**Supplemental Inventory:**

**TableS1-S3:** Table of raw experimental FRET efficiencies used for calculations in Figures 1, 3, and 4.

**Supplemental Videos 1-3:** Cellular time-lapse imaging illustrating changes in receptor clustering following ligand addition (Associated with Figure 5).

**Supplemental Figure S1-S2:** FRET images for control experiments and additional cell line (HEK293).

**Supplemental Figure S3:** Documentation of the extent of Tie1 silencing in shRNA experiments.

**Table S1.** Individual FRET efficiencies for mutant, wild-type, and control receptor experiments listed in Figures 1, 3, and 4. Wild-type receptors are displayed in bold when paired with a complementary mutant or control receptor. Each n represents an independent experiment.

| Receptor-Fluorophore Pair | FRET Efficiencies |
|---|---|
| Tie1-YFP & Tie2-CFP (HEK293) | n=6; 28.27, 21.66, 31.43, 31.71, 40.70, 23.69 |
| Tie1-YFP & Tie2-CFP (U2OS) | n=6; 33.78, 38.53, 22.27, 17.24, 18.58, 34.72 |
| Tie1-YFP & Tie2-CFP (EA.hy 926) | n=6; 22.6, 14.54, 23.48, 22.89, 17.99, 14.31 |
| **Tie1-YFP** & Plexin-CFP | n=6; 0.00, 0.00, 0.00, 0.00, 0.00, 6.10 |
| Plexin-YFP & **Tie2-CFP** | n=3; 0.00, 0.00, 0.00 |
| Tie1-YFP (R91E, K95E, R427E) & **Tie2 CFP** | n=2; 12.40, 10.21 |
| Tie1-YFP (K95E, R260E, R263E) & **Tie2 CFP** | n=5; 30.95, 32.72, 47.21, 43.27, 22.38 |
| Tie1-YFP (R260E, R437E, R438E) & **Tie2 CFP** | n=3, 0.00, 0.00, 0.00 |
| Tie1-YFP (R437E, R438E) & **Tie2 CFP** | n=4; 7.70, 1.65, 8.27, 8.91 |
| **Tie1-YFP** & Tie2 CFP (E53K, D236K, E239K) | n=2; 3.89, 6.35 |
| **Tie1-YFP** & Tie2 CFP (E53K, D60K, D389K) | n=5; 2.59, 4.78, 5.74, 7.06, 2.03 |
| **Tie1-YFP** & Tie2 CFP (E53K, D60K, E109K, D236K, E239K, D389K) | n=6, 6.05, 5.58, 0.00, 3.00, 8.34, 0.00 |
| Tie1-YFP (R260E, R437E, R438E) & Tie2 CFP (E53K, D236K, E239K) | n=2; 19.93, 11.30 |
| Tie1-YFP (R260E, R437E, R438E) & Tie2 CFP (E53K, D60K, E109K, D236K, E239K, D389K) | n=3; 24.77; 28.42, 25.53 |
| Tie1-YFP (R437E, R438E) & Tie2 CFP (E53K, D236K, E239K) | n=3; 22.24, 33.18, 29.74 |

**Table S2.** Individual FRET efficiencies for Figure 5A (Ang-1 addition) time course experiment. Each n represents an independent cell from an independent experiment.

| **Post-addition of Ang1 (500ng/mL)** | **FRET Efficiencies** |
|---|---|
| t=0 | n=6; 27.49, 27.6, 18.96, 20.82, 25.3, 19.92 |
| t=5 | n=5; 11.44, 14.42, 27.35, 8.74, 17.22 |
| t=10 | n=3; 10.67, 4.59, 8.95 |
| t=15 | n=5; 18.25, 16.54, 0.00, 0.00, 0.00 |
| t=20 | n=4; 11.74, 16.27, 0.00, 13.11 |
| t=25 | n=5; 6.55, 6.25, 21.33, 0.00, 7.26 |
| t=30 | n=4; 7.62, 23.11, 1.14, 0.18 |
| t=0 (EA.hy 926) | n=3; 22.6, 14.54, 23.48 |
| t=30 (EA.hy 926) | n=4; 7.94, 5.77, 0.0, 0.0 |

**Table S3.** Individual FRET efficiencies for Figure 5B (Ang-2 addition) time course experiment. Each n represents an independent cell from an independent experiment.

| **Post-addition of Ang2 (500ng/mL)** | **FRET Efficiencies** |
|---|---|
| t=0 | n=5; 27.12, 25.61, 25.29, 29.23, 28.71 |
| t=5 | n=3; 39.73, 19.71, 23.48 |
| t=10 | n=3; 26.29, 21.80, 34.38 |
| t=15 | n=3; 24.66, 26.12, 33.02 |
| t=20 | n=3; 35.71, 30.05, 24.79 |
| t=25 | n=3; 30.90, 23.48, 27.43 |
| t=30 | n=3; 32.17, 33.68, 25.32 |
| t=0 (EA.hy 926) | n=3; 22.89, 17.99, 14.31 |
| t=30 (EA.hy 926) | n=4; 22.51, 14.48, 16.22, 14.5 |

**Supplemental Video S1.** Time lapse imaging of Tie2-YFP within a Tie2-YFP/Tie1-CFP co-expressing U2OS cell following addition of 500ng/mL Ang-2. Individual frames were taken every 30 seconds over a thirty minute period. Notice that lack of significant change in Tie2 localization.

**Supplemental Video S2.** Time lapse imaging of Tie1-CFP within a Tie2-YFP/Tie1-CFP co-expressing U2OS cell following addition of 500ng/mL Ang-2. Individual frames were taken every 30 seconds over a thirty minute period and precisely follow those taken in video S1. Notice that lack of significant change in Tie1 localization.

**Supplemental Video S3.** Time lapse imaging of Tie2-CFP from a representative U2OS cell following addition of 500ng/mL Ang-2. Individual frames were taken every 30 seconds over a thirty minute period. Individual receptor foci form within approximately 10 minutes following ligand addition. Punctate staining of Tie2-YFP continues for the remainder of the experiment. Alternatively, note the absence of change in Tie1 localization.

**Figure S1.** HEK293 FRET proximity assay experiment as described in Figure 1. The majority of the receptor-fluorophore fusions localizes predominantly to the plasma membrane (although a fraction is also observed in the secretory system) and significant FRET is observed.
*For all images CFP will be displayed as cyan and YFP as yellow. Photobleaching experiments were restricted to, and FRET values calculated from, the region within the green box. Both pre- and post-photobleaching images are displayed as 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. Light gray bar in each image indicates 10 μm.

**Figure S2.** Control experiments for FRET proximity assay. A) Tie2-CFP and Plexin A1-YFP or B) Plexin A1-CFP and Tie1-YFP co-expression and association in HEK293 cells. The majority of the receptor-fluorophore fusions localizes predominantly to the plasma

membrane (although a fraction is also observed in the secretory system) yet significant FRET is not observed demonstrating overexpression does not play a significant role in FRET values.

*For all images CFP will be displayed as cyan and YFP as yellow. Photobleaching experiments were restricted to, and FRET values calculated from, the region within the green box. Both pre- and post-photobleaching images are displayed as 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. Light gray bar in each image indicates 10 μm.

**Figure S3.** Tie1 knockdown in EA.hy 926 cells. Crude cell lysates were analyzed by western blot for total Tie1 and Tie2 protein.

**Figure S1.**

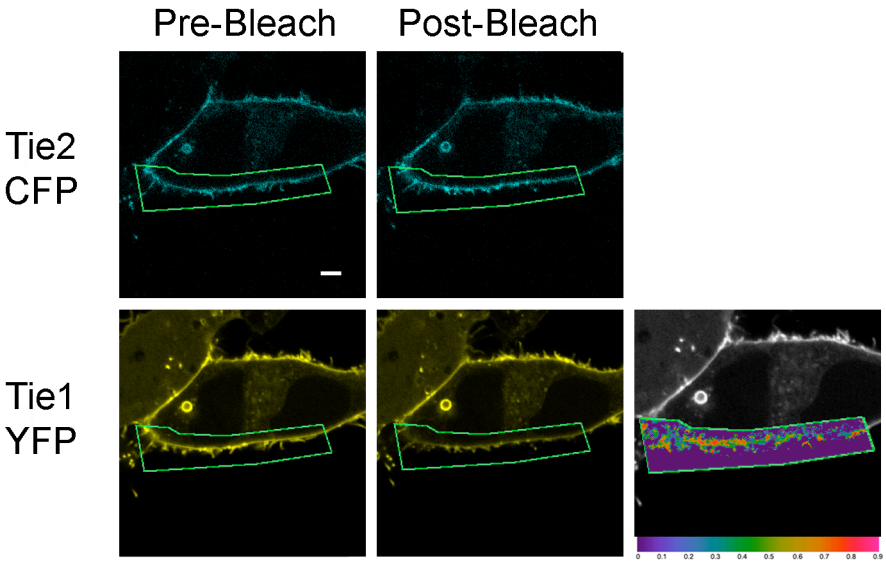
Pre-Bleach
Post-Bleach
Tie2
CFP
Tie1
YFP
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

**Figure S2.**

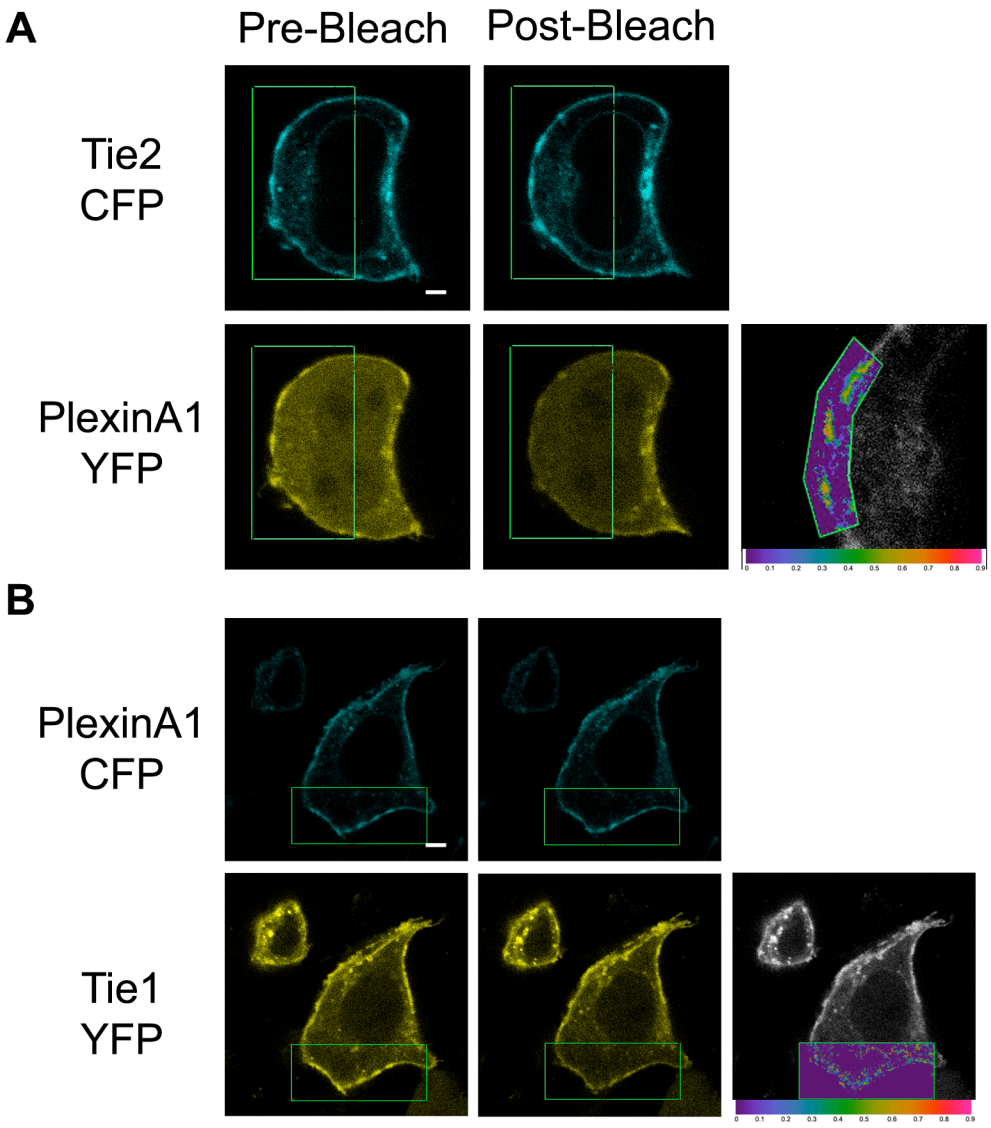
A
Pre-Bleach
Post-Bleach
Tie2
CFP
PlexinA1
YFP
B
PlexinA1
CFP
Tie1
YFP

**Figure S3.**