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Cancer Somatic Mutations Disrupt Functions of the EphA3 Receptor Tyrosine Kinase through Multiple Mechanisms

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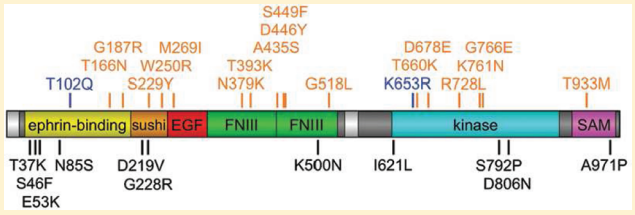
Supporting Information

ABSTRACT: The Eph receptor tyrosine kinases make up an important family of signal transduction molecules that control many cellular processes, including cell adhesion and movement, cell shape, and cell growth. All of these are important aspects of cancer progression, but the relationship between Eph receptors and cancer is complex and not fully understood. Genetic screens of tumor specimens from cancer patients have revealed somatic mutations in many Eph receptors. The most highly mutated Eph receptor is EphA3, but its functional role in cancer is currently not well established. Here we show that many EphA3 mutations identified in lung, colorectal, and hepatocellular cancers, melanoma, and glioblastoma impair kinase activity or ephrin ligand binding and/or decrease the level of receptor cell surface localization. These results suggest that EphA3 has ephrin- and kinase-dependent tumor suppressing activities, which are disrupted by somatic cancer mutations.



The Eph receptors represent the largest family of receptor tyrosine kinases and are divided into two classes: EphA and EphB. There are nine EphA receptors and five EphB receptors in the human genome.[1,2] The EphA receptors prefer to bind the GPI-linked ephrin-A ligands, and the EphB receptors prefer to bind the transmembrane ephrin-B ligands, although interclass binding of ephrins to Eph receptors can occur. EphA and EphB receptors are both type I transmembrane proteins and have a similar multidomain structure. Their extracellular region contains an N-terminal ephrin-binding domain, a cysteine-rich region that can be further subdivided into a sushi domain and an epidermal growth factor (EGF)-like domain, and two fibronectin type III domains (Figure 1). The sushi domain, also known as the complement control protein (CCP) domain, contains approximately 60 amino acids arranged in a β-sandwich structure that is stabilized by two disulfide bonds (http://smart.embl-heidelberg.de/smart/do_annotation.pl?DOMAIN=CCP). The intracellular region of the Eph receptors includes the juxtamembrane segment, the kinase domain, a sterile alpha motif (SAM) domain, and a C-terminal PDZ domain-binding motif.

Ephrin binding induces Eph receptor signaling by promoting oligomerization in concert with several receptor−receptor interaction surfaces located in the ephrin-binding domain, sushi domain, N-terminal fibronectin type III domain, transmembrane domain, and SAM domain.[2−7] The physical proximity of clustered Eph receptor molecules leads to their reciprocal transphosphorylation. Phosphorylation of several intracellular tyrosines enhances Eph kinase activity by disrupting the inhibitory interactions of the juxtamembrane segment with the kinase domain and causing partial ordering of the activation loop.[5,8,9] Furthermore, some of the tyrosine-phosphorylated motifs contribute to Eph receptor signaling by serving as binding sites for cytoplasmic signaling proteins containing Src-homology 2 (SH2) and phosphotyrosine-binding (PTB) domains.

The Eph receptors are expressed in most cell types, including cancer cells and tumor endothelial cells.[1,3,10−12] They can have a dual role in tumorigenesis, leading to tumor promotion or tumor suppression depending on the cellular context, the specific receptor involved, and other poorly understood factors. Recent genetic screens of cancer specimens and cell lines have revealed somatic mutations in essentially all Eph receptors in a wide variety of cancer types.[1] EphA3 is the Eph receptor found to be most frequently mutated in lung cancer, with 19 missense mutations identified so far and a mutation rate of 6% in a study of 188 lung adenocarcinomas.[13−16] EphA3 missense mutations have also been reported in other cancers: six in colorectal cancer,[17,18] three in melanoma,[19,20] four in glioblastoma,[19,21] one in hepatocellular carcinoma,[22] two in pancreatic cancer,[23] one in head and neck squamous cell carcinoma,[24] and four in ovarian cancer[25] (Table 1 of the Supporting Information).

The 40 different mutations are distributed throughout the domains of EphA3 (Figure 1). The frequency of EphA3 mutations in lung cancer is significantly higher than expected by chance, suggesting that EphA3 is a lung cancer "driver" gene, whose mutations play a causal role in cancer development and/or progression.[16] One study suggested that EphA3 is likely to be a protooncogene in lung cancer because it is a receptor tyrosine kinase and was found to be amplified in two lung

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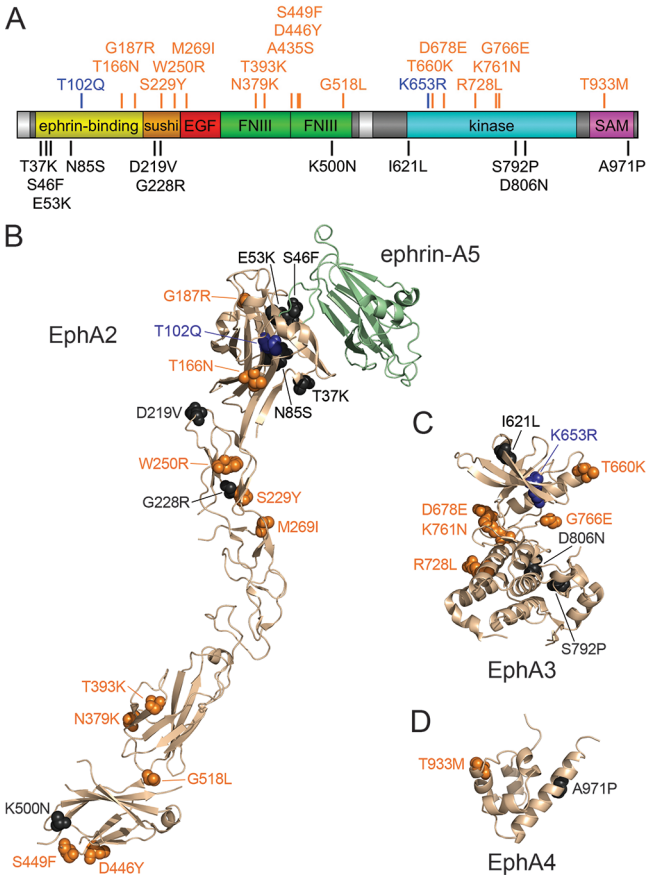

Figure 1. Location of EphA3 somatic cancer mutations in the receptor domain structure. (A) Schematic illustrating the location of the studied mutations with respect to the domains of EphA3. Mutations identified in lung cancer are colored orange, mutations identified in other cancers black, and control mutations known to disrupt ephrin binding (T102Q) or kinase activity (K653R) blue. (B) Crystal structure of the extracellular domain of EphA2 bound to ephrin-A5 (PDB entry 2X11) used as a model to show the location of the EphA3 mutations because the structure of the extracellular region of EphA3 has not been determined. (C) Location of the mutations in the kinase domain of EphA3 (PDB entry 2QOQ). (D) Crystal structure of the SAM domain of EphA4 (PDB entry 1B0X) used as a model to show the location of the EphA3 mutations because the structure of the SAM domain of EphA3 has not been determined. The mutated amino acids are denoted by spheres, orange for mutations found in lung cancer, black for mutations found in other cancers, and blue for the two control mutations. A list of all somatic cancer mutations in EphA3 is provided in Table 1 of the Supporting Information.

cancer specimens.[16] In addition, one of the mutations identified in the EphA3 kinase domain corresponds to an FGF receptor activating mutation. On the other hand, EphA3 was predicted to be a tumor suppressor in head and neck cancer, and the 3p11.2 chromosomal region where the EphA3 gene is located undergoes frequent loss of heterozygosity in different types of cancers.[1,24] Characterization of the functional effects of the mutations found in lung cancer and other cancers could help resolve the role of EphA3 in these cancers.

We therefore generated the 28 mutations that had been reported at the time our study began and systematically examined how they affect several aspects of EphA3 function. We found that many of the mutations disrupt EphA3 autophosphorylation and kinase activity, ephrin binding, and cellular trafficking. These results suggest that the loss of EphA3's ability to signal in response to ephrin ligands may facilitate the development and progression of several cancer types.

EXPERIMENTAL PROCEDURES

EphA3 Cloning and Mutagenesis. The full-length human EphA3 cDNA clone was purchased from Invitrogen (clone MGC:71556; GenBank accession number NP_005224.2) and subcloned into the pEGFP-IRES2 vector (Clontech, Mountain View, CA) between the EcoRI and BamHI restriction sites. The EphA3 mutants were generated using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions. Wild-type and mutant EphA3 alkaline phosphatase (AP) fusion proteins were generated by subcloning cDNAs encoding wild-type and mutant extracellular domains (amino acids 1−537) into the pAPtag-2 vector (GenHunter, Nashville, TN) between BglII and BspEI restriction sites, except for the G228R mutant, which was cloned between the BamHI and BspEI restriction sites.

Cell Lines and Transfection. HEK 293T cells were grown in Dulbecco's modified Eagle's medium (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics. Lipofectamine Plus (Invitrogen, Carlsbad, CA) was used to transfect HEK 293T cells with wild-type and mutant EphA3 cDNA in the pEGFP-IRES2 vector, following the manufacturer's instructions and using various amounts of DNA (1−8 μg) to obtain similar wild-type and mutant EphA3 protein levels (see Figures 2A and 3B).

Immunoblotting. For immunoblotting, cells were lysed in modified RIPA buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA] containing protease and phosphatase inhibitors. Cell lysates were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) followed by immunoblotting with an anti-EphA3 antibody (sc-919, Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-human Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by either an anti-rabbit or an anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) or with a phosphotyrosine antibody conjugated to HRP (610012, BD Biosciences, San Jose CA). Immunoblots were developed with HyGLO chemiluminescence HRP detection reagent (Denville Scientific, Metuchen, NJ).

Production of EphA3 AP. Ten micrograms of wild-type or mutant EphA3 AP plasmid DNA was transiently transfected into HEK 293T cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Culture medium containing secreted EphA3 AP fusion proteins was concentrated using Amicon Ultra-Centrifugal filters (Millipore, Billerica, MA). The concentration of the AP fusion proteins was estimated on the basis of AP activity measurements.[26,27]

Quantification of EphA3−Ephrin-A5 Binding by an Enzyme-Linked Immunosorbent Assay (ELISA). Protein A-coated 96-well plates (Pierce Scientific, Rockford, IL) were used to immobilize ephrin-A5 Fc (0.1 μg/mL) (R&D Systems, Minneapolis, MN) or Fc control (MP Biomedical, Solon, OH) for 1 h at room temperature in 3% BSA in Tris-buffered saline with 0.01% Tween 20 (TBST). Various amounts of culture medium containing concentrated EphA3 AP fusion proteins diluted in TBST were subsequently added for 1 h at room temperature. After three washes with TBST, binding was quantified using *p*-nitrophenyl phosphate (pNPP) (Pierce Scientific) as

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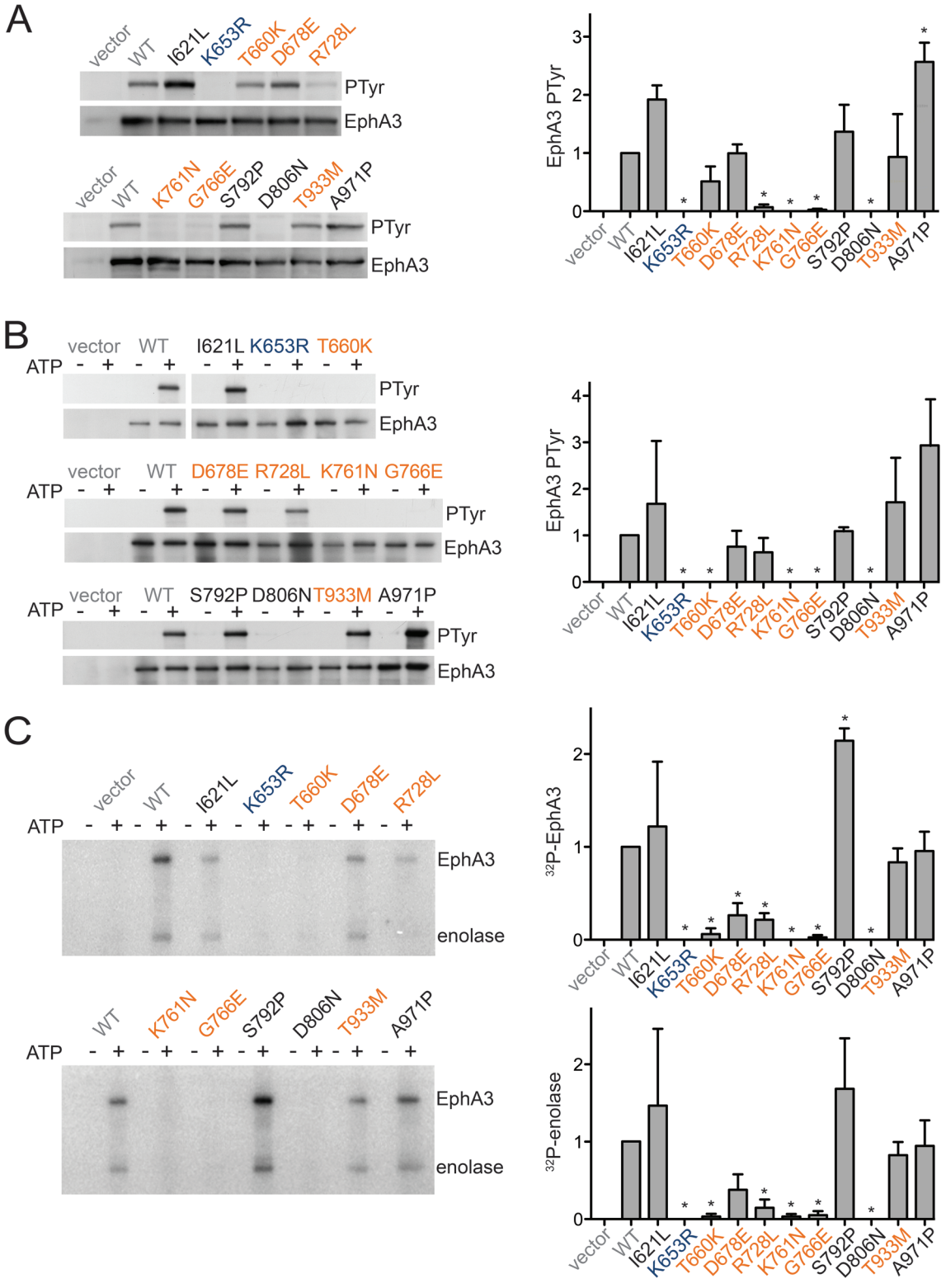

Figure 2. Effects of mutations in the intracellular region of EphA3 on receptor tyrosine phosphorylation. (A) Representative immunoblots showing tyrosine phosphorylation (PTyr) and total levels of wild-type EphA3 and the indicated mutants expressed by transient transfection in HEK 293 cell lysates. The histogram shows averages ± SEM calculated from quantification of three separate experiments and normalized to the wild-type value in each experiment. (B) Representative immunoblots showing autophosphorylation of immunoprecipitated wild-type and mutant EphA3 after in vitro kinase assays with and without the addition of ATP. The histogram shows averages ± SEM calculated from quantification of three separate experiments and normalized to the wild-type value in each experiment. *$P < 0.05$ by Student's paired two-tailed t test comparing phosphorylation of each mutant to that of the wild type. (C) Representative images showing phosphorylation of enolase by immunoprecipitated wild-type and mutant EphA3 as well as autophosphorylation after in vitro kinase assays with and without the addition of [γ-^{32}P]ATP. The histograms show averages ± SEM calculated from quantification of three separate experiments and normalized to the wild-type value in each experiment. *$P < 0.05$ by Student's paired two-tailed t test comparing mutants to the wild type.

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the substrate. Dissociation constants (K_D) were calculated using nonlinear regression and GraphPad Prism (Graphpad Software, Inc., La Jolla, CA).

Assessment of EphA3−Ephrin-A5 Binding by a Pull-Down Assay. Transfected cells were lysed in modified RIPA buffer, and lysates were incubated with 1 μg of ephrin-A5 Fc immobilized on GammaBind Plus Sepharose beads (GE Healthcare, Piscataway, NJ). Complexes were washed three times with RIPA buffer, boiled in sample buffer, separated by SDS−PAGE, and probed by immunoblotting for EphA3.

In Vitro Kinase Assay. HEK 293T cells expressing wild-type or mutant EphA3 were lysed in RIPA buffer without phosphatase inhibitors. EphA3 was immunoprecipitated with 2.5 μg of anti-EphA3 antibody (37-3200, Life Science Technologies-Invitrogen, Carlsbad, CA) and immobilized on GammaBind Plus Sepharose. Immunoprecipitates were then incubated with 25 mM Hepes (pH 7.5), 10 mM $MnCl_2$, 10 $MgCl_2$, 1 mM sodium orthovanadate, 0.1% Triton X-100, and 150 μM ATP with phosphatase inhibitors for 30 min at 30 °C, and reactions were analyzed by immunoblotting for phosphotyrosine and EphA3. Immunoblots were quantified using Image J.

To measure phosphorylation of enolase by EphA3, immunoprecipitates with 1.25 μg of anti-EphA3 antibody (Santa Cruz, sc-919) were washed twice with HNTG buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100] and then twice with kinase reaction buffer [10 mM Hepes (pH 7.5), 25 mM $MgCl_2$, and 10 mM $MnCl_2$]. Rabbit muscle enolase (Sigma) was acid denatured, and 6.25 μg was added to each kinase reaction mixture together with 5 μCi of [γ-^{32}P]ATP. ^{32}P incorporated into enolase and EphA3 after incubation for 30 min at 30 °C was quantified from scans obtained with a Storm phosphorimager using Image J.

Measurement of Ephrin-Binding Domain Folding. Protein-A plates were coated with 0.5 μg/mL anti-EphA3 antibody (sc-919, Santa Cruz Biotechnology) in 3% bovine serum albumin (BSA) in TBST to capture EphA3 from transiently transfected HEK 293T cells lysed in modified RIPA buffer. The amounts of cell lysates used were sufficient to saturate the binding sites of the coated anti-EphA3 antibody, as verified by comparing the results with those obtained with twice as much lysate (data not shown). This ensured that the same amount of EphA3 was immobilized in all the wells. EphA3 with a properly folded ephrin-binding domain was detected using 2 μg/mL IIIA4 anti-EphA3 antibody (from Millipore or a kind gift from A. Boyd) in 3% BSA in TBST, followed by a goat anti-mouse AP antibody (AP124A, Millipore), and binding was quantified using pNPP as the substrate.

Quantification of Cell Surface EphA3. HEK 293T cells expressing wild-type or mutant EphA3 were incubated with EZ-link SulfoNHS-LC-Biotin (Pierce-Thermo Scientific, Rockford, IL) to biotinylate cell surface proteins. Lysates were captured using an anti-EphA3 antibody recognizing an intracellular epitope (Santa Cruz; see the previous section), and biotin labeling was detected using a streptavidin−HRP conjugate (Pierce-Thermo Scientific). The amounts of cell lysates used were sufficient to saturate the binding sites of the coated anti-EphA3 antibody, as verified by comparing the results with those obtained with twice as much lysate (data not shown). This ensured that the same amount of EphA3 was immobilized in all the wells.

RESULTS

Mutations in the EphA3 Intracellular Region Affect Tyrosine Phosphorylation and Kinase Activity. Phosphorylation of cytoplasmic tyrosine residues is a hallmark of Eph receptor activation and downstream signaling.[3] Like other Eph receptors, wild-type EphA3 is constitutively tyrosine phosphorylated when overexpressed by transient transfection in HEK 293 cells (Figure 2A).[28−30] Interestingly, we found that four of the eight mutations examined in the EphA3 kinase domain [R728L, K761N, G766E, and D806N (Figure 1)] essentially abolish EphA3 tyrosine phosphorylation in HEK 293 cells (Figure 2A and Table 1). This effect is similar to that of

Table 1. Effects of EphA3 Mutations[1]

Mutation	Domain	PTyr in cells[2]	*In vitro* kinase activity	Ephrin-A5 binding	IIIA4 antibody binding	Cell surface localization
Wild-type		=	=	=	=	=
T37K	EB[3]	<	nd	=	=	=
S46F	EB	nd	nd	<	<<	<<
E53K	EB	=	nd	<<	=	=
N85S	EB	=	nd	<	=	=
T102Q	EB	<	nd	<<	=	=
T166N	EB	<	nd	<	<<	=
G187R	EB	nd	nd	<<	<<	<<
D219V	Sushi	=	nd	<	=	=
G228R	Sushi	nd	nd	<<	<<	<<
S229Y	Sushi	=	nd	<	<	=
W250R	Sushi	nd	nd	<<	<<	<<
M269I	EGF	nd	nd	<	<<	=
N379K	FNIII-N[4]	=	nd	nd	=	=
T393K	FNIII-N	=	nd	nd	=	=
A435S	FNIII-C[4]	nd	nd	nd	=	=
D446Y	FNIII-C	nd	nd	nd	=	=
S449F	FNIII-C	nd	nd	nd	=	=
K500N	FNIII-C	nd	nd	nd	=	=
G518L	FNIII-C	nd	nd	nd	=	=
I621L	Kinase	=	=	nd	nd	<
K653R	Kinase	<<	<<	nd	nd	=
T660K	Kinase	=	<<	nd	nd	<
D678E	Kinase	=	<	nd	nd	=
R728L	Kinase	<<	<	nd	nd	<
K761N	Kinase	<<	<<	nd	nd	=
G766E	Kinase	<<	<<	nd	nd	<
S792P	Kinase	=	=	nd	nd	=
D806N	Kinase	<<	<<	nd	nd	=
T933M	SAM	=	=	nd	nd	<
A971P	SAM	=	=	nd	nd	=

[1]Legend: =, similar to that of the wild type; <, less than that of the wild type; ≪, much less than that of the wild type. [2]Tyrosine phosphorylation of EphA3 overexpressed in HEK 293 cells. [3]Ephrin-binding domain. [4]FNIII-N, N-terminal fibronectin type III domain; FNIII-C, C-terminal fibronectin type III domain.

the K653R control mutation, which disrupts ATP binding.[29] The two intracellular mutations outside the kinase domain are located in the SAM domain. One of them (A971P) significantly increases the level of tyrosine phosphorylation of EphA3 overexpressed in HEK 293 cells. On the other hand, phosphorylation of the EphA3 I621L, D678E, and S792P kinase domain mutants and the T933M SAM domain mutant is not significantly different from that of the wild type.

The level of EphA3 tyrosine phosphorylation in the cellular environment depends on several factors, including autophosphorylation of the activated receptor, possibly phosphorylation by other tyrosine kinases, and the activity of phosphatases.[3,31−33] To directly examine how the intracellular mutations affect the ability of EphA3 to autophosphorylate, we performed in vitro kinase assays using immunoprecipitated EphA3.[30] Autophosphorylation of the EphA3 T660K, K761N, G766E, and D806N mutants (assessed either by immunoblotting with anti-phosphotyrosine antibodies or by measuring incorporation of ^{32}P) was undetectable, a situation similar to

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that of the control kinase inactive K653R mutant (Figure 2B,C and Table 1). A significant deficiency in autophosphorylation was observed for the D678E and R728L mutants by measuring ^{32}P incorporation but not by immunoblotting with anti-phosphotyrosine antibodies, suggesting a less pronounced impairment of these mutants. The other intracellular region mutants retained the ability to autophosphorylate like or better than the wild type. Phosphorylation of acid-denatured enolase as a substrate in the in vitro kinase assays showed a significant loss of kinase activity for the T660K, R728L, K761N, G766E, and D806N mutants (Figure 2C). Therefore, according to both phosphorylation levels in cells and autophosphorylation in vitro, some of the mutations in the EphA3 kinase domain drastically inhibit receptor tyrosine phosphorylation and kinase activity. However, some intracellular mutations do not affect, or may even increase, the level of EphA3 tyrosine phosphorylation.

Mutations in the Ephrin-Binding Domain and Cysteine-Rich Region of EphA3 Impair Ephrin Ligand Binding. Ephrin-A5 is a preferred ligand for EphA3.[34] The EphA3−ephrin-A5 interaction has been proposed to involve a high-affinity interface in the ephrin-binding domain of EphA3 as well as two lower-affinity interfaces located in the ephrin-binding domain and the neighboring sushi domain,[2,7,35] although direct structural information is not yet available. Twelve of the EphA3 mutations studied are in the ephrin-binding or sushi domain (Figure 1).

To quantitatively assess the effects of the mutations on EphA3−ephrin-A5 binding, we used an ELISA to measure the binding of wild-type or mutant EphA3 AP (the extracellular domain of EphA3 fused to alkaline phosphatase) to immobilized ephrin-A5 Fc (ephrin-A5 fused to the Fc portion of human IgG1) (Figure 3A and Table 1). Although AP fusion proteins are dimeric, which increases their binding avidity,[36] the apparent K_D values obtained with dimeric proteins can be used to compare the binding strength of different Eph receptor−ephrin combinations.[37] A caveat is that the effects of the mutations on binding interactions may be underestimated by using dimeric proteins. The ELISA for binding yielded a 10-fold higher apparent K_D value for the T102Q mutant than for wild-type EphA3, consistent with previous findings that this mutation impairs but does not completely disrupt binding of ephrin-A5 to EphA3.[35] We also observed a drastic effect for the E53K mutation in the high-affinity ephrin-binding interface, which causes an almost 100-fold increase in the K_D compared to that of the wild type, also consistent with the results of a previous random mutagenesis study.[35] The N85S and T166N mutations in the ephrin-binding domain, the D219V and S229Y mutations in the sushi domain, and the M269I mutation in the EGF domain yielded 1.5−2-fold higher apparent K_D values compared to that of wild-type EphA3, suggesting that these mutations cause a smaller impairment of ephrin binding ability. In contrast, the T37K mutation in the ephrin-binding domain did not detectably affect binding of EphA3 to ephrin-A5. The S46F ephrin-binding domain mutant did not reach the same maximal binding as wild-type EphA3, suggesting an unusual effect of the mutation on either ephrin binding or the specific activity of the fused alkaline phosphatase.

The effects of the ephrin-binding domain G187R mutation and the sushi domain G228R and W250R mutations could not be examined in this ELISA because the EphA3 AP proteins with these mutations were poorly expressed and not secreted into the culture medium of the producing HEK 293 cells (data not shown). The three full-length EphA3 mutants, however, were present in soluble form in the lysates of transfected HEK 293 cells. We could therefore assess their binding to ephrin-A5 Fc immobilized on Sepharose beads in pull-down assays (Figure 3B and Table 1). Although these pull-down assays were not sensitive enough to reveal the less prominent effects of some mutations on ephrin binding, they showed a drastic impairment in the association of the G187R, G228R, and W250R mutants with ephrin-A5 Fc.

Conformational abnormalities in the ephrin-binding domain of EphA3 can be detected by using the IIIA4 monoclonal antibody, which binds to the N-terminal region of EphA3 (including the ephrin-binding and sushi domains) and does not recognize the denatured receptor.[35,38,39] The binding of this antibody to full-length wild-type and mutant EphA3 was assessed by immobilizing the receptor in ELISA wells with an antibody that binds to the C-terminal tail. We found that binding of the IIIA4 antibody to the G187R mutant is undetectable (Figure 4A and Table 1), suggesting that this mutation, which is not near the proposed IIIA4 binding site,[35] severely disrupts the conformation of the ephrin-binding domain. The S46F mutation also markedly decreases the level of IIIA4 antibody binding, suggesting that this mutation also has a substantial effect on the folding of the domain. This conformational change may also explain the aberrant ephrin-A5 binding curve observed for this mutant (Figure 3B). The other mutations in the ephrin-binding domain impair IIIA4 antibody binding to a lesser extent or do not have a detectable effect. However, the G228R and W250R mutations in the sushi domain and the M269I mutation in the EGF domain also drastically impair IIIA4 antibody binding. This suggests that conformational defects induced by these mutations in the cysteine-rich region of EphA3 may affect the ephrin-binding domain. In summary, most mutations in the ephrin-binding domain and cysteine-rich region of EphA3 inhibit ephrin binding, albeit to varying degrees. In some cases, this appears to involve conformational defects in the ephrin-binding domain or nearby regions.

Many Mutations Affect EphA3 Cell Surface Localization. Transmembrane and secreted proteins that are not correctly folded remain in the endoplasmic reticulum and are subsequently degraded.[40] Therefore, EphA3 mutants that are not properly folded may not reach the cell surface efficiently and thus be less susceptible to ephrin-dependent activation. To determine whether any of the EphA3 mutations interfere with cell surface localization, we used a cell surface biotinylation assay. After cell surface proteins in transfected HEK 293 cells had been covalently labeled with biotin, wild-type EphA3 and its mutants were immobilized in ELISA wells using a C-terminal antibody. The fraction of EphA3 present on the cell surface was then quantified using a streptavidin−HRP conjugate. This assay revealed that the S46F, G187R, G228R, and W250R mutations cause a significant reduction in the level of cell surface localization (Figure 4B and Table 1), consistent with the severely impaired secretion of the G187R, G228R, and W250R mutant EphA3 AP fusion proteins. Interestingly, several mutations in the kinase domain that inhibit receptor tyrosine phosphorylation and/or kinase activity, including T660K, R728L, and G766E, also somewhat decrease the proportion of EphA3 present on the cell surface (Figure 4B and Table 1). The T933M mutation in the SAM domain also substantially reduces the level of EphA3 cell surface localization. Thus, many cancer mutations impair EphA3 surface localization. However,

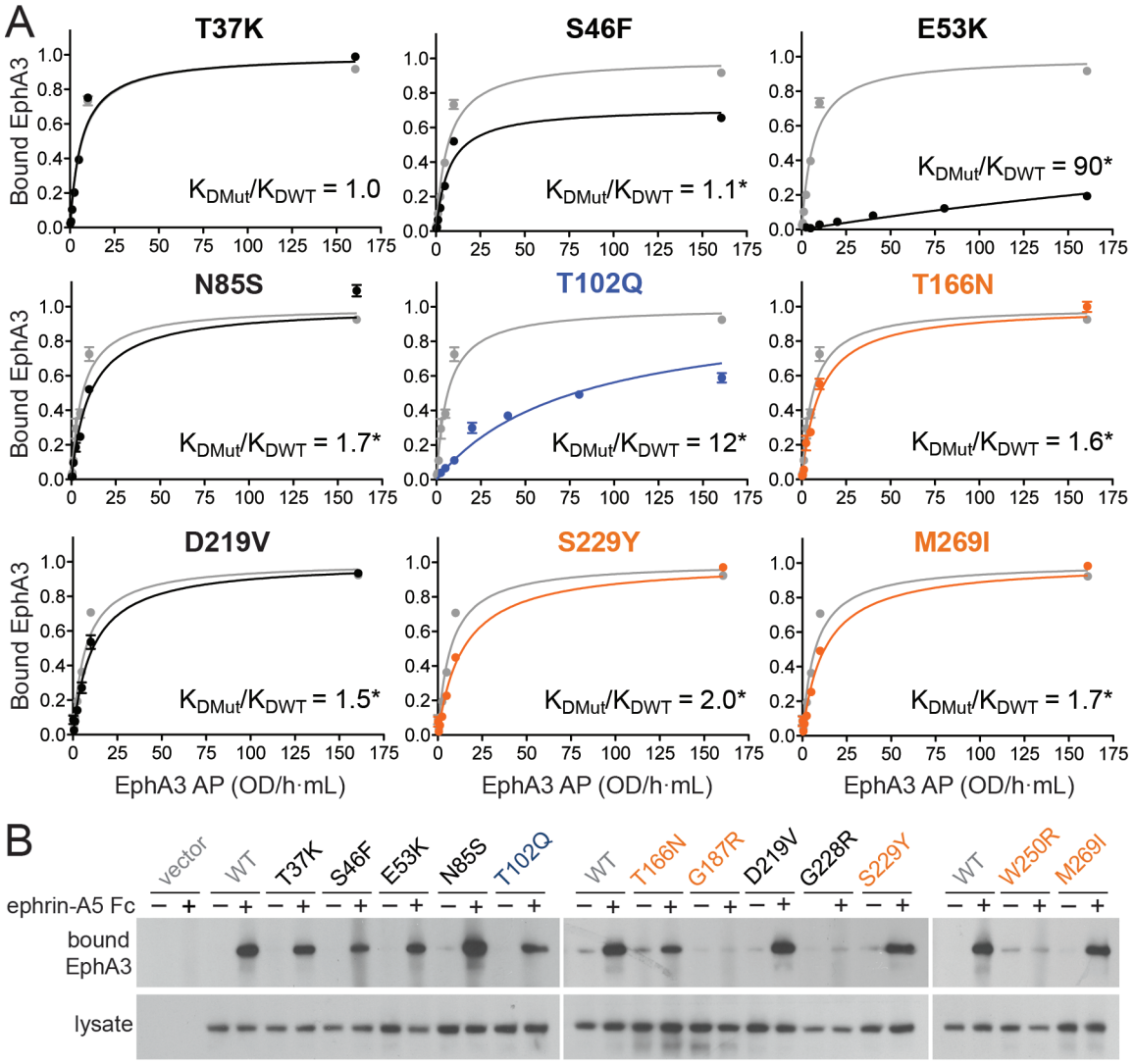

Figure 3. Effects of mutations in the extracellular region of EphA3 on ephrin ligand binding. (A) Curves showing the binding of wild-type and mutant EphA3 AP to ephrin-A5 Fc immobilized in ELISA wells. For each EphA3 AP concentration, averages ± SEM from triplicate measurements from each of two experiments are shown. Curves were fit according to a one-site saturation binding curve. The B_{max} for all mutants except S46F was constrained to that of wild-type EphA3, and the curves were normalized to B_{max} = 1. The ratios between the K_D values for the mutant (K_{DMut}) and wild type (K_{DWT}) are shown. *P < 0.05 for the difference from the K_D value of wild-type EphA3 (measured in the same ELISA plates) by unpaired two-tailed Student's t test using n = 46 (degrees of freedom for each curve). OD/h·mL represents OD_{405} units per hour per mL based on the measurement of alkaline phosphatase activity. (B) Representative immunoblot showing wild-type and mutant EphA3 remaining associated with immobilized ephrin-A5 Fc or Fc as a control (bound EphA3) after being pulled down from lysates of transiently transfected HEK 293 cells. The amount of EphA3 in the lysates is also shown.

we also found that the D219V mutation in the sushi domain, the G518L mutation in the second fibronectin type III domain, and the S792P mutation in the kinase domain slightly increase the proportion of EphA3 on the cell surface. These mutations may cause defects in EphA3 endocytosis and/or turnover.

Some Mutations in the EphA3 Extracellular Region May Affect Oligomerization. Ephrin binding induces Eph receptor oligomerization, which promotes autophosphorylation and downstream signaling.[2,3,7] Several domains in the Eph receptor extracellular region have been proposed to help oligomerization by promoting lateral "cis" interactions between Eph receptor molecules, including the ephrin-binding domain, the sushi domain, and the N-terminal fibronectin type III domain.[2,7] High-level expression of EphA3 transiently transfected in HEK 293 cells leads to constitutive autophophorylation, presumably because the high receptor concentration on the cell surface promotes oligomerization, possibly in conjunction with low levels of endogenous ephrin-A ligand expression (Figures 2A and 4C).[2] Because defects in EphA3 oligomerization and clustering would be expected to impair constitutive autophosphorylation of the receptor in HEK 293 cells, we examined the effects of the T37K, E53K, N85S, T102Q, and T166N mutations in the ephrin-binding domain, the D219V and S229Y mutations in the sushi domain, and the N379K and T393K mutations in the N-terminal fibronectin type III domain. However, among the mutated residues, only N85 is part of a proposed receptor−receptor interface.[2,7] We did not examine the mutations in these domains that severely impair EphA3 surface localization because they could affect autophosphorylation in a manner that is independent of oligomerization. We observed a significantly reduced level of tyrosine phosphorylation of the T37K and T166N mutants, and the control

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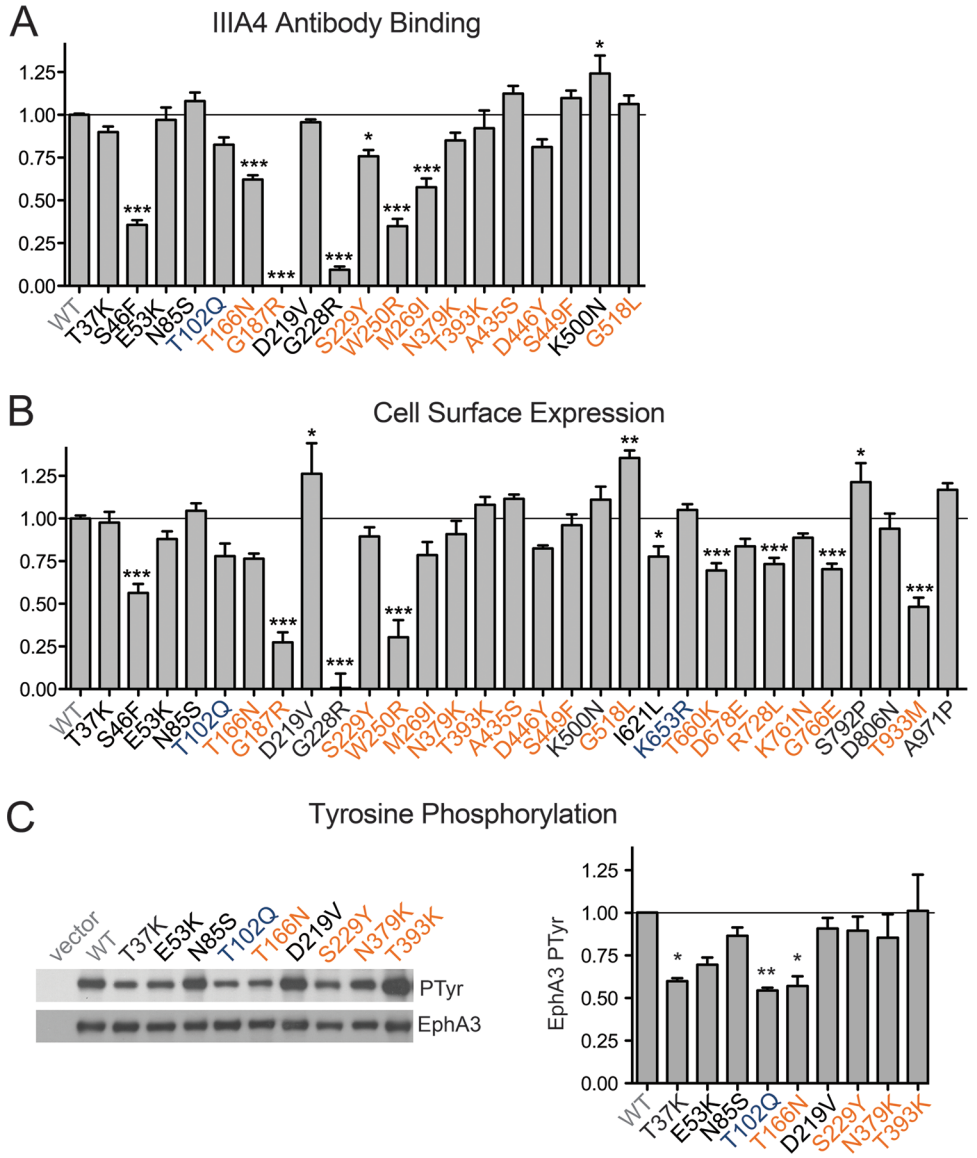

Figure 4. Effects of EphA3 mutations on the conformation of the ephrin-binding domain and cell surface localization. (A) Conformational changes in the ephrin-binding domain of the indicated mutant EphA3 extracellular domains were assessed in an ELISA using the IIIA4 antibody, which has been reported to recognize only the native conformation of the N-terminus of EphA3. Cell lysates were incubated in ELISA wells precoated with an anti-EphA3 antibody; sufficient amounts of lysates were used to ensure maximal EphA3 binding, thus yielding similar EphA3 levels in all the wells. The histogram shows averages ± SEM from triplicate measurements in each of two independent experiments. $*P < 0.05$ and $***P < 0.001$ for comparison to the wild type by one-way ANOVA and Dunnett's post hoc test. (B) Fraction of cell surface wild-type and mutant EphA3 determined in an ELISA following biotinylation of cell surface proteins and detection of the immobilized biotinylated EphA3 with HRP-bound streptavidin. The histogram shows averages ± SEM from triplicate measurements in each of two to four independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ for comparison to the wild type by one-way ANOVA and Dunnett's post hoc test. (C) Representative immunoblots showing tyrosine phosphorylation (PTyr) and total levels of wild-type EphA3 and the indicated mutants expressed by transient transfection in HEK 293 cell lysates. The histogram shows averages ± SEM calculated from quantification of three separate experiments and normalized to the wild-type value in each experiment. $*P < 0.05$ and $**P < 0.01$ for comparison to the wild type by one-way ANOVA and Dunnett's post hoc test.

T102Q mutant, suggesting that these mutations cause a defect in oligomerization (Figure 4C and Table 1). However, we cannot exclude the possibility that the defect in ephrin binding of the T102Q and T166N mutants (Figure 3A) may contribute to the impairment of receptor autophosphorylation in HEK 293 cells.

DISCUSSION

Recent technological advances have made possible large sequencing projects that have identified hundreds of gene mutations in tumor specimens and cancer cell lines.[41−44] This implies that alterations in many genes contribute to cancer development and progression, and not just mutation of a few critical oncogenes and tumor suppressor genes as previously believed. Nonsynonymous mutations more frequent than would be expected by chance are the hallmark of cancer driver genes, which promote tumorigenesis. Although predictions about whether a particular mutation might affect the function of the protein product can be made, follow-up functional studies are needed to conclusively characterize the effects of mutations and understand their clinical implications.[16,42,44,45] Such studies will be particularly informative in the case of the Eph receptor

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tyrosine kinases, which paradoxically have been proposed to have both tumor promoting and tumor suppressing activities.[1,11,46,47] In this study, we have focused on EphA3 somatic mutations identified in various cancers.

EphA3 Expression and Function. The EphA3 receptor is widely expressed during embryonic development, with the highest levels occurring in the nervous system and heart.[48−51] EphA3 is expressed at lower levels in some adult tissues, such as the brain, lung, bladder, prostate, and colon.[52,53] The best characterized roles of EphA3 thus far are in the epithelial−mesenchymal transition in the developing heart[54,55] and in axon guidance in the developing nervous system, where this receptor plays a repulsive role that causes axons to avoid regions of strong ephrin ligand expression.[56−58] EphA3-dependent retraction of cellular processes has also been reported in non-neuronal cells.[28] However, there is only limited information about the signal transduction pathways triggered by EphA3. Binding of the Nck and Crk adaptors and activation of RhoA have been implicated in the cellular repulsive effects.[28,29] Such repulsive signaling pathways could prevent the expansion of normal or tumor cells into ephrin-positive surrounding tissues, perhaps through a mechanism like contact inhibition of locomotion.[59−61] This repulsion may be overcome in tumor cells in which EphA3 is mutated, allowing tumor expansion. However, the role of EphA3 in cancer is not well established, and further studies are needed to more comprehensively elucidate its signaling pathways and biological effects in normal as well as cancer cells. As is the case for other Eph receptors, EphA3 activities in tumors may involve a complex interplay with ephrin ligands as well as crosstalk with other signaling systems.[47,62−64]

Many of the somatic mutations identified in the human EphA3 gene are predicted to strongly affect receptor function based on conservation of the mutated residues in EphA3 from different species and in other members of the Eph receptor family, domain location within the receptor, and type of amino acid changes (Table 1 of the Supporting Information). However, such predictions are not always accurate. Therefore, functional studies are required to conclusively establish how mutations affect receptor activity.

Ephrin-Binding Domain. In the ephrin-binding domain of EphA3, only the E53K mutation involves a residue located in the high-affinity ephrin binding interface (Figure 1). We found that this mutation reduces the apparent ephrin-A5 binding affinity by 100-fold, consistent with the drastic change from a negatively to a positively charged amino acid. The E53K mutation, however, does not appear to affect the overall conformation of the domain (based on the efficient binding of the IIIA4 antibody), in agreement with a previous report.[35] Other mutations in the ephrin-binding domain also impair ephrin-A5 binding, but through a distinct mechanism involving disruption of the native conformation of the domain. This is most pronounced in the G187R mutant, in agreement with the conservation of G187 in all the Eph receptors, which suggests that this glycine plays a critical role that cannot be fulfilled by other amino acids. A further consequence of the G187R mutation is a decrease in the fraction of EphA3 on the cell surface, and thus the impaired ability of the mutated receptor to participate in cell−cell communication. The S46F mutation also appears to disrupt the conformation of the EphA3 ephrin-binding domain and to reduce the level of receptor cell surface localization, although less severely than the G187R mutation.

We observed an only ∼1.5-fold decrease in the apparent ephrin-A5 binding affinity of the T166N mutant, even though this mutation is predicted to have a strong functional effect (Table 1 of the Supporting Information). Because T166 does not participate in a known binding interface (based on structural information from other Eph receptors[2,7,65]), its effect may depend on changes in the overall conformation of the ephrin-binding domain that may also impair the ability of the receptor to efficiently oligomerize. Two other mutations in the ephrin-binding domain, T37K and N85S, involve nonconserved residues that are not predicted to substantially affect receptor function (Table 1 of the Supporting Information). Our characterization indeed did not reveal detectable effects of the T37K mutation, which is also not part of a known binding interface, on the ephrin-A5 binding ability.[2,7,65] However, the reduced level of autophosphorylation of the T37K mutant in HEK 293 cells suggests that the mutation affects EphA3 oligomerization. We did observe a significant but modest (∼1.5-fold) decrease in the ephrin-A5 binding affinity of the N85S mutant. This is consistent with a 2-fold decrease in binding affinity measured by BIAcore surface plamon resonance for the similar EphA3 N85T mutant.[35] Based on the structure of the related EphA2 receptor, N85 is located in a loop that contributes to a stabilizing intramolecular interaction with the sushi domain,[7] and further work will be needed to more accurately assess the effects of this mutation on ephrin binding in the cellular context. Although N85 has also been proposed to participate in a receptor−receptor interface,[2] the N85S mutation does not decrease the level of EphA3 tyrosine phosphorylation in HEK 293 cells, suggesting that it does not impair receptor oligomerization.

Sushi and EGF-like Domains. The G228R and W250R mutations are located in the sushi domain. Similar to G187, residues G228 and W250 are conserved in all Eph receptors, and mutation of these amino acids appears to severely affect the conformation of the EphA3 ephrin-binding domain and drastically reduces levels of ephrin-A5 binding and cell surface expression. It is not clear how these mutations in the sushi domain might affect the conformation of the EphA3 ephrin-binding domain, but our findings suggest that there is communication between the cysteine-rich region and the ephrin-binding domain. Consistent with this, structural characterization of the related EphA2 extracellular domain has revealed an interaction surface between these two domains that is stabilized by six hydrogen or salt bonds.[7] Interestingly, W250 may be located within an important receptor−receptor interaction surface in EphA3 clusters.[2] Mutation of residues within this interface affects the related EphA2 receptor by disrupting the restricted EphA2 cell−cell contact localization observed in HEK 293 cells.[2] However, the dramatic impairment of cell surface expression precludes analysis of EphA3 W250R oligomerization defects through measurements of receptor tyrosine phosphorylation in cells. Finally, we observed an ∼1.5−2-fold decrease in the apparent binding affinity of ephrin for the D219V, S229Y, and M269I mutants, accompanied by modest effects on the conformation of the ephrin-binding domain for the S229Y and M269I mutants. The small effects observed for the D219V mutation are not consistent with the predicted major functional effects (Table 1 of the Supporting Information), suggesting that the mutation may affect aspects of EphA3 function not investigated here. Our results indicate that mutations within the cysteine-rich region can affect ephrin binding and EphA3 surface localization. However, further studies will be necessary to elucidate the precise mechanism underlying the effects of mutations in the cysteine-rich region on the ephrin-binding domain.

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Fibronectin Type III Domains. Several mutations within the two fibronectin type III domains of EphA3, and in particular A435S, K500N, and G518L, are predicted to strongly affect function (Table 1 of the Supporting Information). However, we did not observe severe functional consequences for any mutations in the fibronectin type III domains in our assays. It is conceivable that these mutations may alter aspects of EphA3 function that we did not examine, such as receptor stability. Furthermore, the T393K and A435S mutations could affect glycosylation. The T393K mutation is predicted to abolish a possible glycosylation site at N391 by eliminating the NXT/S motif (where X is any amino acid) required for N-linked glycosylation. This could affect lateral cis interaction with a coexpressed ephrin, in which the EphA2 residue corresponding to N391 has been proposed to be involved. In contrast, the A435S mutation may create a new glycosylation site. Although the glycosylation potential is low for N433,[66] the corresponding residue in EphA2 is known to be glycosylated.[2] The G518 residue in the second fibronectin type III domain also participates in such a predicted ephrin cis interface, and mutation of residues in this interface of EphA2 affects receptor localization at cell−cell contact sites in HEK 293 cells.[2]

Kinase Domain. Several mutations in the kinase domain of EphA3, including K761N, G766E, and D806N, abolish kinase activity. The three mutated residues are conserved in all Eph receptors as well as in tyrosine kinases in general, and the mutations are predicted to strongly affect function. K761 was predicted to be an activating mutation on the basis of the fact that mutation of the corresponding lysine in FGF receptor 2 to asparagine releases autoinhibition by a "molecular brake" in the kinase hinge region.[16,67] Thus, the inactivating effects of the equivalent K761N mutation in EphA3 suggest a distinctive mode of regulation of the Eph receptor kinase domain. G766 is located in the conserved DFG motif within the activation loop, while D806 is located in the C-terminal lobe of the tyrosine kinase domain. The R728L mutation, in the C-terminal lobe of the kinase domain, is also predicted to strongly affect function and indeed shows impaired autophosphorylation in cells and kinase activity in vitro. On the other hand, the T660K mutation abolishes in vitro kinase activity, but the mutant is still phosphorylated after overexpression in HEK 293 cells. This suggests that factors other than receptor autophosphorylation might contribute to phosphorylation of the mutated receptor in cells. The D678E mutation in the N-terminal lobe of the kinase domain may cause a minor defect in kinase activity that is apparent in only some of the assays, while the S792P mutation in the conserved A/SPE motif of the activation loop may increase kinase activity.

We observed decreased levels of cell surface expression of a number of kinase domain mutants. This was the only significant effect observed for the I621L mutation located near the juxtamembrane segment, which is predicted to moderately affect function. The T933M and A971P SAM domain mutations appear to modestly decrease the level of surface localization and increase the level of EphA3 tyrosine phosphorylation in cells, respectively.

Role of EphA3 in Cancer. The inactivating effects of many of the mutations suggest that, in general, wild-type EphA3 suppresses the malignant properties of cancer cells in an ephrin- and kinase-dependent manner. Consistent with tumor suppression by EphA3, several reports demonstrate that the EphA3 promoter is silenced by methylation in colorectal cancer, particularly in association with the oncogenic BRAF V600E mutation, and in hematopoietic tumors.[68,69] In addition, the chromosomal region that encompasses the EphA3 gene often undergoes loss of heterozygosity in lung and other cancers.[24,70−73]

With the exception of the R241G and T933M mutations, the mutations identified involve only one EphA3 allele (Table 1 of the Supporting Information). However, the S46F and E53K mutations were both identified in the MeWo melanoma cell line, and the T393K and R728L mutations were both identified in the same lung tumor specimen, raising the possibility that both EphA3 alleles may be inactivated in these samples, as well. Nevertheless, overall, the EphA3 gene does not have the characteristics of a classical tumor suppressor gene, requiring inactivation of both alleles to affect cancer progression.[43] Perhaps EphA3, like EphA2, has tumor suppressor activities that depend on ephrin and kinase activity but also tumor promoting activities that depend on crosstalk with other signaling molecules and that do not require ephrin binding or kinase activity.[1,47] If this is the case, the mutations that impair ephrin binding and kinase activity may shift the balance toward the tumor promoting effects of EphA3. Alternatively, or in addition, the mutated EphA3 molecules may have dominant negative effects and inhibit the function of the wild-type receptor molecules.

The possibility that EphA3 may also have ephrin- and/or kinase-dependent tumor promoting activities in some cancers or during particular stages of tumorigenesis cannot be excluded. Furthermore, an elevated level of EphA3 expression has been reported in some hematopoietic tumors and melanomas, suggesting that this receptor may promote malignancy in some cancers.[52,73−76] In addition, a recent study has correlated high-level EphA3 expression with shortened survival in colorectal cancer patients.[77] Therefore, it remains unclear how EphA3 expression and signaling might modulate the development and progression of different cancers.

Somatic Mutations in Other Eph Receptors. Remarkably, 14 of the 40 identified EphA3 mutations (35%) occur in samples that also carry mutations in one or more of the other Eph receptor genes (Table 1 of the Supporting Information). This is particularly pronounced in lung cancer, where 58% of the samples with an EphA3 mutation also have at least another Eph receptor mutation. Given the low mutation rates for each Eph receptor, this suggests that cancer cells carrying multiple Eph receptor mutations have a strong selective advantage and that multiple coexpressed Eph receptors may function in concert to inhibit tumorigenesis. It will be interesting to determine if many of the mutations in other Eph receptors are also predominantly inactivating.

All Eph receptors have also been found to be mutated in a variety of cancers.[1] As for EphA3, mutations in other Eph receptors are also widely distributed in the different domains, including the ephrin-binding and kinase domains. The functional effects of only very few of these mutations have been analyzed. The G391R mutation in the first fibronectin type III domain of EphA2 identified in lung cancer has been reported to activate the receptor, resulting in increased activity of the mTORC1 pathway and positive effects on tumorigenesis.[78] On the other hand, the effects of the G228R mutation in EphA3 suggest that the equivalent EphA7 G232R mutation identified in melanoma (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=mutations&ln=EPHA7) also inactivates ephrin binding by disrupting the conformation

of the EphA7 ephrin-binding domain. Furthermore, the effects of the K761N mutation in EphA3 suggest that the equivalent EphA6 K813N mutation identified in stomach cancer (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=mutations&ln=EPHA6) also inactivates the kinase domain. To the best of our knowledge, our study is the first extensive characterization of cancer somatic mutations of an Eph receptor. Although the pathological effects of EphA3 in many cancers are unknown and merit further investigation, our findings shed light on the mechanisms underlying Eph receptor inactivation through somatic mutations.

■ ASSOCIATED CONTENT

Supporting Information

A summary of all EphA3 somatic mutations identified in cancer to date as well as concurrent mutations in other Eph receptors and predicted effects on EphA3 function (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AP, alkaline phosphatase; DTT, dithiothreitol; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; pNPP, *p*-nitrophenyl phosphate; SAM, sterile alpha motif; PDZ, postsynaptic density protein, *Drosophila* disk large tumor suppressor, and zonula occludens-1 protein; SEM, standard error of the mean; PDB, Protein Data Bank.

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