Fibroblast Growth Factor Receptor-induced Phosphorylation of EphrinB1 Modulates Its Interaction with Dishevelled

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The Eph family of receptor tyrosine kinases and their membrane-bound ligands, the ephrins, have been implicated in regulating cell adhesion and migration during development by mediating cell-to-cell signaling events. The transmembrane ephrinB1 protein is a bidirectional signaling molecule that signals through its cytoplasmic domain to promote cellular movements into the eye field, whereas activation of the fibroblast growth factor receptor (FGFR) represses these movements and retinal fate. In *Xenopus* embryos, ephrinB1 plays a role in retinal progenitor cell movement into the eye field through an interaction with the scaffold protein Dishevelled (Dsh). However, the mechanism by which the FGFR may regulate this cell movement is unknown. Here, we present evidence that FGFR-induced repression of retinal fate is dependent upon phosphorylation within the intracellular domain of ephrinB1. We demonstrate that phosphorylation of tyrosines 324 and 325 disrupts the ephrinB1/Dsh interaction, thus modulating retinal progenitor movement that is dependent on the planar cell polarity pathway. These results provide mechanistic insight into how fibroblast growth factor signaling modulates ephrinB1 control of retinal progenitor movement within the eye field.

INTRODUCTION

Vertebrate retinal development consists of a series of steps that progressively restrict the available cell fates, and retinal progenitors need to be positioned within the eye field to receive the local environmental signals that will direct their ultimate fate (Huang and Moody, 1993; Moore and Moody, 1999). The retinal precursor cells that give rise to the vertebrate eye field are specified by several transcription factors that both promote retinal fate and control cell movement of progenitor cells during gastrulation and neurulation (Kenyon *et al.*, 2001). The secreted as well as membrane localized signaling molecules involved in the regulation of vertebrate retinal development provide important signaling cues for eye field formation.

Recent evidence indicates that ephrinB1 signals via its intracellular domain to control retinal progenitor movement into the *Xenopus* eye field by interacting with Dishevelled (Dsh) and coopting the planar cell polarity (PCP) pathway (Moore *et al.*, 2004; Lee *et al.*, 2006). During embryonic development, blocking Dsh translation by using antisense morpholino oligonucleotides prevents retinal progeny from entering the eye field, similar to morpholino-mediated loss of ephrinB1 (Moore *et al.*, 2004; Lee *et al.*, 2006). Fibroblast growth factor (FGF) modulates ephrinB1 signaling to regu-

late the positioning of retinal progenitor cells within the definitive eye field (Moore *et al.*, 2004). Additional signaling molecules such as noncanonical Wnt4 (Maurus *et al.*, 2005) or Wnt11 are involved either through cross-talk or parallel pathways to promote eye field development partly through local antagonism of canonical Wnt signaling and regulation of the cohesion of eye field cells (Cavodeassi *et al.*, 2005).

It has been known for over a decade that ephrinBs are bidirectional signaling molecules that can signal through their intracellular domains to regulate cell–cell boundaries and adhesion (Pasquale, 2005; Sela-Donenfeld and Wilkinson, 2005; Lee *et al.*, 2008; Pasquale, 2008). EphrinB has been shown to interact with Dsh, a scaffold protein in the Wnt signaling pathway (Tanaka *et al.*, 2003; Lee *et al.*, 2006), to promote retinal progenitor movement (Lee *et al.*, 2006). In contrast, FGF receptor (FGFR) signaling antagonizes this activity to restrict movement of these cells within the presumptive eye field, but the mechanism of this regulation remains unknown (Moore *et al.*, 2004; Arvanitis and Davy, 2008).

Here, we use the eye field as a tractable model for understanding how FGFR regulates ephrinB1 control of cell movement. We present evidence that the phosphorylation of specific tyrosine residues in the intracellular domain of ephrinB1 is critical for dissociating the interaction between ephrinB1 and Dsh, providing mechanistic insight into how FGFR restricts ephrinB1 control of retinal progenitor movement via the Dsh/PCP pathway.

MATERIALS AND METHODS

Blastomere Injections

Xenopus embryos were obtained by standard methods (Moody, 2000) and injected with the following mRNAs: β -galactosidase (200 pg), green fluorescent protein (GFP) (250 pg), FGFR1 K562E (KE) (200 pg; Neilson and Friesel,

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Abbreviations used: Dsh, Dishevelled; Eph, erythropoietin producing hepatoma; ephrin, erythropoietin producing hepatoma interactor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; PCP, planar cell polarity.

Figure 1. The C terminus of ephrinB1 mediates binding with Dsh. (A) Immunoprecipitates using anti-ephrinB1 (rabbit), anti-Dsh (rabbit), or anti-c-Myc (rabbit) antibodies in HT 29 human colon carcinoma cell lysates were immunoblotted with anti-Dsh (goat) and anti-ephrinB1 (goat) antibodies. Lysates were analyzed directly by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with indicated antibodies to reveal endogenous expression levels of ephrinB1 and Dsh, respectively. (B) Oocytes were left uninjected (–) or injected (+) with FLAG-tagged ephrinB1WT or ephrinB1Δ6 (10 ng) and HA-tagged Dsh (10 ng) RNAs where indicated. Oocyte lysates were IPed with anti-HA antibody and then immunoblotted with anti-FLAG antibody to detect ephrinB1 proteins.

1996), wild-type and point mutants of ephrinB1 (50 pg, D1.1.1; 250 pg, V1.1.1), Dsh (250 pg), constitutively active (CA) RhoA (50 pg), and dominant-negative (DN) RhoA (250 pg). mRNAs were microinjected into one dorsal (D1.1.1) or ventral animal blastomere (V1.1.1) of the 32-cell stage. Developmental stages were designated according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). All data are representative of at least three experiments.

Cell Fate Analysis

Blastomeres were injected with GFP RNA as a lineage tracer along various specified mRNAs. Tissue sections of stage 37–38 embryos were analyzed for the presence of GFP-labeled cells as described previously (Lee *et al.*, 2006). Embryos with D1.1.1 injections were considered positive when fluorescent progeny contributed >30% of the cells in the retina. Embryos injected in V1.1.1 were considered positive when fluorescent progeny contributed >10% of the cells in the retina.

Tracing of Gastrulation Movements

Blastomeres were injected with β -galactosidase RNA and various mRNAs. Injected embryos were collected at stage 12.5. β -galactosidase assays were performed at 30°C with Red-gal (Research Organics, Cleveland, OH) and were analyzed as described previously (Moore *et al.*, 2004). The frequencies that embryos contained β -galactosidase+ cells in the future eye field in experimental and control groups were compared. Additionally, lateral scatter distances of β -galactosidase+ cells were measured, and experimental versus control groups compared.

Whole-Mount In Situ Hybridization

Embryos were injected with GFP RNA and other specified mRNAs, and GFP expression was used to distinguish the injected side of stage 16 embryos, and then processed for whole-mount in situ hybridization by using standard methods (Moore *et al.*, 2004) with the following probes: *rx1*, *pax6*, and *pax2*. To determine whether marker expression was expanded or reduced, the width or area of their expression on both RNA-injected and uninjected sides were measured using the Spot 4.5 system (Diagnostic Instruments, Sterling Heights, MI).

Immunoprecipitation and Western Blot Analysis

EphB1-Fc (R&D Systems, Minneapolis, MN) was clustered using human immunoglobulin (Ig) as described and added to the HT 29 cell culture medium at a concentration of 2.5 g/ml for 30 min. HT 29 cells, oocytes, embryos, or ectodermal explants were prepared with ice-cold lysis buffer as described previously (Chong *et al.*, 2000). Immunoprecipitations were conducted for 1 h on HT 29 cell extracts or 15 oocytes (embryos) equivalents with antibody raised against Dsh, ephrinB1, c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), hemagglutinin (HA), and FLAG (Applied Biological Materials, Vancouver, BC, Canada) for 1 h and protein-A/G agarose (Santa Cruz Biotechnology) overnight. Washes and immunoblots were performed as described previously (Chong *et al.*, 2000), using anti-FLAG-horseradish peroxidase (HRP)-conjugated (Sigma-Aldrich, St. Louis, MO), anti-HA-HRP-conjugated (Roche Diagnostics, Indianapolis, IN), or anti-ephrinB1, anti-Dsh (Santa Cruz Biotechnology) antibodies.

Immunofluorescence

Xenopus embryos were collected at stage 10.5, and immunofluorescence was carried out as described previously (Dollar *et al.*, 2005). The following primary antibodies were used: anti-GFP antibody (1/400; Roche Diagnostics). Sections were double-stained with a polyclonal anti-HA antibody (1/400; Santa Cruz Biotechnology) to identify ephrinB1-expressing cells, and visualized with fluorescein isothiocyanate-conjugated anti-mouse (1/400; Invitrogen, Carlsbad, CA) or Cy3-conjugated anti-rabbit (1/400; Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies on an Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany).

RESULTS

The C Terminus of EphrinB1 Mediates an Interaction with Dsh

EphrinB1 has been shown to interact with Dsh when both proteins are exogenously expressed in vivo (Lee *et al.*, 2006). Here, we examined whether endogenous ephrinB1 and Dsh proteins interact, using immunoprecipitation (IP) analysis of lysates from HT 29 human colon carcinoma cells that express abundant levels of ephrinB1 and Dsh. Importantly, Dsh is present in ephrinB1 immune-complexes, and in reciprocal IPs ephrinB1 is found in the Dsh immune-complexes (Figure 1A). As expected, control IPs using c-Myc antibodies show little or no coimmunoprecipitation (coIP) of either ephrinB1 or Dsh (Figure 1A). These data indicate that an in vivo interaction exists between ephrinB1 and the Dsh protein. Previous reports show that the C terminus of B-type ephrins contain a postsynaptic density 95/disc-large/zona occludens (PDZ)-binding motif that is important for cell movement and angiogenesis (Lu *et al.*, 2001; Palmer *et al.*, 2002; Davy *et al.*, 2004; Makinen *et al.*, 2005; Lee *et al.*, 2006). We conducted an IP analysis of lysates from oocytes coexpressing Dsh and either wild-type ephrinB1 (ephrinB1WT) or a C terminal deletion construct lacking six amino acids (ephrinB1Δ6). EphrinB1WT and Dsh coIP, but ephrinB1Δ6 fails to interact with Dsh (Figure 1B). These data strongly suggest that the C terminus of ephrinB1 is necessary for a physical interaction with Dsh.

Tyrosine Phosphorylation of the Intracellular Domain of EphrinB1 Disrupts the Interaction with Dsh

EphrinB1 can be phosphorylated in response to binding a cognate Eph receptor (Holland *et al.*, 1996; Bruckner *et al.*,

1997) or the tight junction-associated protein Claudin (Tanaka *et al.*, 2005), or in response to FGFR activation (Chong *et al.*, 2000). We tested whether tyrosine phosphorylation of ephrinB1 affected the association between endogenous ephrinB1 and Dsh. For this analysis human HT 29 cells were used because they display relatively abundant levels of ephrinB1 with low levels of detectable tyrosine phosphorylation (Figure 2A). CoIP analysis of endogenous ephrinB1 or Dsh in HT 29 cells was performed in the presence of the EphB1 ectodomain fused to human Fc (EphB1-Fc). In this case, the EphB1 receptor ectodomain interacts with the extracellular domain of ephrinB1 and induces phosphorylation of the intracellular region of ephrinB1 through a Src family kinase (Holland *et al.*, 1996). The CoIP analysis demonstrated a clear reduction in the ephrinB1/Dsh association (Figure 2A). Similar results were found with exogenously expressed proteins in embryo extracts that also exogenously express the cognate EphB1 receptor lacking a kinase domain [EphB1(Δ C)], which retains the ability to interact with the extracellular domain of ephrinB1 and induce its phosphorylation through a Src family kinase (Figure 2B).

Because ephrinB1 tyrosine phosphorylation is also induced by FGF signaling, active FGFR1 (FGFR1 KE; FGFR1 K562E) was exogenously expressed along with ephrinB1 in *Xenopus* oocytes, resulting in ephrinB1 phosphorylation. As expected, dramatically reduced amounts of ephrinB1 are detected in Dsh immune-complexes (Figure 2C). Reciprocal IP analysis shows a marked reduction in the presence of Dsh in ephrinB1 immune-complexes upon ephrinB1 phosphorylation (Figure 2C). As reported previously (Chong *et al.*, 2000), exogenously expressed ephrinB1 can interact with FGFR1 KE in CoIP analyses (Supplemental Figure S1A), and this interaction is not affected by the presence or absence of Dsh (Supplemental Figure S1A). To confirm that the disruption of the ephrinB1/Dsh complex is due to induction of FGFR1 activation, we expressed wild-type FGFR1 along with ephrinB1 and Dsh in embryos (Supplemental Figure S1B). At stage 8.5, ectodermal explants were dissected and treated with FGF-2 in the presence or absence of FGFR inhibitor PD 173074. Addition of FGF-2 induced FGFR1 activation and the loss of an interaction between ephrinB1 and Dsh in immune-complexes (Supplemental Figure S1B). It is worth noting that application of PD 173074, blocked disruption of the ephrinB1/Dsh interaction (Supplemental Figure S1B). Moreover, phosphorylation of tyrosine 324 in the C terminus of ephrinB1 is inhibited by PD 173074 but not by the Src family inhibitor PP2 (Supplemental Figure S1C). These data indicate that, unlike phosphorylation induced by an interaction with the cognate EphB1-ectodomain, FGF-induced phosphorylation of ephrinB1 is dependent upon FGFR1 activity (Supplemental Figure S1C). Together, these data indicate that the cognate Eph receptor and FGF receptor can block the interaction between ephrinB1 and Dsh through tyrosine phosphorylation of ephrinB1.

Phosphorylation of the Carboxy-Terminal Tyrosines in EphrinB1 Disrupts the Ephrin/Dsh Complex

Because we have established that the C terminus of ephrinB1 is necessary for an interaction with Dsh, whereas tyrosine phosphorylation of ephrinB1 results in disruption of this interaction, we examined whether specific tyrosines within the C terminus of ephrinB1 are important for the phosphorylation-dependent dissociation of the ephrinB1/Dsh complex. Several mutants harboring substitutions of Phe for Tyr in the intracellular domain of ephrinB1 were generated, and coexpressed along with Dsh and FGFR1 KE or a kinase-dead

version of FGFR1 (FGFR1 KD; FGFR1 C289R/K420A), and tested in CoIPs. Interestingly, an ephrinB1 mutant that harbors substitution of Phe for Tyr at positions 324 and 325 (ephrinB1Y324.5F) fails to dissociate from Dsh in the presence of FGFR1 KE (Figure 3A). Similar results were found with exogenously expressed proteins in embryo extracts that also exogenously express the EphB1(Δ C) (Figure 3B). In contrast, an ephrinB1 mutant possessing a Phe substitution at Tyr 310 (ephrinB1Y310F) can dissociate from Dsh in the presence of FGFR1 KE, indicating the specificity for tyrosines 324 and 325 (Supplemental Figure S2A). To confirm that tyrosines 324 and 310 of ephrinB1 are phosphorylated upon FGFR1 activation, a wild-type FGFR1 was expressed in embryos along with either wild-type ephrinB1 or the Y324.5F or Y310F mutant ephrinB1 proteins. Ectodermal explants were dissected and treated with FGF-2 in the presence or absence of FGFR inhibitor and examined by Western analysis by using phospho-specific Tyr 310 or Tyr 324 antibodies. As expected, upon FGF treatment in the absence of FGFR inhibitor, tyrosines 310 and 324 are phosphorylated in wild-type ephrinB1, whereas tyrosine 310 is phosphorylated in the Y324.5F mutant and tyrosine 324 is phosphorylated in the Y310F mutant (Supplemental Figure S1D). A CoIP analysis using mutants of ephrinB1 harboring single Phe for Tyr substitutions at either tyrosine 324 or 325 displays a partial reduction of ephrinB1/Dsh binding compared with wild-type ephrinB1 in the presence of FGFR KE (Supplemental Figure S2B). As expected, the FGFR1 KD control does not disrupt the ephrinB1/Dsh interaction (Figure 3A). Together, these data indicate that phosphorylation of tyrosines 324 and 325 in ephrinB1 prevents or disrupts an interaction between ephrinB1 and Dsh and may represent a critical step in the regulation of ephrinB1/Dsh signaling.

Restriction of Retinal Progenitor Cell Movement by FGFR Is Dependent upon Phosphorylation of Tyrosines 324 and 325 in EphrinB1

Activating the FGF pathway during gastrulation represses cellular movements in the presumptive anterior neural plate, but appropriate overexpression of ephrinB1 can relieve this repression (Moore *et al.*, 2004). To determine whether phosphorylation of tyrosines 324 and 325 of ephrinB1 can influence the movement of retinal progenitor clones during gastrulation, we performed experiments focusing on the D1.1.1 blastomere of a 32-cell stage embryo, which is a major contributor (~50%) to the retina later in development (Moore *et al.*, 2004; Lee *et al.*, 2006). We injected the D1.1.1 blastomere with β -galactosidase RNA alone or along with FGFR1 KE RNA. At stage 12, most control clones are broadly dispersed across the dorsal animal quadrant ($96.7 \pm 5.77\%$), whereas those expressing FGFR1 KE are more tightly confined to the midline ($33.3 \pm 5.55\%$; Figure 4A). Thus, we used a minimal amount (50 pg) of ephrinB1WT or ephrinB1Y324.5F RNA to assess the effect of these tyrosine mutations on FGFR1 KE-induced repression of cell dispersal during gastrulation (Figure 4A). Of particular interest, the ephrinB1Y324.5F mutant shows an enhanced ability to rescue the FGFR1 KE-induced restriction of cell dispersal in the β -galactosidase-expressing cells ($90.9 \pm 3.52\%$) compared with ephrinB1WT ($55.6 \pm 9.64\%$; Figure 4A), suggesting that phosphorylation of these tyrosines reduces ephrinB1-driven cell movement.

Similar results were found for the population of the retina by D1.1.1 progeny (Figure 4B). In this case, we injected the D1.1.1 blastomere with GFP RNA alone, or along with FGFR1 KE RNA, and either ephrinB1WT or ephrinB1Y324.5F mutant RNAs. As expected in GFP-in-

Figure 2. Cognate Eph receptor binding or FGF signaling blocks the interaction between ephrinB1 and Dsh through the tyrosine phosphorylation of the intracellular domain of ephrinB1. (A) EphB1-Fc was clustered using human Ig and added to the HT 29 cell culture medium. Immunoprecipitates using anti-ephrinB1 (rabbit), anti-Dsh (rabbit), or anti-c-Myc (rabbit) antibodies in HT 29 cell lysates were immunoblotted with anti-Dsh (goat), anti-phosphotyrosine-HRP-conjugated, and anti-ephrinB1 (goat) antibodies. Lysates were analyzed directly by SDS-PAGE and immunoblotted with indicated antibodies to reveal endogenous expression levels of ephrinB1 and dishevelled, respectively. (B) Oocytes were left uninjected (–) or injected (+) with ephrinB1-HA (10 ng), Dsh-Myc (10 ng), and EphB1(ΔC)-FLAG (10 ng) RNAs as indicated. Oocyte lysates were IPed with anti-Myc antibody and then immunoblotted with anti-HA antibody to detect ephrinB1 proteins. Oocyte lysates were analyzed directly by SDS-PAGE and immunoblotted with indicated antibodies. (C) Oocytes were left uninjected (–) or injected (+) with ephrinB1-FLAG (10 ng), Dsh-HA (10 ng) and FGFR1 KE (10 ng) RNAs as indicated. Oocyte lysates were IPed with anti-HA (top and second panels) or anti-FLAG (third and fourth panels) antibodies and then immunoblotted with either anti-FLAG or anti-HA antibodies to detect bound proteins. Oocyte lysates were analyzed directly by SDS-PAGE and immunoblotted with indicated antibodies.

jected controls, fluorescent clones are present throughout the retina in a majority of embryos, whereas FGFR1 KE RNA dramatically decreases the percentage of embryos with

D1.1.1 progeny in the retina (Figure 4B). In contrast, the expression of ephrinB1Y324.5F shows a markedly enhanced ability to rescue population of the retina, particularly com-

Figure 3. FGFR1- or Eph-induced phosphorylation of tyrosines 324 and 325 in ephrinB1 disrupts the interaction with Dsh. (A) Oocytes were left uninjected (–) or injected (+) with ephrinB1WT-HA (10 ng) or ephrinB1Y324.5F-HA (10 ng), Dsh-Myc (10 ng) and FGFR1 KE (10 ng), or FGFR1 KD (10 ng) RNAs as indicated. Oocyte lysates were IPed with anti-Myc antibody and then immunoblotted with anti-HA antibody to detect ephrinB1 proteins. Oocyte lysates were analyzed directly by SDS-PAGE and immunoblotted with indicated antibodies. (B) Oocytes were left uninjected (–) or injected (+) with ephrinB1WT-HA (10 ng) or ephrinB1Y324.5F-HA (10 ng), Dsh-Myc (10 ng) and EphB1(ΔC)-FLAG (10 ng) RNAs as indicated. Oocyte lysates were IPed with anti-Myc antibody, then immunoblotted with anti-HA antibody to detect ephrinB1 proteins. Oocyte lysates were analyzed directly by SDS-PAGE and immunoblotted with indicated antibodies.

Figure 4. FGFR1-induced phosphorylation of tyrosines 324 and 325 in ephrinB1 restricts the movement of retinal progenitor cells. (A) The D1.1.1 blastomere was injected with FGFR1 KE (200 pg) and 200 pg of β -galactosidase with or without 50 pg of ephrinB1WT-HA or ephrinB1Y324.5F-HA RNA. Embryos were collected at stage 12.5 and stained for β -galactosidase (red). Histogram represents lateral scatter distances of RNA-injected D1.1.1 progeny as a percentage of controls. All control embryos showed D1.1.1 progeny broadly dispersed across the dorsal animal quadrant, whereas FGFR1 KE-injected embryos were tightly confined to the midline. EphrinB1WT RNA partially rescues cell dispersion that is restricted by FGFR1 KE. In contrast, ephrinB1Y324.5F more prominently rescues the FGFR1 KE-restriction. One hundred percent normal distribution represents the standard lateral distance observed for β -galactosidase expression in control D1.1.1 progeny. Data are shown as mean $\pm$ SD. (B) The D1.1.1 blastomere was injected with FGFR1 KE (200 pg) with 250 pg of GFP with or without 50 pg of ephrinB1WT-HA or ephrinB1Y324.5F-HA RNA. Immunofluorescence was performed on 37–38 stage embryos. The histogram denotes that $>80\%$ of control embryos showed D1.1.1 progeny in the retina, whereas injection of FGFR1 KE results in a significant reduction in the percentage of embryos displaying D1.1.1 progeny in the retina. EphrinB1WT RNA partially rescues the FGFR1 KE-induced block. In contrast, ephrinB1Y324.5F robustly rescues the FGFR1 KE-restricted phenotype. Embryos with D1.1.1 injections were considered positive when fluorescent progeny contributed $>30\%$ of the cells in the retina. White ovals denote retina. Data are shown as mean $\pm$ SD, and the number of embryos per sample (N) is denoted above the histogram.

pared with ephrinB1WT (Figure 4B). Single tyrosine substitution (ephrinB1Y324F or ephrinB1Y325F) mutants display an intermediate ability (between ephrinB1WT and ephrinB1Y324.5F mutant) to rescue the FGFR1-KE-induced block of retinal progenitor cell movement (Supplemental Figure S3). These data are consistent with the biochemical studies and indicate that phosphorylation of both tyrosines 324 and 325 in ephrinB1 restricts movement of retinal progenitor cells by dissociating ephrinB1 from Dsh.

FGFR1-induced Adoption of Ventral Neural Fate Is Dependent upon Phosphorylation of EphrinB1

In progeny of the D1.1.1 blastomere, overexpression of active FGFR1 or knockdown of endogenous ephrinB1 results in an expansion of ventral neural fate at the expense of retinal fate (Moore *et al.*, 2004; Lee *et al.*, 2006). We examined the role played by the tyrosine phosphorylation sites (Y324 and Y325) within ephrinB1 during a rescue of retinal fate in the presence of FGFR1 KE. We performed whole mount in situ hybridization analysis with FGFR1 KE-overexpressing embryos in the presence of ephrinB1WT or the ephrinB1Y324.5F mutant. Consistent with fate mapping studies that show fewer D1.1.1 progeny populating the retina after injection of FGFR1 KE RNA, the expression of two eye field-specific transcription factors, *rx1* and *pax6*, are repressed. In contrast, the *pax2* ventral neural marker is expanded (Figure 5). Interestingly, introduction of the ephrinB1Y324.5F mutant rescues *rx1* and *pax6* expression, and reduces *pax2* to a near normal expression pattern (Figure 5). Moreover, ephrinB1WT displays only a partial rescue of these markers when expressed at comparable levels to ephrinB1Y324.5F (Figure 5). Together, these data strongly suggest that both tyrosine 324 and 325 of ephrinB1 play a critical role in the FGFR1-induced restriction of cell movement, which is confirmed by the alteration of cell fate.

FGFR1-induced Restriction of EphrinB1-driven Cell Movement Is Dependent on Phosphorylation of the Sites Required for Disruption of the Ephrin/Dsh Interaction

We tested the hypothesis that phosphorylation of tyrosines 324 and 325 is necessary for the FGFR1-induced block of ephrinB1-mediated cell movement. Thus, we determined whether active FGFR1 can block ephrinB1-driven dispersal of V1.1.1 progeny, which do not normally contribute to the retina during gastrulation (Moody, 1987). As expected, control cells expressing β -galactosidase alone remain more ventrally located; however, overexpressing ephrinB1WT or the ephrinB1Y324.5F mutant causes V1.1.1 progeny to disperse dorsally across the animal pole at late gastrulation (st.12.5), representing an ephrinB1-driven event (Figure 6A; Moore *et al.*, 2004; Lee *et al.*, 2006). FGFR1 KE blocks ephrinB1WT-induced movement of V1.1.1 progeny but has little effect on ephrinB1Y324.5F-induced movement (Figure 6A), indicating that tyrosines 324 and 325 are required for FGFR1 modulation of ephrinB1-driven cell movements during gastrulation.

To examine whether tyrosines at 324 and 325 in ephrinB1 are also required for an active FGFR1 to prevent V1.1.1 progeny from populating the eye field later in development, we coinjected ephrinB1WT or the ephrinB1Y324.5F mutant along with GFP RNA into V1.1.1. Both wild-type and mutant ephrinB1 can cause V1.1.1 progeny to disperse dorsally into the eye field and populate the retina (Moore *et al.*, 2004; Lee *et al.*, 2006). In the V1.1.1 progeny, expression of FGFR1 KE blocks ephrinB1WT-induced population of the retina but fails to block the ephrinB1Y324.5F-expressing progeny (Figure 6B). These findings demonstrate that the phosphorylation of tyrosines 324 and 325 in ephrinB1 is essential for FGFR1 regulation of ephrinB1-driven cell movement, and they are consistent with the requirement for an interaction with Dsh.

Figure 5. Phosphorylation of both tyrosines is necessary for an FGFR1-induced alteration of cell fate. The D1.1.1 blastomere was injected with 200 pg of FGFR1 KE alone or with 50 pg of wild-type ephrinB1 or the Y324.5F mutant along with 250 pg of GFP RNA. *Xenopus* embryos were collected at stage 16 and subjected to whole mount in situ hybridization. Active FGFR1 reduces eye-specific transcription factors *rx1* and *pax6*. In contrast, ventral neural fate marker *pax2* is expanded on the injected side. A suboptimal amount of ephrinB1WT expression partially rescues FGFR1 KE-induced reduction of eye-specific markers and expansion of the ventral neural marker, but the ephrinB1Y324.5F mutant rescues these markers to a near normal pattern. The asterisk indicates injected side.

FGFR1 Regulates Dsh Localization through the Phosphorylation of EphrinB1

It has been shown that coexpression of wild-type ephrinB1 can relocalize a GFP-Dsh fusion protein from the cytoplasm to the cell membrane (Lee *et al.*, 2006), a hallmark of PCP

signaling (Yang-Snyder *et al.*, 1996). We examined whether the activation of FGFR1 prevents Dsh localization to the membrane in response to ephrinB1, and we also tested whether tyrosines 324 and 325 play a role in Dsh localization. Both blastomeres of two-cell stage embryos were injected with GFP-Dsh RNA along with either ephrinB1WT or ephrinB1Y324.5F mutant RNA in the presence or absence of FGFR1 KE RNA. As expected, expressing ephrinB1WT in gastrula stage ectoderm induces a relocalization of GFP-Dsh from diffuse or punctate cytoplasmic regions to the apical and basolateral domains of the membrane (Lee *et al.*, 2006). Interestingly, this relocalization is blocked by coexpression of FGFR1 KE (Figure 7A). In striking contrast, FGFR1 KE has no effect on the membrane localization of GFP-Dsh induced by expression of the ephrinB1Y324.5F mutant (Figure 7A). These results are consistent with the physical interaction and functional data, and indicate that the FGFR1 regulates Dsh localization through the phosphorylation of tyrosines 324 and 325 on ephrinB1 and thus may regulate PCP signaling through this mechanism.

FGFR1-induced Restriction of Retinal Population Is Circumvented by Activating the PCP Pathway Downstream of EphrinB1

We demonstrated previously that Dsh mediates ephrinB1 signaling in the eye field through the PCP pathway (Lee *et al.*, 2006). To further test whether the FGFR1-induced inhibition of retinal population was due to its influence on ephrinB1, we activated the PCP pathway downstream of ephrinB1 in the presence of an active FGFR1. Overexpression of Dsh in D1.1.1 rescues the FGFR1 KE-induced block to retinal population, as evidenced by the appearance of GFP-expressing D1.1.1 progeny cells in the retina (Figure 7B). Moreover, introduction of an active form of RhoA (CA RhoA), which is a critical downstream mediator of PCP signaling (Strutt *et al.*, 1997), also rescues the FGFR1 KE-induced block to retinal population, whereas an inactive form of RhoA (DN RhoA) fails (Figure 7B). Collectively, these data suggest that FGFR1 activity exerts an inhibitory effect on retinal progenitor movement by phosphorylating

Figure 6. Phosphorylation on tyrosines 324 and 325 is necessary for blocking ephrinB1-driven movement of cells during gastrulation. The V1.1.1 blastomere was injected with 250 pg of ephrinB1WT-HA or ephrinB1Y324.5F-HA RNA with or without FGFR1 KE (400 pg) and 200 pg of β -galactosidase or 250 pg of GFP RNA (A and B, respectively). (A) Embryos were collected at stage 12.5 and stained for β -galactosidase (red). Histogram represents the percentage of embryos displaying ventral to dorsal (V-D) movements of RNA-injected V1.1.1 progeny. All control embryos display a tight restriction of V1.1.1 progeny to the ventral side, whereas ephrinB1WT or ephrinB1Y324.5F-injected embryos show V1.1.1 progeny dispersed to dorsal regions. FGFR1 KE RNA restricts ephrinB1WT-induced cell movement, whereas ephrinB1Y324.5F-expressing cells migrated dorsally. Embryos are considered positive for V-D movements when 30% of progeny cells cross the ventral-dorsal midline. Data are shown as mean $\pm$ SD, and the number of embryos per sample (N) is denoted above the histogram. (B) Embryos were collected at stage 37–38, sectioned, and immunofluorescence was performed. The histogram denotes that below 10% of

control embryos showed V1.1.1 progeny in the retina. Injection of ephrinB1WT or ephrinB1Y324.5F mutant RNA results in a significant induction in the percentage of embryos displaying V1.1.1 progeny in the retina. FGFR1 KE RNA restricts ephrinB1WT-induced cell movement but it does not restrict ephrinB1Y324.5F-expressing cells. Embryos injected in V1.1.1 were considered positive when fluorescent progeny contributed >10% of the cells in the retina. White ovals denote retina. Data are shown as mean $\pm$ SD.

Figure 7. FGFR1-induced phosphorylation of tyrosines 324 and 325 in ephrinB1 is necessary to block PCP signaling. (A) GFP-Dsh RNA (250 pg) was injected singly or with RNAs encoding HA-tagged ephrinB1WT (400 pg) or ephrinB1Y324.5F (400 pg) in the presence of FGFR1 KE (500 pg) into each blastomere of two-cell stage embryos and collected at stage 10.5. Embryos were sectioned and immunostained for ephrinB1 (HA) or GFP-Dsh (GFP). Fluorescence microscopy shows cytoplasmic localization when GFP-Dsh is expressed alone. In contrast, coexpression of GFP-Dsh with ephrinB1WT or ephrinB1Y324.5F relocalizes Dsh to the membrane. Moreover, FGFR1 KE disrupts ephrinB1WT-induced membrane localization of Dsh but fails to disrupt ephrinB1Y324.5F-induced Dsh relocalization. (B) The D1.1.1 blastomere was injected with 200 pg of FGFR1 KE and 250 pg of Dsh or 50 pg of CA RhoA or 250 pg of DN RhoA RNA along with 250 pg of GFP RNA. At stage 37–38, the retina was examined, and a significant rescue by either Dsh or CA RhoA was observed as evidenced by the percentage of FGFR1 KE-injected embryos with fluorescent D1.1.1 retinal clones in the retina. White ovals denote retina. Data are shown as mean $\pm$ SD.

the C terminus of ephrinB1, and this inhibition can be circumvented by activating downstream members of the PCP pathway.

DISCUSSION

FGF family members control morphogenetic movements and cell motility during gastrulation (Slack *et al.*, 1988; Amaya *et al.*, 1991; Amaya *et al.*, 1993; Rossant *et al.*, 1997; Wacker *et al.*, 1998; Yang *et al.*, 2002; Chuai *et al.*, 2006), and they modulate cell adhesive interactions (Kinoshita *et al.*, 1993). Targeted disruption of FGFR1 or FGF-8 results in mice with severe gastrulation defects, including abnormalities in the migration of cells within the primitive streak (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994; Sun *et al.*, 1999). Although FGF signaling promotes gastrulation movements of mesoderm through the streak, it seems to inhibit movement of retinal progenitor cells (Moore *et al.*, 2004). We found previously that constitutive activation of FGFR1 or FGFR2 in the embryonic lineage that gives rise to the anterior neural plate prevents cellular dispersal during late gastrulation and early neural plate formation (Moore *et al.*, 2004). This lack of cell movement results in cells expressing ventral neural markers rather than retinal markers. Moreover, acquisition of a retinal fate requires dispersal of cells in the anterior neural plate, and activation of FGFR signaling represses this cellular dispersal (Moore *et al.*, 2004).

FGFs can interact with adhesion molecules involved in morphogenetic movements, including neural cell adhesion molecules (Doherty and Walsh, 1996) and ephrins (Chong *et al.*, 2000). Ephrins and their tyrosine kinase receptors (Ephs) are important for multiple developmental processes involving cell migration and morphogenetic movements (Jones *et al.*, 1998; Pasquale, 2005; Sela-Donenfeld and Wilkinson, 2005; Arvanitis and Davy, 2008; Pasquale, 2008). These processes include guidance of axonal growth, segmentation of the hindbrain and somites, vasculogenesis, neural crest migration, and the control of gastrulation movements (Poliakov *et al.*, 2004). In *Xenopus*, ephrinB1 is expressed in the anterior ectoderm during gastrulation (Jones *et al.*, 1997) and later in the anterior neural plate overlapping the eye field (Moore *et al.*, 2004). Thus, it displays appropriate temporal and spatial expression to modulate the early morphogenetic movements of retinal precursors. Moreover, ephrinB1 is re-

quired normally for retinogenic cells to acquire a retinal fate (Moore *et al.*, 2004; Lee *et al.*, 2006), and ephrinB1 overexpression is able to rescue the inhibition of retinal fate caused by activated FGFR signaling (Moore *et al.*, 2004). These previous studies indicate that ephrinB1 signaling promotes retinogenic cell movements in the anterior ectoderm in opposition to FGFR signaling. Additionally, activation of the endogenous FGFR can induce the tyrosine phosphorylation of ephrinB1 in embryonic chick retina (Chong *et al.*, 2000). Also, in *Xenopus* embryos, the FGFR can interact with ephrinB1 and induce its phosphorylation both in vitro and in vivo (Chong *et al.*, 2000; Supplemental Figure S1A).

Based on this interaction, we proposed that during normal development, cells are recruited to the eye field by activation of the ephrinB1 signaling pathway, which allows them to disperse (Moore *et al.*, 2004; Lee *et al.*, 2006). An interaction with an active FGFR is likely to occur along the anterior borders of the anterior neural plate based on the expression domains of FGFR2 and FGFR4 (Golub *et al.*, 2000). This interaction may suppress this cell movement and thereby repress a retinal fate. In *Xenopus* embryos, it has been shown that Dsh associates with ephrinB1 and mediates ephrinB1 signaling via members of the PCP pathway during eye field formation (Lee *et al.*, 2006). Although these studies have provided some insight into how ephrinB1 promotes movement of retinal progenitors, the mechanism by which FGFR modulates this signaling and thus results in restriction of retinal progenitor movement is unknown.

Here, we demonstrate that ephrinB1 can be dissociated from Dsh through the tyrosine phosphorylation of ephrinB1. Using the extracellular domain of the cognate Eph receptor, we show that tyrosine phosphorylation of endogenous ephrinB1 in HT 29 cells disrupts its interaction with the endogenous Dsh protein (Figure 2A). We also show that the active FGFR1 can induce the same event using exogenously expressed proteins in *Xenopus* oocytes (Figure 2C) and that mutations in both tyrosines 324 and 325 prevent disruption of the complex with Dsh (Figure 3A). Interestingly, Kalo *et al.* (2001) derived an in vivo phosphorylation profile of ephrinB1 in chick retina, and identified a phosphorylated peptide containing the equivalent of these two tyrosines. It is worth noting that these two tyrosines are found in a C terminal PDZ binding motif of ephrinB1 and that phosphorylation of this region has been reported to have little effect

on the binding of some interactors, such as PDZ-RGS3 (Lu *et al.*, 2001) or Fap-1 (Lin *et al.*, 1999; Palmer *et al.*, 2002), but it does reduce binding to syntenin (Lin *et al.*, 1999; Palmer *et al.*, 2002). Thus, there is specificity regarding how tyrosine phosphorylation may affect recruitment and retention of binding partners to the ephrinB1 C terminus.

In our study we reveal the functional significance of C-terminal tyrosine phosphorylation, show FGFR1-induced restriction of retinal progenitor cell movement and the corresponding reduction of retinal markers are dependent upon tyrosines 324 and 325 in ephrinB1 (Figures 4, A and B, and 5, respectively). Moreover, if ephrinB1 is overexpressed in a ventral blastomere (V1.1.1) that normally does not give rise to retinal tissue, a portion of the progeny will migrate into the retinal field. This ephrinB1-driven cell movement can be restricted by FGFR1-induced phosphorylation, but is dependent upon tyrosines 324 and 325 (Figure 6, A and B).

Finally, ephrinB1 activation of the PCP signaling pathway is mediated by its interaction with Dsh (Lee *et al.*, 2006), and coexpression of wild-type ephrinB1 can relocalize a GFP-Dsh fusion protein from the cytoplasm to the cell membrane (Lee *et al.*, 2006), indicating that the PCP pathway is activated (Yang-Snyder *et al.*, 1996). In this report, we show that an active FGFR1 blocks this PCP hallmark event but not when the C terminal tyrosines within ephrinB1 are mutated (Figure 7A), indicating a requirement for phosphorylation on tyrosines 324 and 325. These data show a clear correlation between FGFR1-induced dissociation of the ephrinB1/Dsh interaction and disruption of PCP signaling as evidenced by Dsh relocalization. Additional evidence supporting a block of PCP signaling at the level of ephrinB1 phosphorylation, comes from experiments showing that overexpression of Dsh or expression of an activated RhoA restores retinal population by D1.1.1 progeny, even in the presence of an active FGFR1 (Figure 7B). In summary, our findings demonstrate that FGFR1-induced phosphorylation of ephrinB1 on tyrosines 324 and 325 disrupts the ephrinB1/Dsh interaction, leading to a loss of ephrinB1-induced PCP activation.

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