

Tie1-Tie2 Interactions Mediate Functional Differences between Angiopoietin Ligands

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SUMMARY

The Tie family of endothelial-specific receptor tyrosine kinases is essential for cell proliferation, migration, and survival during angiogenesis. Despite considerable similarity, experiments with Tie1- or Tie2-deficient mice highlight distinct functions for these receptors *in vivo*. The Tie2 receptor is further unique with respect to its structurally homologous ligands. Angiopoietin-2 and -3 can function as agonists or antagonists; angiopoietin-1 and -4 are constitutive agonists. To address the role of Tie1 in angiopoietin-mediated Tie2 signaling and determine the basis for the behavior of the individual angiopoietins, we used an *in vivo* FRET-based proximity assay to monitor Tie1 and -2 localization and association. We provide evidence for Tie1-Tie2 complex formation on the cell surface and identify molecular surface areas essential for receptor-receptor recognition. We further demonstrate that the Tie1-Tie2 interactions are dynamic, inhibitory, and differentially modulated by angiopoietin-1 and -2. Based on the available data, we propose a unified model for angiopoietin-induced Tie2 signaling.

INTRODUCTION

Tie1 and Tie2 are type 1 transmembrane protein receptor tyrosine kinases (RTKs) (Ward and Dumont, 2002; Yancopoulos et al., 2000). Along with the vascular endothelial growth factor (VEGF) receptor, these are the only known endothelial cell-specific RTKs. Studies with dominant-negative and null mice reveal that loss of *tie2* function results in embryonic death because of a failure of the vasculature system to expand (Dumont et al., 1994; Sato et al., 1995). Thus, it was proposed that *tie2* is not required for the differentiation of endothelial cells, but rather for their maintenance and proliferation (Dumont et al., 1994). Mice lacking *tie1* also die *in utero*, most likely a result of pulmonary edema (Puri et al., 1995). Although the vasculature remains intact, the integrity of vessel endothelial cells is compro-

mised. Accordingly, *tie1*-null mice display defects in vessel integrity, demonstrated by localized hemorrhaging and the presence of an underdeveloped heart. However, the exact role of Tie1 in angiogenesis remains unknown. Considerable evidence also identifies the angiopoietins and the Tie2 receptor as important regulators of tumor-induced angiogenesis and, therefore, cancer growth and metastasis (Lin et al., 1998; Lin et al., 1997; Oliner et al., 2004).

The two receptors are remarkably similar, with two amino-terminal immunoglobulin (Ig) domains, followed by three epidermal growth factor (EGF) repeats, a third Ig domain, and finally, three fibronectin type III repeats in the extracellular region. Both Ties also contain a catalytic carboxyl-terminal tyrosine kinase domain responsible for intracellular signaling. Although binding studies originally identified both the Ig and EGF domains of Tie2 as necessary and sufficient for angiopoietin binding (Barton et al., 2005; Fiedler et al., 2003), more recent mutagenesis and crystallographic data have revealed the ligand-binding site as the second Ig domain (Barton et al., 2006).

Initially described as an orphan receptor (Dumont et al., 1992), Tie2 was subsequently shown to interact with all four of the angiopoietins (Ang1–Ang4) (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The different angiopoietins, although having high sequence homology, elicit different responses from the RTK Tie2. Indeed, Ang1 is a constitutive receptor agonist, while Ang2 is a context-dependent one (Davis et al., 1996; Gale et al., 2002; Maisonpierre et al., 1997). The hypothesis that differential presentation and binding of the various angiopoietins to Tie2 is responsible for their distinct biological effects appears unlikely in light of previous evidence that Ang1 and -2 bind to the same region of Tie2 (Barton et al., 2005; Barton et al., 2006; Maisonpierre et al., 1997).

Now, it appears more likely that other cell-specific surface receptors exist that could help transduce the angiopoietin signals and modulate their functional potential. This hypothesis was recently supported by several reports defining a potential role for Tie1 in Tie2 signaling (Kim et al., 2006; Saharinen et al., 2005; Yuan et al., 2007). Tie1, although a close sequence homolog of Tie2, does not interact directly with the angiopoietins, and its *in vivo* ligands are yet to be identified (Maisonpierre et al., 1997). Nevertheless, the angiopoietins may affect Tie1 function (Kim et al., 2006; Saharinen et al., 2005; Yuan et al., 2007). As observed by coimmunoprecipitation analysis, Tie1

and -2 appear to associate on the cell surface with receptor phosphorylation correlating with activation. A recent study using catalytically inactive Tie2 demonstrated that Tie1 phosphorylation can occur in *trans*, and is dependent on a functional Tie2 (Kim et al., 2006; Yuan et al., 2007).

Although great strides have been made in the elucidation of the structural and molecular mechanisms of the Ang-Tie signaling, many essential interactions and functional roles have yet to be determined. Of fundamental importance is the precise role of the Tie1 receptor in Tie2 signaling. It remains unclear when and how Tie1 and -2 associate on the cell surface. Furthermore, the ultimate biological significance of these interactions has yet to be determined, particularly with regard to the specific signaling characteristics of the individual angiotensins.

To better understand the role and mechanism of action of Tie1 in angiotensin-mediated signaling, we examined Tie1 and -2 receptor localization and association in the presence and absence of angiotensin ligand on the cell surface via a FRET-based proximity assay. Based upon our studies, we propose a new model for the role of the Tie1 in angiotensin-induced Tie2 signaling.

RESULTS

Pre-existent Tie1-Tie2 Complexes Observed by FRET Imaging on the Cell Surface

To examine the potential role of Tie1 as a coreceptor and evaluate Tie1-Tie2 interactions on the cell surface, we monitored receptor-receptor interactions in vivo by tagging the individual proteins with CFP and YFP, and following their localization and association during both agonist and antagonist angiotensin signaling by confocal microscopy coupled with fluorescence (Förster) resonance energy transfer (FRET). Specifically, for assessing Tie1 and -2 receptor spatial proximity during angiotensin signaling, we exploited the FRET methodology recently utilized by the Tsien and Springer groups to analyze the role of lipid modifications in membrane partitioning and integrin signaling, respectively (Kim et al., 2003; Zacharias et al., 2002). We fused the *monomeric* enhanced green fluorescent protein variants mCFP and mYFP to the carboxyl termini of Tie2 and Tie1, respectively, in place of the catalytic tyrosine kinase domain. As energy transfer between donor (CFP) and acceptor (YFP) only occurs over short distances (≤ 10 nm), emission of YFP following excitation of CFP is observed only when Tie1 and -2 are in close proximity, presumably as a receptor/coreceptor complex (illustrated conceptually in Figure 1A). FRET efficiency is calculated by subtracting the background FRET (determined by the acceptor-photobleaching method) from the experimental FRET efficiency (Wouters et al., 2001).

Through the use of our FRET-based proximity assay, we clearly observe Tie1-Tie2 association on the cell surface, indicating that the two receptors are within less than 100 Å of one another. Interestingly, our observations are in the absence of angiotensin ligand, further demonstrating that Tie1 and -2 are in a pre-existent complex prior to ligand recognition. Figures 1B–1C illustrates a representative image of the fluorescence intensity and membrane localization of Tie2-CFP/Tie1-YFP, which we will denote as wild-type for our discussions. Both

receptors localize with uniform diffuse staining, predominantly on the plasma membrane. Using the acceptor-photobleaching technique for measuring FRET efficiency, we observed Tie1-Tie2 association with an average overall efficiency of 30%, 27%, and 19% under optimal conditions in human embryonic kidney (HEK) 293, U2OS, and EA.hy 926 cells, respectively (Figures 1B and 1C; see also Figure S1 in the Supplemental Information available with this article online). In accordance with the variables identified by Springer et al. (Kim et al., 2003), the length of linker between receptor transmembrane domain and amino-terminus of fluorescent protein was varied to identify the optimal combination of receptor length, and fluorophore-receptor pair (data not shown). Furthermore, for our experiments, HEK293 and U2OS cells were chosen in addition to EA.hy 926 endothelial cells, based upon their lack of endogenous Tie receptors, as well as ease of transfection and cellular imaging (Yuan et al., 2007).

We found, as expected, that increased linker lengths significantly decreased overall FRET efficiency (Kim et al., 2003). Among several constructs tested, one pair with 11 and 10 residues for Tie1 and -2, respectively, within the linker consistently yielded reliable results (data not shown) in all three independent cell lines (EA.hy 926, HEK293, and U2OS). Importantly, both CFP and YFP variants carry the A206K, L221K, and F223R mutations thought to significantly decrease the chance of fluorescent protein multimerization (Zacharias et al., 2002). As a control, we also coexpressed each receptor with both fluorophores to account for any nonspecific interactions that may occur between them. No FRET signal was observed under these circumstances (data not shown). Similarly, to evaluate possible effects of overexpression, Tie receptors were coexpressed with the functionally unrelated receptor plexin-A1 (both CFP and YFP variants) (Figures S2A and S2B). Under these circumstances, we observed between 0% and 2% FRET efficiency. The five experimental conditions are graphically compared in Figure 1D and summarized in Table S1. Together, these findings validate the overall specificity of the assay, and reveal the conservation of Tie receptor interactions within both epithelial and endothelial cell lines, while simultaneously demonstrating that protein overexpression does not contribute significantly to FRET efficiency.

Homology Modeling of Tie1

To understand the potential structural differences between Tie1 and -2 that mediate their distinct biological properties and identify structural elements that may contribute to Tie1-Tie2 interactions, we modeled the structure of Tie1 using the experimentally determined 2.5 Å Tie2 structure (Barton et al., 2006) and the program MODELER (Marti-Renom et al., 2000). Tie1 and -2 are highly homologous—sharing 39% amino acid identity—and, not surprisingly Tie1 can be easily modeled on the structure of Tie2. A schematic representation of the molecular surfaces of Tie2 and of the Tie1 model, color coded according to electrostatic potential and hydrophobicity of the exposed amino acid side chains, is presented in Figure 2. The hydrophobic surface features of Tie1 and -2 are very similar overall. Interestingly, two patches of exposed hydrophobic residues, indicated with arrows, are present at the tip of Tie2, but are absent in the equivalent Tie1 region. Indeed, these overlap with the binding site of the Tie2-specific ligand Ang2, indicated with a green circle

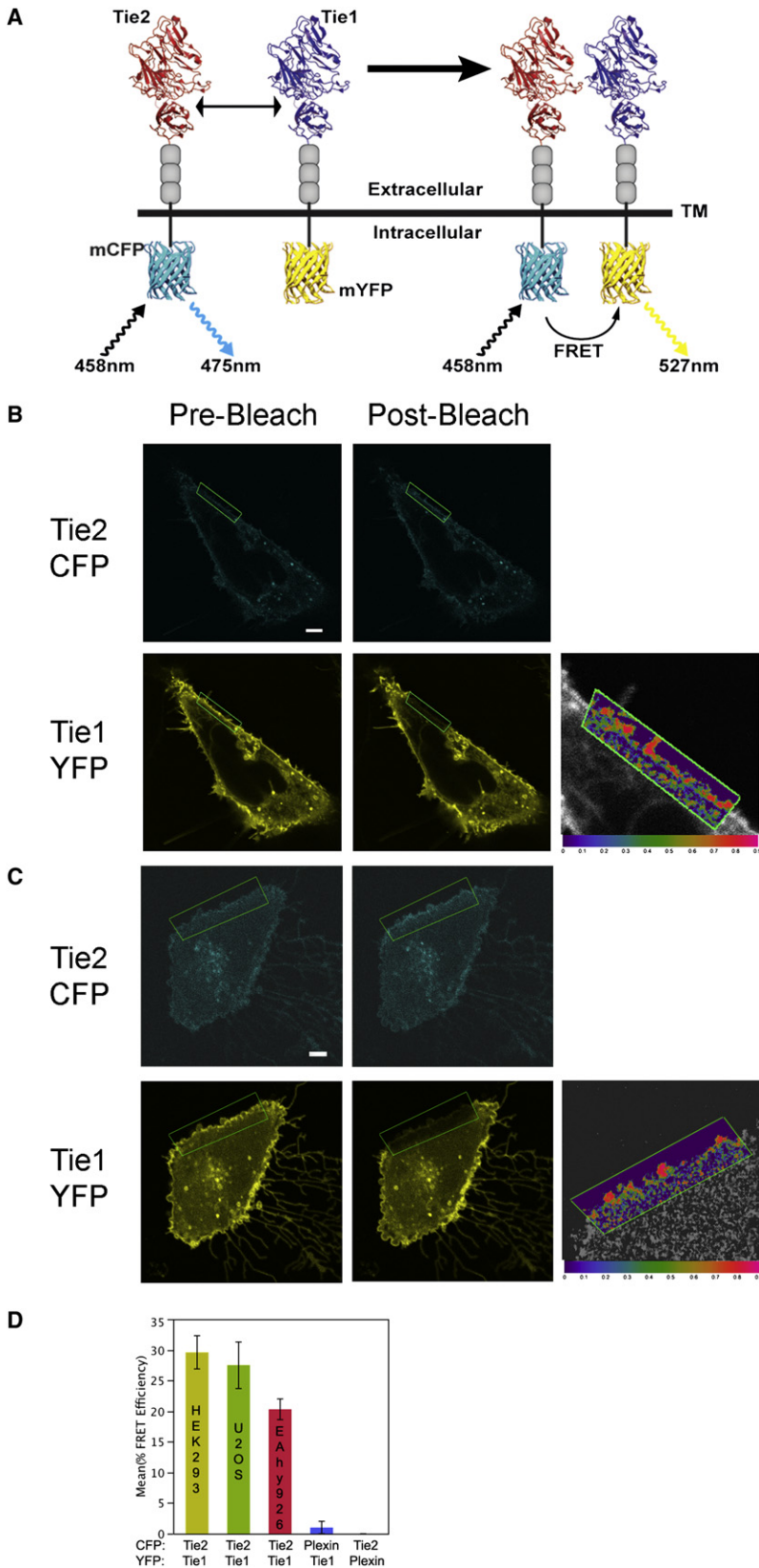


Figure 1. Association of Tie1 and -2 on the Cell Surface as Analyzed by the FRET-Based Spatial Proximity Assay

(A) Schematic representation of the spatial proximity assay used to monitor Tie1-Tie2 interactions in vivo. Receptor association allows measurable FRET between CFP and YFP via confocal microscopy. Receptor dimerization is shown for simplicity.

(B and C) Tie1-YFP and Tie2-CFP coexpression and association in U2OS (B) and EA.hy 926 (C) cells (see also Figure S1 for coexpression and localization in HEK293 cells). The majority of the receptor-fluorophore fusion localizes predominantly to the plasma membrane (although a fraction is also observed in the secretory system).

(D) Graphical representation of average FRET values for wild-type Tie1 and -2, as well as control receptors (control experiments were conducted in U2OS cells and displayed in Figure S2). Data are represented as mean \pm SEM. For all images CFP is displayed as cyan and YFP as yellow.

Photobleaching experiments were restricted to, and FRET values calculated from, the region within the green box in (B) and (C). Both pre- and postphotobleaching images are displayed in the left and right panels, respectively. FRET efficiency is displayed as an absolute range from high (red, 1.0) to low (purple, 0.0) on a magnified overlay of a CFP/YFP merged image for orientation purposes only. Scale bars, 10 μ m.

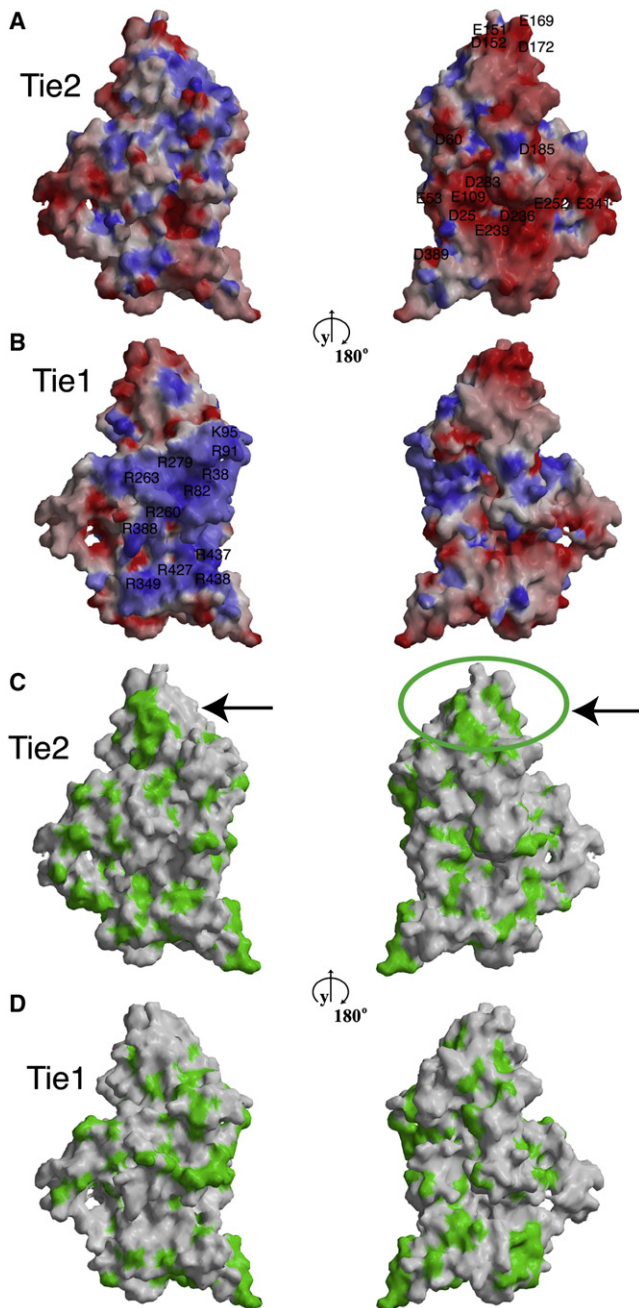


Figure 2. Comparison of the Molecular Surface Properties of Tie1 and -2

Molecular surface representation of Tie2 (A–C) and of the modeled Tie1 (B–D) structure, color-coded according to electrostatic surface potential (A and B) or hydrophobicity (C and D). Red and blue represent electrostatic potentials in the range of -11 to $+11$ $k_B T$, where k_B is the Boltzman constant and T is the temperature (293K). Green represents surface residues with hydrophobic side chains. The left and right panels are related by 180° rotation about the y axis. Arrows point to the previously determined ligand-binding region within the tip of the second Ig domain in Tie2.

(Barton et al., 2006), providing the structural explanation for the distinct ligand-binding properties of the two Tie receptors.

Comparison of the surface electrostatic potentials of Tie1 and -2 (Figures 2A and 2B) reveals that Tie2 has a slight negative overall charge, with a theoretical pI value, if isolated in solution, of 6.9. Tie1, on the other hand, has a positive overall charge, with a corresponding pI value of 9.3. Tie2 has one expansive negatively charged surface area, which results from the approximation of several exposed aspartic and glutamic acids in Ig1 and EGF1, including D25, E53, D60, E109, D236, D239, D252, and D283 (Figure 2A, right). The corresponding Tie1 surface area is overall neutral in charge. Tie1, on the other hand, contains one large positively charged area on its surface, located on the opposite side of the molecule (Figure 2B, left). Its electrostatic surface potential results from the approximation of several arginines and lysines in Ig1, EGF2, and Ig3, including R38, R82, K95, R91, R260, R279, R263, R349, R388, R427, R437, and R438.

Identification of Residues Involved in Tie1-Tie2 Interactions

The presence of large patches of oppositely charged molecular surfaces in Tie1 and -2 suggest that these areas might be involved in Tie1-Tie2 recognition. To evaluate their roles in mediating receptor-receptor interactions, we utilized site-directed mutagenesis in combination with the proximity assay utilized in Figure 1. Various “regions” within the charged patches were targeted by mutagenesis through the construction of Tie variants containing multiple amino acid substitutions, and analyzed for their ability to associate via FRET. Our experience has shown that single-site mutations often have little or no effect on interactions involving relatively large protein-protein interfaces (Barton et al., 2003; Barton et al., 2005; Barton et al., 2006), and, therefore, we made multiple mutations simultaneously (typically two or three), following a charge reversal strategy (replaced basic residues with acidic amino acids, and vice versa). As all of these mutations are in surface-exposed residues, they likely do not affect the overall folding of the proteins. In agreement, all mutant receptors are well expressed, processed, and localize correctly to the cell surface (Figures 3A and 3B and data not shown). A list of all seven mutant receptor combinations, including averaged FRET efficiencies, is included in Table S1 and schematically displayed in Figure 3C.

Upon examination of our individual receptor mutants, it is clear that we have identified several charged surface residues involved in their association. Indeed, three of the four Tie1 mutants (B1, C1, and D1) and all three Tie2 variants (A2, B2, and C2) exhibit significantly lower FRET efficiencies than the wild-type receptors. A representative image of the receptor pair, Tie1-YFP-B1/wild-type-Tie2-CFP, is displayed in Figure 3A. Even under ideal conditions, we failed to observe significant FRET when these two receptors are coexpressed in either HEK293 or U2OS cell lines (0% FRET efficiency). Similarly, Tie2-CFP-A2 exhibits proper localization to the membrane, yet fails to associate with wild-type Tie1-YFP to any significant extent (5% FRET efficiency [Figure 3B]). Together, the data suggest a complex interaction between Tie1 and -2 involving a broad surface area within their basic and acidic regions, respectively. Indeed, several different mutant receptor combinations

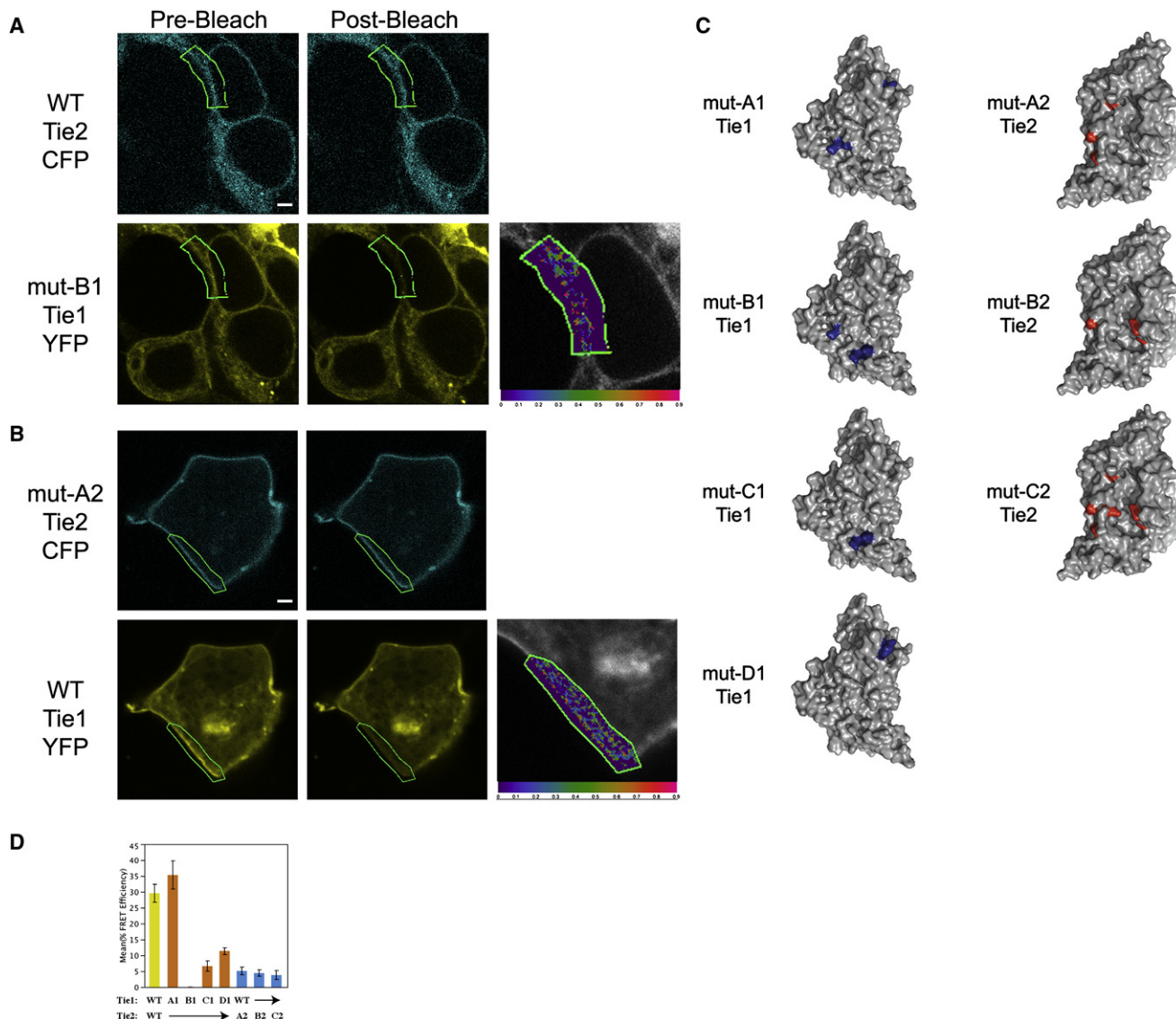


Figure 3. Complementary Electrostatic Surface Patches Mediate Tie1-Tie2 Association

(A and B) HEK293 cells were transfected with (A) wild-type Tie2-CFP and mutant Tie1-YFP B1, or (B) wild-type Tie1-YFP and mutant Tie2-CFP A2, and monitored by confocal microscopy. Individual mutations are abbreviated for clarity and are: A1, Tie1 (K95E, R260E, R263E); B1, Tie1 (R260E, R437E, R438E); C1, Tie1 (R437E, R438E); D1, Tie1 (R91E, K95E, R427E); A2, Tie2 (E53K, D236K, E239K); B2, Tie2 (E53K, D60K, D389K); and C2, Tie2 (E53K, D60K, E109K, D236K, E239K, D389K). Images are as described in Figure 1.

(C) Molecular surface representation of Tie2 and modeled Tie1 structure, color-coded according to their corresponding mutations. Images are as described in Figure 1.

(D) The average FRET efficiencies from the pairing of wild-type and mutant receptors. Data are represented as means \pm SEM.

with a wild-type coreceptor result in the loss of receptor-receptor association, as illustrated by low or absent FRET efficiencies, demonstrating that the charged surface patches play a role in the Tie1-Tie2 association (see Figure 3D and Table S1). Moreover, analysis of the Tie1 mutants suggests that residues R260, R437, and R438 in the bottom half of the ectodomain (see Figures 2B and 3C) play a central role in receptor association (compare mutants A1, B1, and C1 in Figure 3D). Finally, the identification of mutant-wild-type receptor pairs that fail to

demonstrate appreciable FRET further demonstrates the exquisite specificity of this proximity assay.

Tie1 and -2 Interactions Are Direct

To validate the notion that Tie1-Tie2 interactions are direct, and not mediated by an unidentified binding partner, we assayed for receptor complementation—as illustrated by the restoration of wild-type FRET efficiencies—between two individual mutant receptors. HEK293 cells were cotransfected with all nine

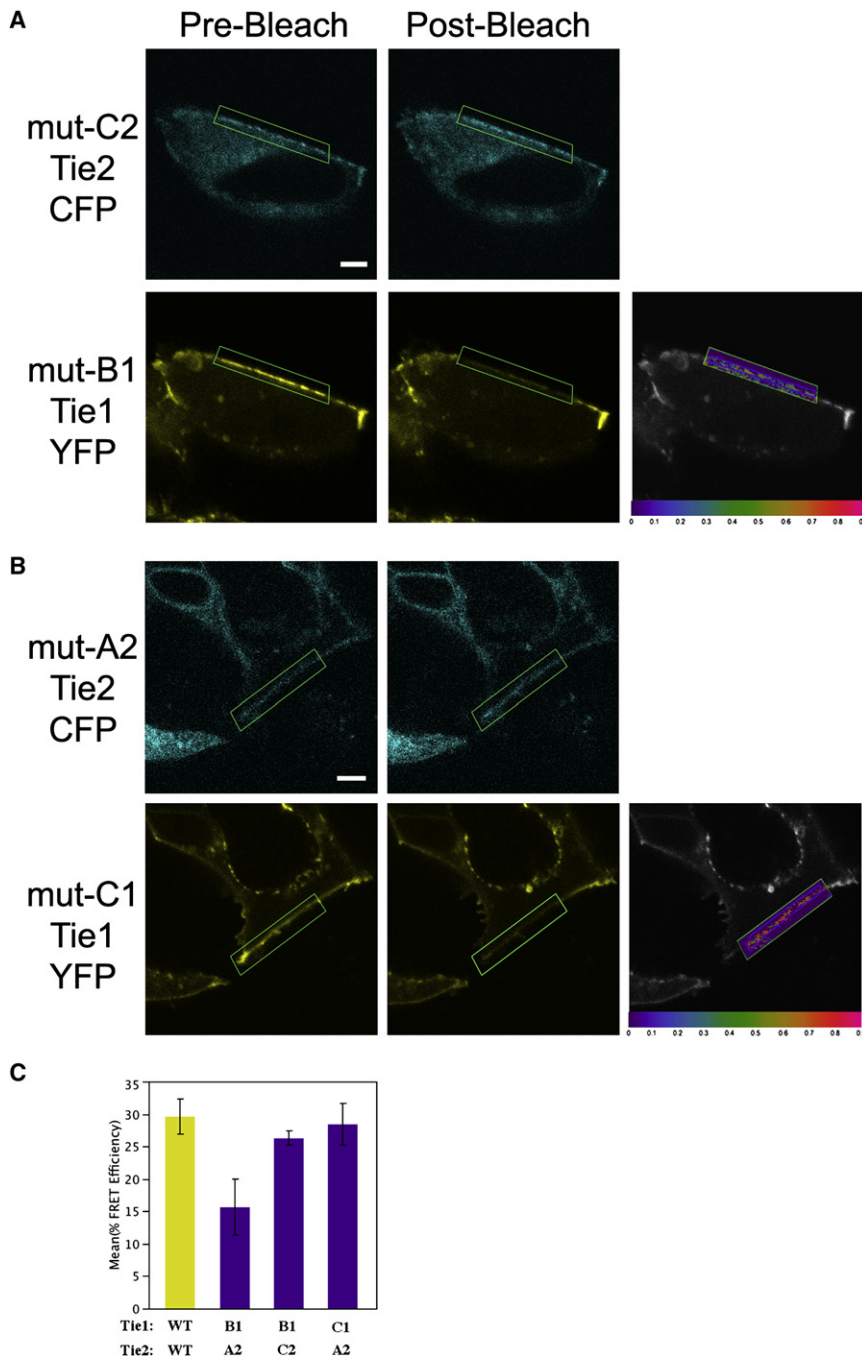


Figure 4. Tie1 and -2 Form a Direct Interaction as Demonstrated by Allelic Suppression

HEK293 cells were transfected with the mutant receptor pairs Tie1-YFP B1 and Tie2-CFP C2 (A) or Tie1-YFP C1 and Tie2-CFP A2 (B), and monitored by confocal microscopy. (C) The average FRET efficiencies from experiments in (A) and (B). Data are represented as means \pm SEM. Images are as described in Figure 1.

receptors is incapable of interacting with their counterpart wild-type receptor or other mutant receptors, yet when coexpressed, display FRET efficiencies nearly identical to that of wild-type receptors (Figures 4A and 4B). Averaged FRET efficiencies from several independent experiments for each receptor pair are graphed and compared to wild-type in Figure 4C. FRET efficiencies between wild-type receptors and these two mutant pairs are not significantly different. This demonstration of “allelic suppression” excludes the possibility of an unknown binding partner, and provides strong support for a direct interaction between Tie1 and -2 involving the complementary charged surface areas within the receptor ectodomains.

Different Angiotensins Differentially Modulate the Tie1-Tie2 Interaction

Despite the high level of sequence identity between Tie2 and -1, only Tie2 can form high-affinity complexes with all four known angiotensins, while Tie1 does not bind any of them (Davis et al., 2003; Ramsauer and D’Amore, 2002). Nevertheless, the identification of pre-existing Tie1-Tie2 complexes suggests that Tie1 plays a role in Tie2 signaling. Interestingly, our crystal structure of Tie2 bound to Ang2 (Barton et al., 2006) and to Ang1 (unpublished data) show that all

possible combinations of mutant Tie1 and -2 receptors. Given the size and complexity of the potential interface involved, only a subset of receptor combinations would be predicted to have molecular surfaces of sufficient complementarity to rescue the FRET efficiency to near-wild-type levels. Indeed, from all tested receptor combinations, we identified only two mutant receptor pairs: Tie1-YFP-B1/Tie2-CFP-C2, and Tie1-YFP-C1/Tie2-CFP-A2, which display significant receptor association (representative FRET efficiencies for each receptor pair are shown in Figure 4C and Table S1). Individually, each of these mutated

angiotensins bind Tie2 in a similar conformation, excluding the possibility of differential Tie2 activation resulting from alternate Tie2/angiotensin structural arrangements. However, our structural analysis does suggest that the different angiotensin ligands could present distinct molecular surfaces outside of the receptor-binding interface that could influence the Tie1-Tie2 receptor complexes (Barton et al., 2005). Based upon our findings, we hypothesized that the observed direct Tie2-Tie1 interactions are inhibitory, and the ability, or inability, of individual angiotensins to effectively destabilize the Tie1-Tie2 complexes at

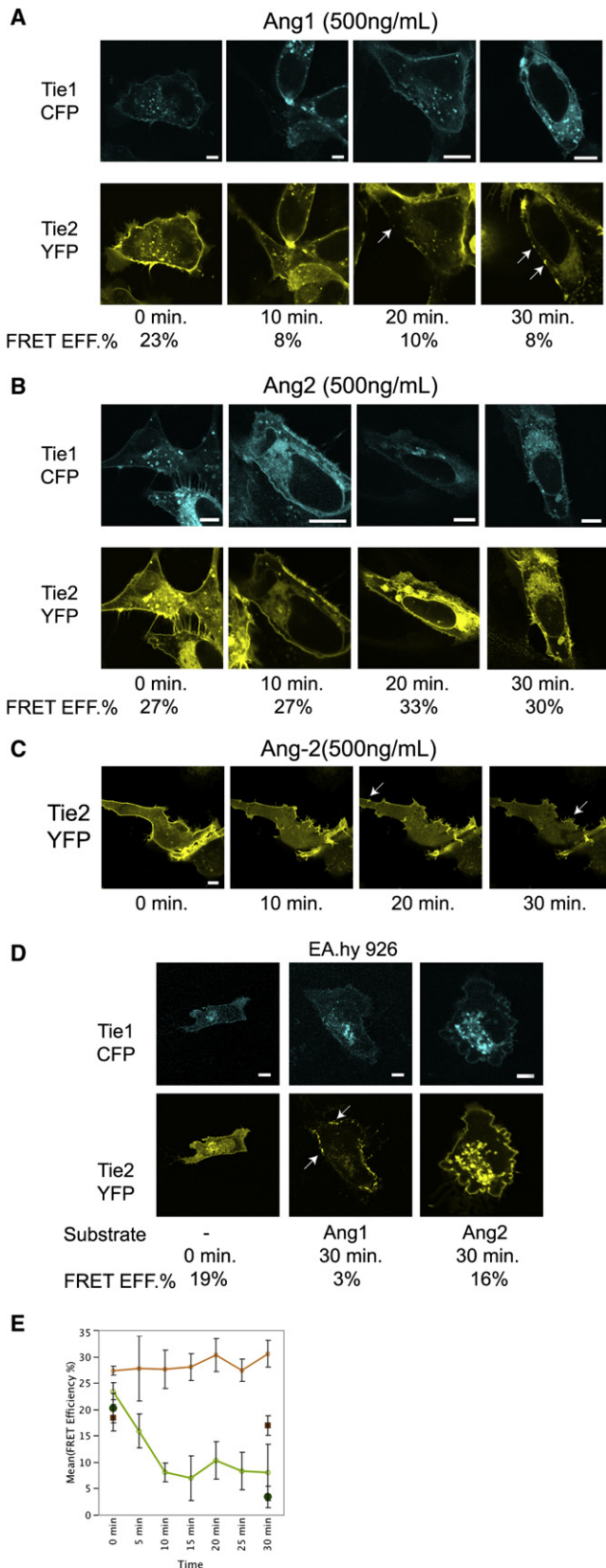


Figure 5. Ang1, but Not Ang2, Promotes Tie2 Clustering and Dissociation of Tie1-Tie2 Complexes

(A and B) U2OS cells were transfected with both Tie2-YFP and Tie1-CFP and monitored by confocal microscopy over a period of 30 min. At time 0, either 500 ng/ml of Ang1 (A) or 500 ng/ml of Ang2 (B) was added to the growth media. Listed below each representative image are averaged FRET values correlating to the listed time point. Arrowheads indicate regions of clustered Tie2.

(C) U2OS cells were singly transfected with Tie2-YFP and monitored by confocal microscopy over a period of 30 min following addition of 500 ng/ml of Ang2 to the growth media.

(D) EA.hy 926 cells were transfected with both Tie2-YFP and Tie1-CFP and monitored by confocal microscopy over a period of 30 min. At time 0, either 500 ng/ml of Ang1 (middle panels), or 500 ng/ml of Ang2 (right panels) was added to the growth media. Listed below each representative image are averaged FRET values correlating to the listed time point.

(E) The average FRET efficiencies from experiments in (A)–(C). Green and orange lines represent Ang1 or Ang2 addition for U2OS cells, respectively, while closed green circles and closed orange squares represent Ang1 or Ang2 addition for EA.hy 926 cells, respectively. Data are represented as means \pm SEM.

the cell surface would define their respective agonistic or antagonistic roles.

To assess the role of Tie1 in Tie2 clustering and activation following angiopoietin exposure, receptor association, localization, and activation was monitored in the presence of Ang1 or -2. Specifically, Tie1-CFP and Tie2-YFP were expressed in U2OS and EA.hy 926 cells and stimulated with varying concentrations of Ang1 or -2, and followed by confocal microscopy over the course of 30 or 60 min. As shown in [Figure 5A](#) (and via time-lapse imaging in the [Supplemental Information](#)), addition of Ang1 to the culture media led to a drastic reduction of Tie1-Tie2 association in U2OS cells within 10 min, as measured by FRET. This timeframe corresponds to previously determined rates of Tie2 activation ([Bogdanovic et al., 2006](#); [Yuan et al., 2007](#)). Furthermore, the loss in FRET signal was followed by a dramatic change in Tie2 localization (compare times 0 and 20 min in the lower panel of [Figure 5A](#)). Specifically, the majority of the membrane-associated Tie2 migrates and forms discrete foci, reminiscent of the Tie2 punctate staining observed by others in human lung microvascular endothelial cells ([Fukuhara et al., 2008](#); [Saharinen et al., 2008](#)). At 30 min after Ang1 addition, cells that contain discrete Tie2 foci are still observed (lower panel of [Figure 5A](#)). As expected from our FRET analysis, Tie1 localization remains undisturbed following Ang1 stimulation (compare bottom and top panels of [Figure 5A](#)). EA.hy 926 endothelial cells under similar conditions behave correspondingly. In these cells, Tie2 readily localizes to the membrane, yet transforms to punctate staining with similar kinetics to HEK293 cells upon addition of Ang1 ([Figure 5D](#), middle panels). FRET efficiency rapidly decreases (from 19% to 3%), further demonstrating disruption of Tie1-Tie2 interactions.

Alternatively, when Tie1- and Tie2-expressing U2OS or EA.hy 926 cells are treated with similar concentrations of Ang2, no changes in the Tie1-Tie2 association and/or Tie2 localization are observed (compare [Figures 5A](#) and [5B](#) and right bottom panels in [Figure 5D](#)). FRET efficiencies and receptor localization do not significantly change over a 30-min period, as graphically illustrated in [Figure 5E](#). This is in sharp contrast with the ability of

Ang2 to induce changes in Tie2 localization in the absence of Tie1. Under these conditions, Tie2 displays punctate staining within 10–20 min, identical to that observed for Ang1 (Figure 5C and Supplemental Movies S1–S3). Thus, our data demonstrate a unique difference between individual angiopoietins to affect Tie1-Tie2 association and subsequent Tie2 clustering.

Tie1 Is an Inhibitory Coreceptor

To further assess the physiological role of Tie1, particularly with its capability to manipulate Tie2 activation, we followed Tie2 phosphorylation in the presence or absence of Tie1 and angiopoietin in both HEK293 and human umbilical vein endothelial cells (HUVECs). Initially, HEK293 cells, which lack endogenous Tie receptors, were transiently transfected with full-length myc-tagged Tie2, full-length HA-tagged Tie1, Tie1-CFP lacking a tyrosine kinase domain, or a combination of the three. As seen in Figure 6A, western blotting of crude lysates with anti-Tie1 and anti-Tie2 antibodies, respectively, clearly demonstrate robust Tie1 and -2 expression. Interestingly, ectopically expressed Tie2 displays a low level of basal activation in 293 cells as observed by the anti-phosphotyrosine 992 Tie2 antibody (Figure 6A, top panel). However, as predicted, addition of Tie1 dramatically decreased Tie2 basal activation (~50% relative to Tie2 alone). Interestingly, addition of chimeric Tie1-CFP lacking an intracellular tyrosine kinase domain also decreases Tie2 activation to levels comparable to those observed with full-length Tie1 (Figure 6A). This finding further demonstrates that Tie ectodomain interactions are necessary and sufficient for Tie2 inhibition.

Using an alternative approach to examine the role of endogenous Tie1, HUVECs were infected with recombinant lentivirus encoding a nonspecific (control) or Tie1-specific miR-shRNA. Stable cell lines were selected with puromycin and Tie receptor expression, and receptor activation was analyzed following ligand addition by means of western blot analysis. In an shRNA control cell line, which displays wild-type levels of Tie1, Tie2 activation can be readily observed within 15 min following the addition of Ang1 (Figure 6B). In contrast, and in agreement with the work of others, addition of Ang2 fails to significantly alter receptor phosphorylation and activation (Davis et al., 2003; Maisonpierre et al., 1997).

Alternatively, HUVEC Tie1-shRNA stable cell lines, which express significantly (approximately 70%) less Tie1 as seen in control (non-specific shRNA) cells, behave considerably different. In the absence Tie1, and in agreement with our findings in epithelial cells, Tie2 displays approximately 2 fold greater basal activation than within control cells (Figure 6B). Furthermore, despite the observation that Tie2 appears partially active in Tie1 silenced cells, addition of Ang1 also leads to ~3.5 fold greater receptor activation. However, in the absence of Tie1, Ang2 is now also capable of stimulating Tie2 activation. Indeed, despite the observation that Tie2 appears partially active in Tie1 silenced cells, addition of Ang1 or -2 leads to greater receptor activation (~3.5- and 2-fold, respectively). Cumulatively, these observations are consistent with an inhibitory role for endogenous Tie1, and indicate that its presence is necessary to distinguish the agonist/antagonist role for Ang2.

The Tie2 Kinase Domain Is Unnecessary for Receptor Clustering and Tie1 Interaction

Despite a clear demonstration by FRET analysis of Tie receptor ectodomain interactions, it remained unclear if the intracellular kinase domain could influence receptor localization and clustering in response to ligand. Therefore, to evaluate the role of the Tie2 tyrosine kinase domain in Tie interactions, a full-length version of Tie2 with a carboxyl terminal mCherry domain was expressed in control and Tie1 silenced EA.hy 926 cells (the extent of Tie1 knockdown can be observed in Figure S3). Under these conditions, endogenous Tie1 will interact with, and influence, Tie2 localization, if indeed the tyrosine kinase domains are essential. Accordingly, Tie2-mCherry localization was monitored by confocal microscopy following both Ang1 and -2 addition. Figure 6C includes images of cells prior to, 30 min after, and 60 min after ligand addition. As observed with Tie2-CFP chimeras lacking the tyrosine kinase domain, full-length Tie2-mCherry exhibits punctate staining within 30 min in the presence of Ang1 in both control and Tie1 silenced cells. Similarly, Tie2 clustering is observed within 60 min of addition of Ang2 in the absence of Tie1 (Figure 6C, bottom panels). A slight difference in time between the observed clustering in Ang1- and Ang2-induced cells is likely, due to the residual Tie1 (~25% of wild-type levels) that remains in Tie1 knockdown cells. Although these studies do not rule out other potential roles for the Tie tyrosine kinase domains, these findings demonstrate that kinase domain interactions do not significantly influence the clustering and localization of Tie2.

DISCUSSION

Until now, the molecular basis for the Tie1 and the differential angiopoietin functions has remained elusive. However, using a powerful *in vivo* proximity assay, we now identify a clear role for Tie1 in Tie2 signaling, and demonstrate a direct, inhibitory interaction between these receptors. We further identify, via structure-based, site-directed mutagenesis, the precise molecular surface regions of the Tie1 and -2 ectodomains, which mediate this critical interaction. Finally, we reveal that this interaction is dynamic and differentially modulated by the different angiopoietin ligands, thereby providing a molecular mechanism for the observed differences in angiopoietin function.

Based upon previous studies and the data presented here, we propose a model for Tie2 signaling, as illustrated in Figure 7. Specifically, in cells expressing both Tie1 and -2, the receptors form heterodimers within endothelial cells via ectodomain electrostatic interactions that inhibit Tie2 activation and clustering. Binding of Ang1 to Tie2 promotes heterodimer dissociation, Tie2 clustering, and signaling initiation. On the other hand, Ang2 is unable to dissociate the inhibitory Tie2-Tie1 complexes upon binding Tie2 and, therefore, does not induce Tie2 clustering and signaling, yet behaves as a competitive antagonist by blocking further binding of Ang1. Alternatively, for cells that do not express Tie1, *all* angiopoietins promote Tie2 clustering and activation. Our model explicitly proposes that the balance of Tie1 and -2 expression modulates the functional potential of Ang2, and by analogy, vascular homeostasis.

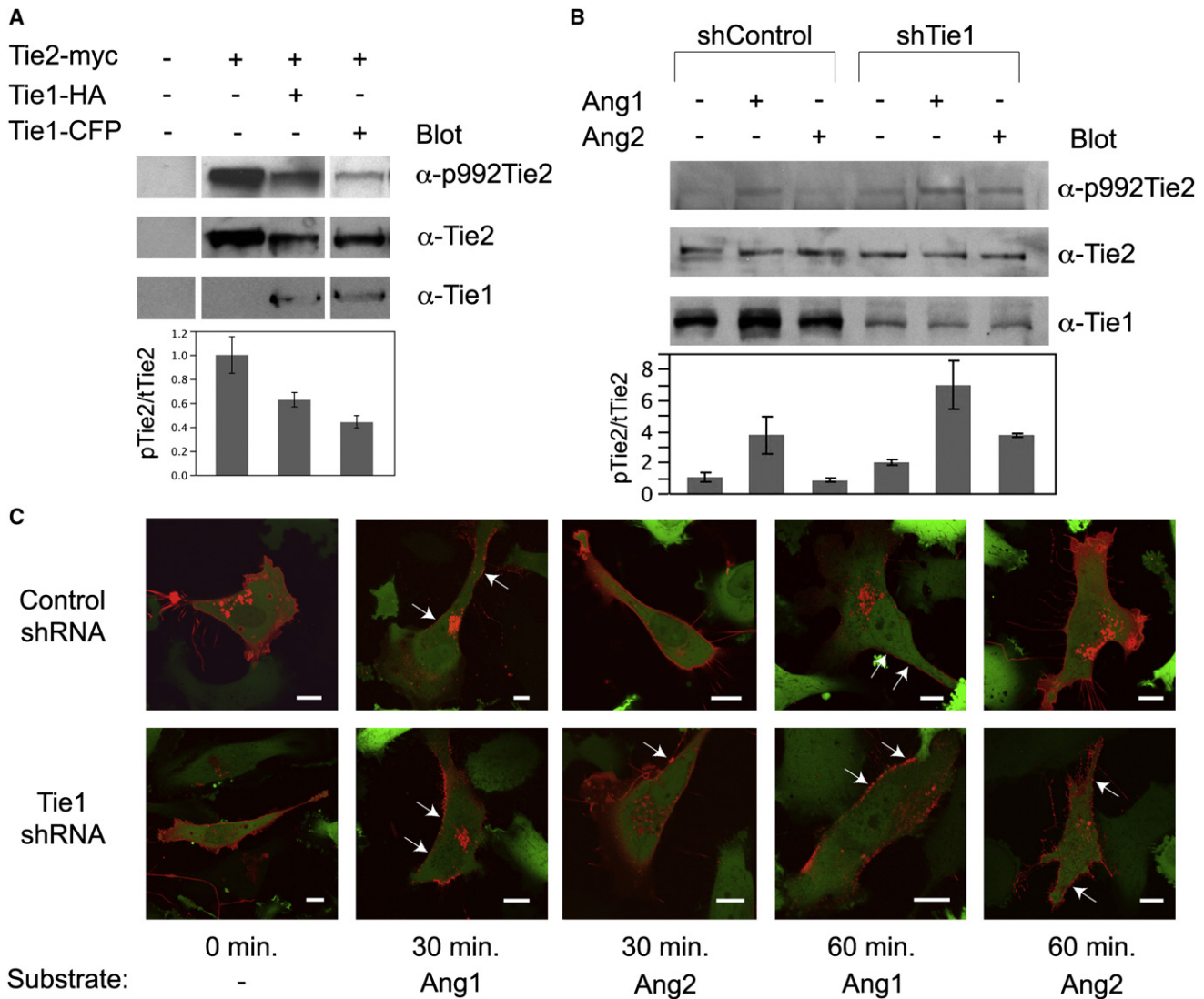


Figure 6. Tie1 Is an Inhibitory Coreceptor for Tie2

(A) HEK293 cells were transfected with full-length Tie2-myc alone or in combination with full-length Tie1-HA, or Tie1-CFP, harvested, and lysates probed with anti-Tie1, anti-Tie2, and anti-phosphotyrosine 992 Tie2. The relative ratio of phosphorylated Tie2 (top panel) to total Tie2 (middle panel) was determined, and the calculated value was arbitrarily set equal to 1.0 for Tie2 expressed alone. Values and associated standard errors were calculated from at least three independent experiments from multiple western blots, and are graphically displayed below each respective lane. Displayed samples are from nonadjacent lanes of the same blot.

(B) HUVECs were infected with an shRNA control or Tie1 knockdown lentivirus and selected with puromycin for stable integration (see FigureS3 for extent of Tie1 silencing). Asynchronous cultures were subjected to 4 hours of serum withdrawal, followed by stimulation with vehicle (buffer), 500 ng/ml Ang1, or 500 ng/ml Ang2. At 15 min after addition of ligand, cells were harvested and subjected to western blotting with anti-Tie2 (middle panel), anti-Tie1 (bottom panel), and the phosphotyrosine specific anti-Tie2 (992) (top panel), as indicated. The relative ratio of phosphorylated Tie2 (top panel) over total Tie2 (middle panel) was determined, and the calculated value was arbitrarily set equal to 1.0 for unstimulated Tie2 from shControl cells. Values are graphically displayed below each respective lane.

(C) Control (top panels) or Tie1 knockdown (bottom panels) stable EA.hy 926 cells were transiently transfected with full-length Tie2 fused to mCherry and imaged via confocal microscopy 48 hr posttransfection. Cells were stimulated with Ang1 or -2, and mCherry expression was followed over the course of 60 min. GFP expression (green) indicates stable viral integration, and was overlaid onto mCherry (red) for orientation purposes. Arrows indicate areas of punctate Tie2 staining. Scale bars, 10 μm.

While it is known that Tie2 is expressed ubiquitously throughout the vascular endothelium (Dumont et al., 1992, 1994), Tie1 expression is significantly restricted to vascular bifurcations and branching points following embryogenesis (Porat

et al., 2004). Furthermore, Tie1 is transcriptionally upregulated by hypoxia, VEGF stimulation, and in areas of wound healing and tumor growth and development (i.e., sites of neovascularization) (Korhonen et al., 1992; McCarthy et al., 1998). Therefore,

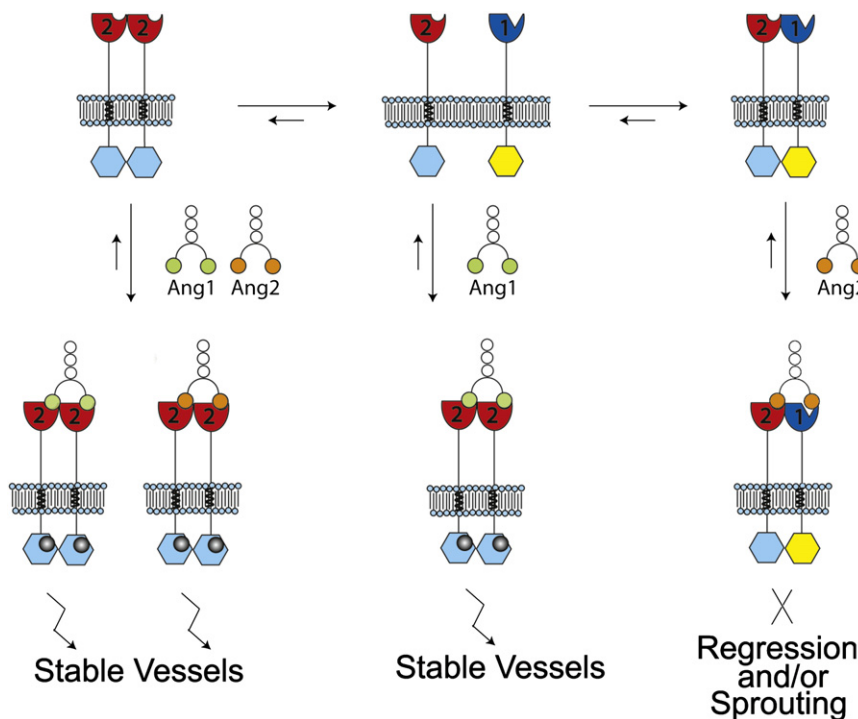


Figure 7. Model for Angiotensin-Mediated Tie2 Signaling

Expression of Tie2 in the absence of Tie1 at sites of vessel quiescence and maturity. In the absence of Tie1, Tie2 can be activated with either Ang1 or -2. Both ligands stimulate receptor clustering, tyrosine kinase activity, and downstream signaling events, effectively become unresponsive to vessel sprouting and branching cues. Within sites of active angiogenesis, Tie1 and -2 associate to form a complex prior to ligand stimulation. Upon addition of Ang2, Tie1 and -2 association and localization remain unchanged. Under these conditions, Ang2 fails to activate the Tie2 receptor and opposes the activation of downstream signaling generated by Ang1. However, upon addition of Ang1, the opposite is observed. Ang1 stimulates Tie2 clustering, tyrosine kinase activity, and downstream signaling events similar to those observed in the absence of Tie1. Angiotensins are depicted as dimers for illustration purposes, although they are known to exist as higher-order clusters.

the adult vasculature is composed of regions of alternating (high and low, or absent) Tie1 expression. Regions that are actively involved in angiogenesis, and require the antagonistic function of Ang2, are, interestingly, the very same regions where Tie1 expression is observed. Under these conditions, the majority of Tie1 and -2 form signaling-incompetent heterotypic complexes on the cell surface (Figure 7). Their association is dynamic and mediated via an electrostatic interaction between charged residues within their ectodomains, as well as the presence or absence of activating ligand (i.e., Ang1). Cells expressing both receptors are responsive to the survival, migration, and chemotactic cues caused by activation and inactivation of Tie2 via Ang1 and -2 and are, therefore, able to promote the required vessel branching and sprouting necessary for angiogenesis.

Alternatively, Tie1 is absent in vascular regions that are stable and quiescent, such as mature vessels. The absence of Tie1 negates the functional differences between Ang1 and -2 and allows endothelial cells to respond to either ligand in a similar fashion (both as agonist), and foster the same phenotypic response—quiescence and survival (Figure 7). In this regard, Tie1 serves as a selectivity factor, designating when and how Ang2 functions. In general, this could provide greater cellular adhesion with the underlying vessel support cells, and provide yet another mechanism to stabilize the adult vasculature and prevent aberrant vessel sprouting and branching that could lead to pathogenesis.

Consistent with our proposed model, in the absence of Tie1 (Figure 6), Tie2 exhibits basal phosphorylation and remains partially activated, despite the absence of ligand, yet it can become further clustered and activated by both the agonist, Ang1, as well as the antagonist, Ang2 (Davis et al., 2003; Maison-pierre et al., 1997). Alternatively, in the presence of Tie1, the

ligand independence and basal Tie2 activity are attenuated (Figures 6A and 6B). In agreement, loss of Tie1 (via shRNA) eliminates the functional differences between Ang1 and -2 in HUVEC (Figure 6B). Under these conditions, endothelial cells respond to both ligands similarly. In agreement, Yuan et al. (2007) observed that siRNA toward Tie1 significantly increased the downstream activation of Tie2 signaling components, therefore concluding that Tie1, in its normal role, antagonizes Tie2 function. An analogous conclusion was drawn by Patan (1998) following histological assessment of Tie1 and -2 knockout mouse phenotypes. In addition, Kim et al. (2006) demonstrate Ang2-induced Tie2 activation following downregulation of Tie1 via siRNA in HUVECs, and Nguyen et al. (2007) observed differential responses of lymphatic versus venous or arterial endothelial cells to Ang1 and -2. In line with this observation, our model would predict that lymphatic endothelial cells, thought to be the primary target of the Ang2 agonist (as shown by knockout and overexpression experiments), would display lower levels of Tie1 protein, although this has yet to be investigated.

Some have observed Tie2 to preferentially localize at sites of cell-cell contacts (Fukuhara et al., 2008; Saharinen et al., 2008). Indeed, our expression constructs, with and without the intracellular tyrosine kinase domain, demonstrate related localization patterns, and we, therefore, conclude that clustering to cell-cell junctions is likely mediated by ectodomain interactions, and not through the Tie2 kinase domain. While Tie2 clustering to cell junctions presents an appealing means to localize specific signaling events, the exact mechanism by which this occurs remains unclear.

It is interesting to note that our model for Tie2 activation differs from others. Using Tie1 truncation mutants and a TrkA/Tie2 fusion protein, Marron et al. (2000), for example, suggest an

association between Tie1 and -2 that is mediated by their cytoplasmic kinase domains, while we observe a dynamic interaction between these receptors in the absence of the catalytic kinase domains and any subsequent downstream signaling events. Moreover, our structure-based receptor mutagenesis, combined with localization and FRET analysis, strongly supports receptor interactions through their extracellular domains. Interestingly, despite having documented receptor association, Marron et al. (2000) did not observe significant phosphorylation of Tie1. However, during the course of Tie2 activation, others have observed Tie1 phosphorylation (Saharinen et al., 2005; Yuan et al., 2007). It appears, in some circumstances, that Tie1 phosphorylation correlates with Tie2 activation, although it remains unknown whether this is as a result of stable or of transient interactions caused by high local concentrations of Tie2 (i.e., within signaling clusters) (Milner et al., 2009; Saharinen et al., 2005). Furthermore, the physiological significance of Tie1 phosphorylation remains unknown.

Finally, it should be noted that our studies also contrast with those by Saharinen et al. (2008) in which Tie1 and -2 association is proposed to occur in the presence of Ang1, as suggested by colocalization experiments. Previous reports by the same group indicate stimulation of Tie1-Tie2 coimmunoprecipitation following Ang1 exposure (Saharinen et al., 2005). Although we observed dramatic changes in Tie2 localization, clustering, and association with Tie1 upon binding of Ang1, we do not see a concomitant change in Tie1 localization. Furthermore, the extent of Tie2 clustering that we observed is significantly greater, and FRET analysis reveals nearly immediate dissociation of the Tie1-Tie2 complexes upon receptor stimulation. Since our analysis of receptor complex formation is at a significantly higher resolution (<10 nm versus >1 μ m) and more direct, we feel that the lack of change in Tie1 localization and loss of FRET documents Ang1-induced Tie2-Tie1 complex dissociation rather than association. Furthermore, our data and proposed function of Tie1 correlate with the observed phenotypes of Tie and Ang knock-out mice, Tie receptor expression patterns, and recent data (including data presented here), confirming the inhibitory effects of Tie1 on Tie2 activation (Kim et al., 2006; Patan, 1998; Yuan et al., 2007). Collectively, our results indicate that the balance of expression and dynamic interaction between Tie1 and Tie2 provides an effective means of controlling receptor activation, and, by analogy, vascular homeostasis, using a *single* set of structurally similar ligands. The exquisite level of molecular control clearly highlights the importance of restricting Tie2 activation in order to maintain vascular homeostasis and to prevent pathogenesis; therefore, the Tie1-Tie2 interface may serve as an attractive therapeutic target, and may be more relevant than the currently scrutinized Ang2-Tie2 interaction.

EXPERIMENTAL PROCEDURES

Homology Model of Tie1

The Tie1 homology structure was modeled with the program MODELER (Marti-Renom et al., 2000) and the experimentally determined 2.5 Å Tie2 ligand-binding domain crystal structure (PDB 2GY5) (Barton et al., 2006). Although initial alignments between Tie1 and -2 were prepared with CLUSTALX (Larkin et al., 2007), use of the sequence/secondary structure alignment implementation in MODELER yielded better results (as assessed

below), and was therefore adopted for final calculations. Four distinct models were calculated that satisfied basic spatial and stereochemical restraints; however, the model used for interpretation and illustration purposes had the lowest discrete optimized protein energy assessment score and MODELER objective function (Shen and Sali, 2006). Stereochemical analysis via PROCHECK (CCP4, 1994) revealed main chain parameters better than or within the typical range of values for *experimentally* determined protein structures at 2.5 Å resolution.

Cloning and Gene Expression

The sequences encoding the human Tie1 (IMAGE 5,767,075) and Tie2 (IMAGE 5,228,999) genes were cloned as interchangeable receptor-fluorophore fusion cassettes in both pcDNA3.1(+) hygromycin and neomycin resistance vectors for expression in HEK293 cells and human osteosarcoma cell line U2OS. Briefly, NheI, and EcoRI sites were appended to the amino and carboxyterminus via PCR with the following oligonucleotides: Tie1-Nhe, GCTAGCATGGT CTGGCGGGTGCCC; Tie2-Nhe, GCTAGCATGGACTCTTTAGCCAGCCTTAG; Tie1-EcoRI, GAATTCGGTGCGTCTCCGATGCAGGCAGC; and Tie2-EcoRI, GAATTCTCTCTCTTTGCACATTTGCCCTC (restriction sites are underlined and initiating methionine is in bold letters). After insertion of receptor DNA into the pcDNA vectors, GFP cassettes were cloned downstream and in-frame with EcoRI and XhoI, yielding an open reading frame consisting of Tie receptor fused to either C- or YFP. Monomeric CFP and YFP variants (containing dimeric suppression mutations—obtained from Dr. T. Springer) were utilized as templates for fluorophore incorporation via PCR with the following oligonucleotides: C/YFP-EcoRI, GAATTCATGGTGAGCAAGGGCGAGGAG; and C/YFP-XhoI, CTCGAGTTATCTAGATCCGGTGGATCC. Although several initial constructs were constructed and tested for optimal linker length between receptor and fluorophore, ultimately, one receptor pair was chosen for further study in which the Tie receptors were fused to either CFP or YFP after residues 796 and 780 of Tie1 and Tie2, respectively. This left the transmembrane domain intact, as well as 10 or 11 additional cytoplasmic residues, but eliminated the carboxyl-terminal tyrosine kinase catalytic domain. Altering fluorophores on individual receptors did not appear to make any significant difference, and were, therefore, used interchangeably.

Full-length Tie2-mCherry was constructed by fusing the fluorophore to the carboxy terminus of Tie2 via PCR stitching with the partially complementary oligonucleotides: mCherry/TKD, GTTCTGTGAAGAAGCGGCCGGTGCATCTGGTTCTATGGCCATCATCAAGGAG; TKD/mCherry, CTCCTTGATGATGGCCATAGAACCAGATGCACCGGCCGCTTCTTCAGCAGAAC; and mCherry Xho, GCTCACTCGAGCTACTTGTACAGCTCGTCC. The final construct was cloned as an NheI-XhoI fragment into pcDNA3.1(+) hygromycin.

Mutations within Tie1 and -2 coding regions were introduced by site-directed mutagenesis (Quikchange Multi; Stratagene) following manufacturer's recommendations. To confirm the presence of the desired mutations, both DNA strands were sequenced by standard di-deoxy sequencing chemistry (Cornell University Bioresource Center).

Cell Manipulations and Transfections

HEK293, U2OS, and EA.hy 926 (a gift from Dr. C.-J. Edgell) cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. HUVECs were grown in EBM II media according to manufacturer recommendations (Clonetics). Cells were consistently transfected at 80%–90% confluence in 35 mm glass-bottom culture dishes (MatTek) with Lipofectamine 2000 (HEK293), FuGENE HD (U2OS), or FuGENE 6 (EA.hy 926), according to manufacturers' recommendations (Invitrogen and Roche). For coexpression experiments, equimolar concentrations of Tie1 and -2 vector DNA were used.

shRNA Knockdown of Tie1

Recombinant lentivirus encoding a Tie1 or control shRNA was constructed according to the manufacturer's recommendations (Open Biosystems). Stable EA.hy 926 or early-passage HUVECs were selected in the presence of 0.8 μ g/ml puromycin 48 hr after viral infection. After 7 days, stable cells were monitored for Tie1 and -2 expression via western blot analysis (antibodies listed below).

Tie2 Activation Assays

For analysis of Tie2 activation in HEK293, cells were transfected with combinations of full-length Tie1-HA, and/or Tie2-myc tagged vectors. At 48 hr after transfection, cells were lysed in HBST (20 mM HEPES [pH 7.4], 150 mM NaCl, 0.1% Triton X-100) in the presence of 0.5 mM sodium orthovanadate, and subjected to western blotting. For Tie2 activation in HUVECs and EA.hy cells, postconfluent cells were serum starved for 2 hr prior to the addition of 500 ng/ml Ang1, -2, or vehicle (PBS). At 15 min prior to ligand addition, sodium orthovanadate (Sigma) was added to 1 mM in the culture medium. At 30 and 60 min after ligand addition, cells were harvested as stated above. Endogenous Tie1 and -2 were analyzed with anti-Tie2 (Santa Cruz Biotechnology), anti-phosphotyrosine-specific 992 Tie2 (R&D Systems), or anti-Tie1 (R&D Systems and Santa Cruz Biotechnology). Quantitative values for band intensities were obtained from western blots with the program ImageJ (Abramoff et al., 2004). Briefly, the integrated pixel intensity was determined for each band of interest with an identically sized rectangular masking box. The background was similarly determined from an identical region of the blot from lanes lacking the protein of interest—except in cases where no such lane control could be used. Under these conditions, background was calculated from a blank region above each band of interest. Finally, the background was subtracted uniformly from the experimental values to obtain the final raw values. All statistical calculations were determined with JMP version 7.0 software (SAS Institute).

Cellular Imaging

The spatial proximity assay was based on the work of Dr. R. Tsien (Zacharias et al., 2002) and Dr. T. Springer and colleagues (Kim et al., 2003). Live cell imaging was performed 24–48 hr posttransfection on a Leica TCS-SP2 AOBs confocal laser scanning microscope equipped with a blue diode (405 nm), argon (458, 476, 488, 514 nm), green HeNe (543 nm), orange HeNe (594 nm), and red HeNe (633 nm) lasers, an HCX PI Apo 63x/1.3 n.a. glycerin-immersion objective lens, a motorized XY stage (Märzhäuser), and an environmentally controlled (temperature, humidity, and CO₂) stage incubator (PeCon). Fluorophore-receptor fusions were imaged with excitation and emission wavelengths of 458 nm and 514 nm for CFP and YFP, respectively, and fluorescence emissions were detected with SP window settings of 465–505 nm and 525–600 nm (for CFP and YFP, respectively). FRET efficiencies were determined by the acceptor photobleaching methodology with Leica version 2.61 software. Briefly, regions of interest (ROIs) were chosen for analysis based on extent of fluorophore expression, localization, and uniformity. For acceptor photobleaching, YFP within the ROI (green box within images) was consistently photobleached (with the AOTF ramped up to 100% transmission of the 514 nm laser line) from 50% to 70% reduction in fluorescence intensity, as monitored by Leica software. Care was taken to exclude cells for analysis that displayed significantly higher, or lower, fluorescent intensity than the “average” cell. To eliminate bias, cells were also chosen based on similar levels of CFP and YFP fusion protein. Similar to the work of Kim et al. (2003), cells that displayed significant drift in the x-y focal plane were discarded for FRET analysis. Due to significant cellular drift, discrete regions, rather than whole cells, were subjected to photobleaching to accelerate FRET analysis. Pre- and postbleach images were recorded for both donor (CFP) and acceptor (YFP), and FRET efficiency was calculated as: $FRET_{Eff} = (D_{post} - D_{pre}) / D_{post}$ for all $D_{post} > D_{pre}$, where D_{pre} and D_{post} is the donor intensity before and after photobleaching, respectively. JMP software was used for all statistical analyses. mCherry-receptor fusions and GFP were imaged as stated above, with the exception that excitation wavelengths were 594 nm and 488 nm, respectively, and fluorescence emissions were detected with SP window settings of 605–700 nm and 500–560 nm (for mCherry and GFP, respectively).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and three movies and can be found with this article online at doi:10.1016/j.molcel.2010.02.007.

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