can contribute to the functional outcome of Eph activation, we asked whether Eph receptors themselves possess intrinsically distinct properties that generate different cell responses. Cell rounding assays are widely used model systems for repulsive signaling and have been used to study Eph signaling upon stimulation with preclustered ephrins. Cytoskeleton collapse, cell rounding and de-adherence result from Eph kinase activation. Because ligand binding affinity and kinetics could contribute to functional outcome, we first sought to control for this factor. To do so, we investigated the relative binding affinities of ephrinA5 to EphA2 and EphA4 in surface plasmon resonance assays. We found that ephrinA5 binds with similar affinity to EphA2 and EphA4 (data not shown), consistent with previous reports based on enzyme-linked immunosorbent assays. We engineered constructs in which full-length, transmembrane mouse EphA4 and human EphA2 receptors were fused to a fluorescent protein tag (mVenus) and expressed them in HeLa or COS7 cells (Online Methods). Transiently transfected cells expressed EphA2 and EphA4 at comparable levels after ~20 h (Supplementary Fig. 1). We found that EphA4-transfected cells, as compared to that of EphA2-transfected cells, undergo significantly stronger rounding in response to preclustered ephrinA5-Fc (Fig. 1b, Supplementary Fig. 2 and Supplementary Movies 1 and 2). We did not detect endogenous EphA2 or EphA4 in our HeLa cell cultures (Fig. 1b and Supplementary Fig. 1), a result consistent with the low level of collapse observed with untransfected control cells. Conversely, in a stripe assay, we found that EphA2-transfected HeLa cells adhere strongly to ephrinA5-Fc–coated stripes, whereas EphA4-transfected cells distribute more randomly between control and ephrinA5-Fc–coated stripes (Fig. 1c), What determines these Eph-specific signaling responses?

EphA4 ligand-free and ligand-bound crystal structures

To dissect the molecular properties that distinguish the EphA4 ectodomain (EphA4ecto) from the EphA2 ectodomain (EphA2ecto), we solved four crystal structures for the complete EphA4ecto in its ligand-free form (to 3.65 Å resolution), in complex with ephrinB3 RBD (one crystal, 4.65 Å) or with ephrinA5 RBD (two crystals: lysine-methylated, 4.0 Å and native, 4.95 Å). Data statistics and the highest-resolution shells using the CC1/2 criteria or data quality of I/σI > 2 are provided in Table 1. We expressed human EphA4ecto and ephrin RBD proteins in HEK293S GnTI- cells, crystallized them and phased the structures by molecular replacement (Online Methods and Supplementary Note). The unusually high solvent content of the crystals (76–88%) in conjunction with solvent flattening methods produced good-quality electron density maps despite the generally low resolution of the diffraction data sets (Table 1 and Supplementary Fig. 3). We maintained strong stereochemical restraints for refinement of the ligand-free EphA4ecto structure and the lysine-methylated EphA4ecto ephrinA5 RBD complex. For the native EphA4ecto–ephrinA5 RBD and EphA4ecto–ephrinB3 RBD complexes, we limited refinement to rigid body and translation-libration-screw motion (TLS) of the individual domains; stereochemical outlier residues present in high-resolution molecular replacement models were not altered for these two lowest-resolution structures. All four crystal structures have greater than 95% of residues in the favored regions of the Ramachandran plot (and 0.0–0.4% in disallowed regions), as assessed by MolProbity.
The domain arrangement within EphA4ecto is similar to that of EphA2ecto (refs. 14, 15), with sequential LBD, sushi, EGF, FN1 and FN2 domains resulting in an oblong molecule with an ~90° kink at the FN1–FN2 linkage (Fig. 2a). Unlike EphA2ecto (refs. 14, 15), EphA4 shows conservation of the FN1–FN2 linker conformation and of the relative orientation of the FN1 and FN2 domains in all four crystal structures, a result suggesting rigidity (Supplementary Fig. 4). The amino acid sequences of EphA2 and EphA4 in the FN1–FN2 linker region are not conserved and include a potential N-linked glycosylation site in EphA2 but not EphA4. Also, the EphA4 FN2 domain packs closely against the FN1 domain by using loop residues 518–520 (Ala-Ala-Gly). Two of these residues are replaced by bulkier and more hydrophilic amino acids for the corresponding residues 512–514 in EphA2 (Gln-Glu-Gly). Taken together, these differences may underlie the reduction in interdomain rigidity for this region in EphA2 relative to EphA4. The functional consequences of these differences in rigidity are unclear, but given the proximity of FN2 to the plasma membrane they may affect receptor-membrane interactions and orientation at the cell surface.

All reported structures of EphA2ecto revealed continuous array-like arrangements within the crystal lattice, compatible with EphA2 clustering in cis on the cell membrane14,15 (Supplementary Fig. 5). Here we show that EphA4ecto does not form extended EphA2-like arrays in the crystal but does form defined units. The EphA4ecto units compatible with in-cis interaction in the context of a cell surface are composed of two, three, or six copies of EphA4 in a dimeric or circular arrangement (Fig. 2b). These structural data suggest that the mode of clustering observed in EphA2 cannot be extrapolated to all Eph-family members.

Superposition of the EphA4ecto structure (ligand free or bound to ephrin) on EphA2ecto as arranged in its characteristic arrays does not lead to obvious clashes. We conclude that surface properties, rather than differences in the overall shape, determine the mode of Eph ectodomain clustering. EphA2ecto arrays form through Eph-Eph interactions mediated by two specific surface patches, one centered on the FN2 domains resulting in an oblong molecule with an ~90° kink at the FN1–FN2 linkage (Fig. 2a). Unlike EphA2ecto (refs. 14, 15), EphA4 shows conservation of the FN1–FN2 linker conformation and of the relative orientation of the FN1 and FN2 domains in all four crystal structures, a result suggesting rigidity (Supplementary Fig. 4). The amino acid sequences of EphA2 and EphA4 in the FN1–FN2 linker region are not conserved and include a potential N-linked glycosylation site in EphA2 but not EphA4. Also, the EphA4 FN2 domain packs closely against the FN1 domain by using loop residues 518–520 (Ala-Ala-Gly). Two of these residues are replaced by bulkier and more hydrophilic amino acids for the corresponding residues 512–514 in EphA2 (Gln-Glu-Gly). Taken together, these differences may underlie the reduction in interdomain rigidity for this region in EphA2 relative to EphA4. The functional consequences of these differences in rigidity are unclear, but given the proximity of FN2 to the plasma membrane they may affect receptor-membrane interactions and orientation at the cell surface.

All reported structures of EphA2ecto revealed continuous array-like arrangements within the crystal lattice, compatible with EphA2 clustering in cis on the cell membrane14,15 (Supplementary Fig. 5). Here we show that EphA4ecto does not form extended EphA2-like arrays in the crystal but does form defined units. The EphA4ecto units compatible with in-cis interaction in the context of a cell surface are composed of two, three, or six copies of EphA4 in a dimeric or circular arrangement (Fig. 2b). These structural data suggest that the mode of clustering observed in EphA2 cannot be extrapolated to all Eph-family members.

Superposition of the EphA4ecto structure (ligand free or bound to ephrin) on EphA2ecto as arranged in its characteristic arrays does not lead to obvious clashes. We conclude that surface properties, rather than differences in the overall shape, determine the mode of Eph ectodomain clustering. EphA2ecto arrays form through Eph-Eph interactions mediated by two specific surface patches, one centered on the
HI loop of the LBD and one on the sushi domain14,15 (Supplementary Figs. 5 and 6). The structures reported here show that the EphA4 sushi domain can also mediate Eph-Eph interaction by using the equivalent surface patch, often through dimerization as found in EphA2ecto structures14,15. In contrast, the less-conserved HI-loop region on the LBD mediates no EphA4-EphA4 contacts in three of the four EphA4ecto structures presented (Fig. 3 and Supplementary Figs. 6 and 7). It provides Eph-Eph contacts only in the crystal structure derived from lysine-methylated EphA4eme-ephrinA5 RBD complex (Supplementary Fig. 8), but as the EphA4 HI loop contains a lysine (Fig. 3b), it may have non-native properties in this sample. Taken together, the data show that, of the two EphA2 surfaces required to build up EphA2ecto arrays, only one, henceforth termed the sushi dimerization surface, is functionally conserved in EphA4 as an Eph-Eph interaction surface. The structural results further suggest that one such interaction surface is not sufficient to support the formation of EphA2-like arrays. Might Eph response to ligand be modulated by the structurally encoded clustering properties of the receptors’ ectodomains?

Figure 3 Properties of the EphA4 HI-loop area and sushi dimerization region. (a) Surface of EphA4ecto in complex with ephrinB3 RBD, shown in two color schemes. Top, domains are colored separately, as in Figure 2. Bottom, colors are: white, EphA4; gray, ephrinB3; magenta, HI loop and adjacent regions implicated in clustering for EphA2 (ref. 14); cyan, sushi dimerization surface. (b-e) Zoomed-in view of the EphA4 HI-loop region (magenta) and sushi dimerization surfaces (cyan). In b, Residues are shown as sticks. Arrowheads mark Lys133. Asterisks mark Glu134 (equivalent to Gly131 in EphA2, which is mutated to tyrosine in EphA2 HI) and the surface residues Leu254, Val255 and Ile257 on the sushi domain (mutated to aspartate in EphA4su, EphA2su and EphA2nd-su). In c, colors are according to sequence conservation among human EphAs, ranging from black (highly conserved) to white (not conserved). The HI-loop area and sushi dimerization surface are encircled. In d, colors are according to the frequency of residues mediating lattice contacts in the four crystal structures presented, ranging from black (involved in all lattices) to white (not involved in any lattices). In e, colors are according to electrostatic potential, calculated for vacuum. Blue, positive charge; red, negative charge; white, neutral; units in PyMoL adjusted to ±63.

Eph ectodomain clustering characteristics and function

To test whether differences in the clustering properties of the EphA2 and EphA4 ectodomains have a functional role in the cell, we first produced chimeras of the full-length, mVenus-tagged transmembrane receptors in which the ectodomains of EphA2 and EphA4 were switched (Fig. 4a). Expression levels in HeLa cells were similar for mutant and wild-type receptors (Supplementary Fig. 1). Indeed, the cell rounding assays showed that the EphA4 ectodomain produced more collapse than did the EphA2 ectodomain (Fig. 4b), indicating that the Eph ectodomain is the major determinant defining the response. However, switching the ectodomains of EphA2 and EphA4 did not fully switch the receptor functionality, thus suggesting that the identity of the transmembrane helix and/or the cytoplasmic region also contribute to the cell response, possibly through distinct steric or protein-interaction properties in these regions. Next we sought to test whether the ectodomain-dependent functional differences relate to their clustering properties.

To directly characterize the clustering properties of fluorescently tagged EphA2 and EphA4 in COS7 cells, we used time-lapse and localization microscopy techniques in living and fixed cells, respectively. Our COS7 cells expressed no EphA4 and moderate levels of endogenous EphA2 (Supplementary Fig. 1). We found that in live-cell imaging, EphA2 clusters formed at the cell surface within minutes after the addition of preclustered ephrinA5-Fc, initially with increased abundance of clusters at the cell periphery (Fig. 4c and Supplementary Fig. 9). At 15–30 min after stimulation, EphA2 was found in large endocytic vesicles, and the cell membrane was essentially cleared of the receptor (Supplementary Fig. 9 and data not shown). In contrast, EphA4 formed relatively small clusters and vesicles at the cell surface under the same conditions (Fig. 4c and Supplementary Fig. 9).

To gain quantitative information on the Eph clusters, we analyzed fixed cells, implementing one of the recently developed methods of super-resolution fluorescence microscopy. Spectral position determination microscopy (SPDM)29 is a form of localization microscopy (also termed direct stochastic optical reconstruction microscopy or dSTORM29) applicable for standard fluorophores such as mVenus. An intense laser excitation of the sample shifts all the fluorophores into a long-lived dark state. The stochastic recovery of individual molecules to the relatively short-lived fluorescent state then separates their signals over time (and with sequential image acquisition also provides separation in space), thus allowing the positions of single molecules to be determined with high accuracy. We collected data sets of individual molecular positions of mVenus-tagged wild-type and chimeric Ephs in paraformaldehyde-fixed samples of COS7 cells (nonstimulated or 10 min after addition of preclustered ephrinA5-Fc). We chose cells showing similar Eph expression levels and analyzed the distribution of distances between receptor positions. To detect deviations from a spatially homogeneous distribution (that is, clustering) we used Ripley’s L function30, which essentially shows the normalized distribution of distances between the single-molecule positions (Online Methods). A random distribution will approximately follow the horizontal zero axis with constant dispersion only if the data follow a homogeneous Poisson process. Signals above zero therefore reflect both the amount of clustering (y values) and cluster diameter (x values). Further analysis of the receptor clusters was performed with a method31 based on a threshold of local molecule density to identify and characterize individual clusters in the data sets (Online Methods). The chimeric Eph receptors
Figure 4 Eph ectodomains can control Eph clustering and function. (a) Schematic representation of transmembrane Eph constructs. Blue, EphA2; red, EphA4. Chimeric proteins were engineered by switching ectodomains (A4A2 or A2A4). Asterisks mark point mutants in the HI loop, sushi dimerization surface, and major ephrin-binding sites. All constructs contain an N-terminal Flag tag and a C-terminal mVenus or mCherry tag. (b) HeLa cell rounding responses measured 10 min after ephrinA5-Fc stimulation. Plotted are averaged ratios of the adherent-cell surface after and before stimulation. Statistical significance was determined by one-way ANOVA and Tukey’s post hoc test. Error bars, s.e.m. **P < 0.01; ***P < 0.001; ****P < 0.0001 for three independent biological experiments; n > 100. (c) Confocal time-lapse images of Eph clustering induced by ephrinA5-Fc stimulation in COS7 cells. (d) Single-molecule experiments performed with localization microscopy. Normalized Ripley’s L functions (L(r)−r) are plotted over the distances (r in nm) between the molecules. Increased L(r)−r at particular distances reflect the amount of clustering and the cluster sizes. (e) Further quantification of data presented in d. The percentage of detected molecules present in clusters is shown. Clusters were defined as molecules having at least 20 neighboring molecules within a radius of 50 nm (Supplementary Fig. 10). (f) Cell rounding in response to preclustered ephrinA5-Fc and cell adhesion to ephrinA5-Fc containing stripes in transfected HeLa cells. Cyan, EphA2; pink, EphA4; gray, EphA2 su. Statistical significance was determined by one-way ANOVA and Tukey’s post hoc test. Error bars, s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

revealed that the ectodomain strongly drives the clustering properties (Fig. 4d,e and Supplementary Figs. 9 and 10). Notably, small clusters for EphA4 correlated with small units found in the EphA4 ecto crystals, whereas larger clusters correlated with the extended arrays described for EphA2 ecto.

The molecular determinants of clustering

To identify whether the molecular determinants responsible for the differing functional effects were those mediating Eph-Eph interactions in the lattice assemblies of the crystal structures, we designed a panel of mutant Eph-mVenus–tagged constructs. EphA2 su and EphA4 su each contain the multiple mutations L254D V255D I257D in the sushi dimerization surface. To target the EphA2 HI loop that mediates Eph-Eph contacts specifically in EphA2 ecto crystal structures, we used the G131Y mutant EphA2 HI.

In localization microscopy, we found that mutations in the HI loop (construct EphA2 HI) or in the sushi dimerization surface (construct EphA2 su2) reduced EphA2 clustering to a level closer to that of EphA4 (Fig. 4d,e). To further validate this result, we introduced a second EphA2 sushi mutant, EphA2 su2, containing an N-type glycosylation site in the sushi dimerization surface (H246N A248S). The bulky sugar in this region of EphA2 su2 reduced clustering of the receptor even further as compared to that for EphA2 su (Fig. 4d). EphA2 su2 had EphA4-like characteristics also in cell rounding and stripe assays (Fig. 4f). Thus, targeting the array-forming abilities of EphA2 can convert its function. The equivalent mutant EphA4 su also showed reduced clustering compared to that of wild-type EphA4 (Fig. 4d). However, the overall clustering response of wild-type EphA4 was low, and this further reduction was not significant.

In sequence comparisons between Ephs, the sushi dimerization surface is highly conserved, and this suggests that this surface may mediate a conserved (Eph-Eph) interaction mode across all family members, with the possible exception of EphB4 (Supplementary Fig. 11). In contrast, sequence variation in the HI loop is high. Nevertheless, Gly131, which we show here is important in EphA2 clustering, is conserved in all EphAs except EphA4 (Supplementary Fig. 11). It remains to be seen whether the other EphA receptors have EphA2- or EphA4-like clustering characteristics.

Ligand-independent recruitment to clusters

Previously reported fluorescence microscopy data for EphA3 (ref. 32) suggested that ephrin-independent Eph-Eph association can increase the size of clusters. To test whether...
the strong EphA2 clustering response observed in COS7 cells involves Eph-Eph association independently of ephrin binding, we coexpressed EphA2 (mCherry tagged) and the non-ephrin-binding mutant EphA2\(^{nb}\) (mVenus tagged). EphA2\(^{nb}\) contains mutation A190N L192S. In agreement with the crystallographic results, EphA2\(^{nb}\) was not recruited into clusters of wild-type EphA2 (Fig. 5a). When expressed on its own, EphA2\(^{nb}\) did not cluster in response to ephrinA5-Fc addition (data not shown). To find out whether the recruitment of EphA2\(^{nb}\) into EphA2 clusters depended on the array-forming Eph-Eph interaction surfaces, we coexpressed EphA2 with the non-ephrin-binding mutant EphA2\(^{nb}\), which combined the mutations in the sushi dimerization surface (L1254D V2255D I257D) and in the ephrin-binding site (A190N L192S). In agreement with the crystallographic results, EphA2\(^{nb}\) was not recruited into wild-type EphA2 clusters (Fig. 5b). Supplementary Movies 3–10 show time-lapse experiments of Eph clustering experiments.

**DISCUSSION**

The different members of the Eph receptor family are responsible for a myriad of often opposing functions including cell-cell adhesion and repulsion\(^1,4\). EphA4 is best known for its role as a classical guidance receptor in the neuronal system, in which it controls cell dynamics and motility mostly through repulsive signaling\(^{16–19}\). EphA2 has been extensively studied for its function in controlling cell adhesion, motility and invasiveness in a variety of tissues, especially in many cancers\(^4\). Most previous efforts to dissect the different functionalities of Ephs have focused either on the ephrin binding specificities\(^1,33\) or the intracellular interactions with downstream signaling proteins\(^1,34\). A recent study suggested that EphA2 clustering propensities can affect function, although the underlying mechanisms remained unclear\(^35\). We are now able to address this issue by structural comparison of Eph ectodomain assemblies. We found that Ephs within the same class, EphA2 and EphA4, have distinct ectodomain properties that can determine differential clustering, albeit with the caveat that a simple switch of ectodomains does not entirely switch the receptor functionality.

These results demonstrate that, together with the functional contributions of the intracellular domains, the distinctive extracellular-clustering characteristics can modulate signaling responses of the full-length receptor. The HI loop and its adjacent regions on the LBD contain some of these receptor-specific features. Early structural studies using EphB2 LBD and ephrinB2 RBD implicated this region (then termed the ‘specificity loop’) in secondary Eph-ephrin interactions that give rise to circular Eph–ephrin heterotetramers\(^12\). The functional importance of Eph surfaces mediating heterotetramerization were further confirmed in a mutagenesis study\(^13\). More recently, we and others presented structural data for complete EphA2 ectodomains and found that for these, the EphA2 HI loop is part of a large homotypic Eph-Eph interaction surface contributing to EphA2 array formation in cis. These results led to a new array-based model for Eph clustering\(^14,15\). The data reported here reveal that there is no single generic arrangement for Eph cluster formation but rather that the different modes of interaction involving the HI-loop region reflect a diverse functional repertoire. The sushi dimerization surface is the only surface showing a conserved (Eph-Eph) interaction mode. In the absence of additional Eph- or ephrin-mediated interactions, this surface can give rise only to dimeric but not multimeric arrangements. It is therefore necessary but not sufficient for array-like clustering.

Our results suggest a model in which different Eph ectodomain interaction properties are capable of dramatic modulation of signaling outputs to provide an additional level of control. This model allows for structural fine-tuning of functional output in cells coexpressing different Eph receptors, given the receptors’ abilities to form heterooligomeric clusters\(^36,37\), presumably through the conserved sushi dimerization surface. The Ephs may thus exemplify a new mechanism whereby a relatively small number of distinct receptors can control the vast number of diverse signaling processes associated with the complexity we find in higher organisms.

EphA2 is notorious for its oncogenic effects in many cancers\(^4,5,35\). These effects have been, at least in part, linked to Eph kinase–independent clustering properties\(^38\) as well as more generally to the level of the receptor-ligand clustering and activation\(^6\). In the results reported here, we demonstrated that, by targeting surfaces involved in EphA2 clustering, we can convert EphA2 activity to resemble repulsion-mediating EphA4. The correlation of EphA2 clustering ability with breast cancer cell malignancy\(^35\) renders this mechanism for altering cellular response therapeutically relevant. Classically, efforts in targeting Eph functions in cancers have aimed at the kinase and ligand binding functions of the receptor or at regulating the overall expression levels\(^4\). Our results highlight the potential of a new approach in which Eph receptor function can be controlled by modulation of its clustering properties.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structural factors have been deposited in the Protein Data Bank, under the following accession codes: EphA4 ectodomain, 4BK4; EphA4 ectodomain in complex with ephrinA5, methylated sample, 4BK5; EphA4 ectodomain in complex with ephrinA5 4BA; and EphA4 ectodomain in complex with ephrinB3, 4BKE.

**Note:** Supplementary information is available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank Y. Zhao and W. Lu for protein expression and M. Jones and T.S. Walter for technical support. We are grateful to T. Gaitanos for help with confocal microscopy data acquisition, I. Davis and I.M. Dobble for discussion and assistance at the single-molecule–localization facility in the Micron Advanced Bioimaging Unit, J. Erl and M. Ponsier for assistance with cell-based assays, K.J. Morris for providing access to MetaMorph software and R.M. Esonuf for aiding in protein structure analysis. We thank the staff of the Diamond Light Source for assistance with diffraction data collection and K. Diederichs for help with data integration. This research was funded by a Cancer Research United Kingdom grant to E.Y.J. (grant A10979). Localization microscopy facilities in the Micron Advanced Bioimaging Unit were funded by the Wellcome Trust (grant 091911). E.S. was funded by an Intra-European Fellowship (Marie Curie); D.d.T.R. was funded by an European Molecular Biology Organization long-term fellowship; N.M. is supported by a Wellcome Trust D.Phil. studentship and A.R.A. was supported as a United Kingdom Medical Research Council Career Development Award Fellow. Partial funding was provided by the Deutsche Forshungsgemeinschaft (SFB870) to R. Klein and Wellcome Trust grant 090532/Z/09/Z supporting the Wellcome Trust Centre for Human Genetics.

**AUTHOR CONTRIBUTIONS**

E.S. performed protein crystallization, structure analysis, cell rounding assays and Eph clustering experiments. A.S. contributed to time-lapse imaging experiments. D.d.T.R. performed stripe assays. R. Kaufman conducted localization microscopy data acquisition and analysis. N.M. contributed to protein crystallization. K.H. performed Eph crystal mounting for data collection. A.R.A., R. Klein and E.Y.J. contributed to discussion at all stages of the project. All authors contributed to writing of the manuscript.

**COMPETING FINANCIAL INTERESTS**

The Authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
ARTICLES

1. Klein, R. Eph/ephrin signalling during development. Development 139, 4105–4109 (2012).

2. Battie, E. & Wilkinson, D.G. Molecular mechanisms of cell segregation and boundary formation in development and tumorigenesis. Cold Spring Harb. Perspect. Biol. 4, 211–226 (2012).

3. Pitulescu, M.E. & Adams, R.H. Eph/ephrin molecules: a hub for signaling and endocytosis. Genes Dev. 24, 2480–2492 (2010).

4. Pasquale, E.B. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat. Rev. Cancer 10, 165–180 (2010).

5. Brantley–Sieders, D.M. Clinical relevance of Ephs and ephrins in cancer: lessons from breast, colorectal, and lung cancer profiling. Semin. Cell Dev. Biol. 23, 102–108 (2012).

6. Nievergall, E., Lackmann, M. & Janes, P.W. Eph-dependent cell-cell adhesion and segregation in development and cancer. Cell Mol. Life Sci. 69, 1813–1842 (2012).

7. Gale, N.W. et al. Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. Neuron 17, 9–19 (1996).

8. Bowden, T.A. et al. Structural plasticity of eph receptor A4 facilitates cross-class ephrin signaling. Structure 17, 1386–1397 (2009).

9. Noberini, R., Rubio de la Torre, E. & Pasquale, E.B. Profiling Eph receptor expression in cells and tissues: a targeted mass spectrometry approach. Cell Adh. Migr. 6, 102–112 (2012).

10. Himanen, J.P. Ectodomain structures of Eph receptors. Semin. Cell Dev. Biol. 23, 35–42 (2012).

11. Janes, P.W., Nievergall, E. & Lackmann, M. Concepts and consequences of Eph receptor clustering. Semin. Cell Dev. Biol. 23, 43–50 (2012).

12. Himanen, J.P. et al. Crystal structure of an Eph receptor-ephrin complex. Nature 414, 933–938 (2001).

13. Smith, F.M. et al. Dissecting the EphA3/Ephrin-A5 interactions using a novel functional mutagenesis screen. J. Biol. Chem. 279, 9522–9531 (2004).

14. Seiradake, E., Harlos, K., Sutton, G., Aricescu, A.R. & Jones, E.Y. An extracellular steric seeding mechanism for Eph–ephrin signaling platform assembly. Nat. Struct. Mol. Biol. 17, 398–402 (2010).

15. Himanen, J.P. et al. Architecture of Eph receptor clusters. Proc. Natl. Acad. Sci. USA 107, 10860–10865 (2010).

16. Egea, J. et al. Regulation of EphA4 kinase activity is required for a subset of axon guidance decisions suggesting a key role for receptor clustering in Eph function. Neuron 47, 515–528 (2005).

17. Dufour, A. et al. Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes. Neuron 39, 453–465 (2003).

18. Kuillander, K. et al. Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. Science 299, 1889–1892 (2003).

19. Kao, T.J., Law, C. & Kania, A. Eph and ephrin signaling: lessons learned from spinal motor neurons. Semin. Cell Dev. Biol. 23, 83–91 (2012).

20. Wang, L., Klein, R., Zheng, B. & Marquardt, T. Anatomical coupling of sensory and motor nerve trajectory via axon tracking. Neuron 71, 263–272 (2011).

21. Britts, P.A., Lu, Q. & Flanagan, J.G. Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. Cell 110, 223–235 (2002).

22. Hafer, C. et al. Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. Clin. Chem. 50, 490–499 (2004).

23. Cooper, M.A. et al. Loss of ephrin-A5 function disrupts lens fiber cell packing and leads to cataract. Proc. Natl. Acad. Sci. USA 105, 16620–16625 (2008).

24. Qin, R. et al. Structural characterization of the EphA4–Ephrin-B2 complex reveals new features enabling Eph–ephrin binding promiscuity. J. Biol. Chem. 285, 644–654 (2010).

25. Poliakov, A., Cotrina, M. & Wilkinson, D.G. Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. Dev. Cell 7, 465–480 (2004).

26. Bush, E. & Diederichs, K. Linking crystallographic model and data quality. Science 336, 1030–1033 (2012).

27. Davis, I.W. et al. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res. 35, W375–W383 (2007).

28. Lemmer, P. et al. SPDM: light microscopy with single-molecule resolution at the nanoscale. Appl. Phys. B 93, 1–12 (2008).

29. Heilemann, M. et al. Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. Angew. Chem. Int. Ed. Engl. 47, 6172–6176 (2008).

30. Ripley, B.D. Modeling spatial patterns. J. Roy. Stat. Soc. B 39, 172–212 (1977).

31. Kaufmann, R., Mueller, P., Hildenbrand, G., Hausmann, M. & Cremers, C. Analysis of HerZhu membrane protein clusters in different types of breast cancer cells using localization microscopy. J. Microsc. 242, 46–54 (2010).

32. Wimmer-Kleikamp, S.H., Janes, P.W., Squire, A., Bastiaens, P.I. & Lackmann, M. Recruitment of Eph receptors into signaling clusters does not require ephrin contact. J. Cell Biol. 164, 661–666 (2004).

33. Tripplett, J.W. & Feldheim, D.A. Eph and ephrin signaling in the formation of topographic maps. Semin. Cell Dev. Biol. 23, 7–15 (2012).

34. Astin, J.W. et al. Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells. Nat. Cell Biol. 12, 1041–1052 (2010).

35. Salaita, K. et al. Restriction of receptor movement alters cellular response: physical force sensing by EphA2. Science 327, 1380–1385 (2010).

36. Janes, P.W. et al. Eph receptor function is modulated by heterooligomerization of A- and B-type Eph receptors. J. Cell Biol. 195, 1033–1043 (2011).

37. Freywald, A., Sharpe, N. & Rolfsman, C.M. The kinase-null EphB6 receptor undergoes transphosphorylation in a complex with EphB1. J. Biol. Chem. 277, 3823–3828 (2002).

38. Miao, H. 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 16, 9–20 (2009).
ONLINE METHODS

Vectors and cloning. Transmembrane constructs encoding human EphA2 (residues 27–976, UniProt P295317) and mouse EphA4 (residues 28–986, UniProt Q035137) were fused to an N-terminal Flag tag (TGGYKDQDDDK). Chimeric construct A2A4 was produced by fusion of EphA2 ectodomain (residues 27–534) to EphA4 transmembrane and intracellular regions (residues 548–986) and an N-terminal Flag tag. Chimeric construct A2A4 was produced by fusion of EphA4 ectodomain (residues 28–547) to EphA2 transmembrane and intracellular regions (residues 535–976) and an N-terminal Flag tag. Point mutants were generated by PCR techniques. All transmembrane Eph constructs were cloned into the AgeI–KpnI cloning site of a modified pHLsec vector49 encoding an N-terminal secretion signal sequence and a C-terminal mVenus or mCherry tag followed by a polyhistidine tag. Secreted proteins were produced by mixture of the concentrated EphA4ecto and ephrinB2 RBD solution (1.8 M ammonium phosphate and 100 mM HEPES, pH 7.4) and human ephrin RBDs (ephrinA5, residues 27–166, UniProt P52583; ephrinB3, residues 27–169, UniProt Q15768; and ephrinB2, residues 27–167, UniProt P52799) were cloned into the AgeI–KpnI sites of a pHLsec vector49 encoding an N-terminal secretion signal sequence and a C-terminal polyhistidine tag.

Protein purification and crystallization. We expressed EphA4ecto, ephrinA5 RBD and ephrinB3 RBD transiently50 in HeLaK293S cells51. Proteins were purified separately from cell culture medium by Ni-affinity and size-exclusion chromatography. Complexes were formed by mixing either before or after deglycosylation with endoglycosidase F1 (refs. 39,41) and subsequent lysine methylation by established protocols52 (Supplementary Note). All crystals were grown at 20 °C in sitting drops by vapor diffusion43. Crystals of ligand-free EphA4ecto were produced by mixture of the concentrated EphA4ecto and ephrinB2 RBD solution (Supplementary Note) in a 1:1 ratio (v/v) with crystallization solution 1 (1.8 M ammonium phosphate and 100 mM HEPES, pH 7.4). Crystals of lysine-methylated EphA4ecto, in complex with ephrinA5 RBD grew by mixture of concentrated sample 1:1 (v/v) with crystallization solution 2 (1.8 M ammonium phosphate and 100 mM HEPES, pH 7.4). Native crystals of EphA4ecto in complex with ephrinA5 RBD grew by mixture of concentrated sample 1:1 (v/v) with crystallization solution 3 (0.01 M MgSO4, 1.8 M LiCl, and 0.05 M sodium cacodylate, pH 6.5) and 4% benzamidine-HCl (Hampton). Native crystals of EphA4ecto in complex with ephrinB3 RBD grew by mixture of concentrated sample 1:1 (v/v) with crystallization solution 4 (0.08 M magnesium acetate tetrahydrate, 0.03 M cacodylate, pH 6.5, and 15% polyethylene glycol 400) and 0.1 M spermine (Hampton).

Structure determination. Diffraction data were collected at the Diamond Light Source (beamlines I24, I04 and I04-1) at 100 K. Crystals were flash frozen after brief dipping into reservoir solution supplemented with 15–25% glycerol. Diffraction data were processed with XIA2 (ref. 44), XD2MOSFILM46 and CCP4 suite47. The structure of ligand-free EphA4ecto was solved from subdomains of the previously published EphA2ecto model (PDB 2X1014) as inputs for molecular replacement in PHASER48. The resultant model was improved with the MR_ROSETTA49 implementation in PHENIX50. Electron-density modification was done with PARROT51, manual improvement of the model in COOT52 and final rounds of refinement in autoBUSTER53, with strong stereochemical restraints and TLS. The resultant model was used to phase the data for lysine-methylated EphA4ecto in complex with ephrinA5 RBD. The structure of ephrinA5 RBD, as found in complex with EphA2ecto (PDB 2X114), was placed manually by superposition. Individual domains were refined with rigid-body refinement in PHASER50, and minor adjustments were performed by hand in COOT52. Electron-density modification was done with PARROT51. A final round of refinement was done in autoBUSTER52, with strong stereochemical restraints and TLS. The resultant model was used to phase the data for native EphA4ecto in complex with ephrinA5 RBD. We used the refinement program in PHENIX50 and REFMAC5 (ref. 54) for rigid-body and TLS refinement of individual domains. Electron-density modification was done with PARROT51. The structure of EphA4ecto in complex with ephrinB3 RBD was solved from the model for ligand-free EphA4ecto and the model for ephrinB3 RBD as found in complex with the Nipah virus G attachment glycoprotein (PDB 3D1255). Rigid-body and TLS refinement were done in PHENIX50 and BUSTER56 and electron-density modification in PARROT51.

HeLa cell culture assay. HeLa cells were grown in live-cell imaging chambers (Lab-Tek, cat. no. 155380) at 37 °C in Dulbecco’s Modified Eagle’s Medium (DMEM, 4.5 g/l glucose, + 1-glutamine, no pyruvate, Gibco) supplemented with 1× 1-glutamine (PAA), 10% fetal calf serum (HyClone) and 1× penicillin and streptomycin (PAA). Cells were transfected with Fugene or X-tremeGene HP DNA transfection reagent (Roche) according to the recommended protocol, with 0.2 μg plasmid DNA per milliliter. After 12–15 h, the medium was changed to imaging medium (DMEM, PAN Biotech GmbH, cat. no. P04-05545) supplemented with 1× 1-glutamine, 0.5% fetal calf serum (HyClone) and 1× penicillin and streptomycin (PAA) and the cells incubated at 37 °C for 2–6 h. EphrinA5-Fc (R&D, cat. no. 374-EA) at 0.2 mg ml⁻¹ in PBS was mixed with goat anti-human IgG Fcy (Jackson ImmunoResearch, cat. no. 109-005-098) and imaging medium in a 5:1:28 ratio and incubated for 2 h at room temperature. For stimulation, the ephrinA5-Fc mixture was further diluted with prewarmed imaging medium and added to the cells in a final concentration of 2 μg ephrinA5-Fc and 0.04 μg anti-IgG per ml. Cells were imaged with a Zeiss Axiovert 200M microscope equipped with a temperature-controlled carbon dioxide incubation chamber set to 37 °C, 65% humidity and 5% CO2. Illumination was provided by an X-Cite lamp (series 120, Lumen Dynamics Group), and images were recorded by a Coolspool HQ camera (Photometrics). Sequential images were acquired before and every 6 min following preclustered ephrin-Fc addition. MetaMorph imaging software ( Molecular Devices) was used to analyze cell cluster responses and to assemble movies.

Costimulation assay. COS7 cells were transfected and stimulated with ephrinA5-Fc and anti-human IgG as described above for HeLa cells. Cells were imaged with a confocal spinning-disc microscope (Zeiss AXIO Observer Z1) and temperature-controlled incubation chamber (Zeiss) set to 37 °C, before and every 4 min after stimulation at 63× magnification. Z-stack slices of 0.5 μm were taken. MetaMorph imaging software was used to pick and project three in-focus Z-planes per image and to assemble movies.

Localisation microscopy–based analysis. COS7 cells were cultured and transfected with mVenues-tagged Eph constructs as described in previous sections. We prepared paraformaldehyde-fixed samples of nonstimulated COST cells and of COST cells after 10 min of stimulation with ephrinA5-Fc and anti-IgG. Samples were mounted on regular glass slides with fluorescence mounting medium (Dako). An OMX (optical microscope experimental, V2, API) was modified to enable localisation microscopy with conventional fluorescent proteins56. The intensity of the 488-nm laser was adjusted to ~14 kW/cm² in the object plane to drive the fluorophores into a long-lived dark state; a stochastic recovery of individual molecules to the fluorescent state was recorded by image sequences of 2,000 frames and an integration time of 50 ms per frame. The positions of single molecules were calculated with an estimated mean localization accuracy of ~21 nm with a maximum likelihood–based algorithm57 that we adapted to the OMX hardware configuration. The resulting data sets were sorted according to their mean point densities (number of molecules recorded per square micrometer). To control for the effect of Eph expression level on clustering, only data sets with similar mean point densities (~170/μm²) were selected for further analyses. To analyze the distances between neighboring molecule positions, we used Ripley’s L function58, which takes into account distances expected for a homogenous distribution and allows interpretation of the strength of clustering (amplitude) and gives an indication of the size and size variance of clusters (position along the x axis, width of the distribution). Receptor clusters were identified and analyzed by a previously described algorithm53 based on a threshold value of local point density. For the present analysis the value defining what was regarded as a cluster was set to >20 molecule positions within a radius of 30 μm (~2.670 points/μm²).

StripE assay. EphiR A5-Fc protein (12.5 μg/ml; R&D cat. no. 374-EA) was preclustered (ratio 1:3) with Alexa594-conjugated anti-hFc antibody (Invitrogen, cat. no. A11014) in PBS for 30 min. Clustered ephrinA5-Fc was injected into matrices (90-μm width) placed on 60-mm dishes35, to result in the first red fluorescent stripes. After 30-min incubation at 37 °C, dishes were washed with PBS, and matrices were carefully removed. The dishes were further coated with 12.5 μg/ml of Fc (Jackson, cat. no. 009-000-008) protein preclustered with anti-hFc (Jackson, cat. no. 109-005-098; no fluorescent dye) for 30 min at 37 °C. After that, stripes were washed three times with PBS. HeLa cells, transfected with Fugene HD transfection reagent (Roche) according to the manufacturer’s instructions, with 0.6 μg per well (six-well format), were cultured for 24 h on the
stripes (30,000 cells per carpet). Cells were fixed with 4% PFA in PBS for 20 min at RT and washed with PBS. The mVenus tag in the Eph constructs was used to visualize transfected cells. Images of cells and stripes were acquired with an Axioplan epifluorescent microscope (Zeiss) at 10× magnification. Images containing stripes were further separated into two parts (on red or black stripes). The total numbers of Venus+ pixels on red stripes (ephrinA5-Fc) were quantified with ImageJ.

46. Leslie, A.G. The integration of macromolecular diffraction data. Acta Crystallogr. D Biol. Crystallogr. 62, 48–57 (2006).
47. Collaborative Computational Project 4. The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. 50, 760–763 (1994).
48. McCoy, A.J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
49. DiMaio, F., Tyka, M.D., Baker, M.L., Chiu, W. & Baker, D. Refinement of protein structures into low-resolution density maps using rosetta. J. Mol. Biol. 392, 181–190 (2009).
50. Zwart, P.H. et al. Automated structure solution with the PHENIX suite. Methods Mol. Biol. 426, 419–435 (2008).
51. Zhang, K.Y., Cowtan, K. & Main, P. Combining constraints for electron-density modification. Methods Enzymol. 277, 53–64 (1997).
52. McCoy, A.J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
53. Xu, K. et al. Host cell recognition by the henipaviruses: crystal structures of the Nipah G attachment glycoprotein and its complex with ephrin-B3. Proc. Natl. Acad. Sci. USA 105, 9953–9958 (2008).
54. Adams, P.D. et al. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr. D Biol. Crystallogr. 58, 1948–1954 (2002).
55. Winter, G. xia2: an expert system for macromolecular crystallography data reduction. J. Appl. Crystallogr. 43, 186–190 (2010).