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Involvement of EphA2-mediated tyrosine phosphorylation of Shp2 in Shp2-regulated activation of extracellular signal-regulated kinase

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Shp2 is a positive regulator for Erk activation downstream of receptor tyrosine kinases for growth factors. It has been controversial how Shp2 induces Erk activation. We here demonstrate that EphA2 is responsible for Shp2-mediated Erk activation by phosphorylating Tyr542 and Tyr580 of Shp2 in the cells stimulated with growth factors. In NMuMG mammary epithelial cells stimulated with hepatocyte growth factor (HGF), HGF-dependent Erk phosphorylation was prolonged only in the presence of EphA2. This Erk activation paralleled the phosphorylation of Tyr542/580 of Shp2 and the association of Grb2 with Shp2, suggesting the positive signal involving Grb2 signal to activate Ras-Erk pathway. Immunohistochemical studies of mammary cancer specimens revealed that the cancer progression was associated with both Tyr580 phosphorylation of Shp2 and increased expression of EphA2, which were also correlated with increased Erk phosphorylation. Overexpression of either Shp2Thr468Met (a phosphatase-defective mutant found in Lentigines, Electrocardiographic abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retardation of growth and sensorineural Deafness (LEOPARD) syndrome) or Shp2Asn308Asp (a phosphatase-active mutant found in Noonan syndrome) with EphA2 exhibited comparable activation of Erk and stronger activation than wild-type Shp2, suggesting the phosphatase-independent Erk activation. Expression of Shp2Thr468Met with Tyr542/580Phe mutations resulted in the suppression of Erk activation. Phosphatase-active and -inactive, and wild-type Shp2s bound equally to Grb2, suggesting that phosphorylation of Tyr542/580 of Shp2 was essential but not sufficient for Shp2-mediated Erk activation. We found that Gab1 (Grb2-associated binder 1) was involved in the mutant Shp2-mediated Erk activation. Zebrafish injected with Shp2Thr468Met mRNA showed cardiac edema, whereas those depleted of EphA2b showed less phenotype, suggesting that EphA2 might partly account for the phenotype of LEOPARD syndrome. Collectively, tyrosine phosphorylation of Shp2 by EphA2 contributes to the phosphatase-independent Shp2-mediated activation of Erk and might be involved in Shp2-associated diseases.

INTRODUCTION

Shp2, encoded by the *PTPN11* gene, is a protein tyrosine phosphatase that acts as a positive regulator of Ras-Erk pathway.[1–3] Shp2 contains two tandem Src homology 2 (SH2) domains, a protein tyrosine phosphatase domain and tyrosine phosphorylation sites.[1–3] Shp2 is thought to be inactive by forming intramolecular folding under unstimulated conditions, whereas it becomes active when the amino-terminal SH2 domains bind to phosphorylated molecules, including Grb2-associated binder (Gab), insulin receptor substrate and fibroblast growth factor receptor substrate, by forming an open conformation.[2,3] It remains unclear how Shp2 is involved in the activation of Erk signal and whether its phosphatase activity of Shp2 is essential for the activation of Ras-Erk signal. Phosphatase-active mutation of Shp2 (a genetic mutation of *PTPN11* encoding Asn308Asp, hereafter referred to as N308D) is found in patients with Noonan syndrome, whereas phosphatase-defective mutation

(Thr468Met, hereafter referred to as T468M) is found in those with Lentigines, Electrocardiographic abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retardation of growth and sensorineural Deafness (LEOPARD) syndrome.[4,5] The patients with Noonan syndrome and those with LEOPARD syndrome exhibit the overlapping symptoms, including craniofacial abnormalities, cardiac defects and growth retardation. In addition, leukemia diseases are often found in the patients with those syndromes.[4,5] Cardiomyocytes derived from induced pluripotent stem cells from the patient with LEOPARD syndrome show an increased activation of Erk,[6] suggesting the phosphatase-independent role of Shp2 in the Erk activation.

Growth factors promote cell proliferation *via* their specific tyrosine kinase receptors by activating Ras-Erk signaling. Autophosphorylated tyrosine kinase receptors provide the binding sites for adaptor molecules containing SH2 domain and for scaffold adaptor molecules to activate Sos-Ras-Erk signaling pathway.

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Although the Ras-Erk activation is transient, Shp2 is reported to be a modulator that prolongs the activation of Erk.[7] Hepatocyte growth factor (HGF) induces phosphorylation of Met receptor and subsequent recruitment of an adaptor scaffolding protein, Gab1. Furthermore, phosphorylated Gab1 becomes associated with SH2 domains of Shp2.[1–3] This association of Shp2 with Gab1 leads to the Ras-Erk activation.[1–3] In addition, it has been suggested that the phosphorylated Tyr542 and Tyr580 recruit Grb2/Sos complex to activate Ras-Erk signaling.[2,7,8] Recently, it was reported that ZAP70, a non-receptor tyrosine kinase, enhances phosphorylation of Shp2 at Tyr580 and activates Erk.[9] However, it is still unclear how growth factor stimuli induce tyrosine phosphorylation of Shp2.

Activation of Ras-Erk signaling pathway is observed in cancer progression as well as in the diseases called 'Rasopathies', including Noonan syndrome, LEOPARD syndrome, Costello syndrome and neurofibromatosis type 1.[4,5] The diseases with genetic mutations of the genes encoding the molecules constituting the Ras-Erk signaling pathway, such as SOS1, KRAS, HRAS, NRAS, RAF1, BRAF, PTPN11, MEK1/2, SHOC2, CBL, SPREAD1 and NF1, are also included in Rasopathies. Active mutations of receptor tyrosine kinases for growth factors inducing Ras-Erk activation, such as EGFR, ErbB2, FGFR and c-KIT, are found in patients with a wide variety of cancers,[10] suggesting the significant role of the Erk signal in cancer promotion. Furthermore, the overexpression and the contribution to Erk activation of another receptor tyrosine kinase, EphA2, have been reported in many types of cancers.[11,12]

EphA2 is a member of the EphA family of receptor tyrosine kinases consisting of nine EphAs (EphA1–A8 and A10) for membrane-anchored ephrinA ligands.[13,14] Paradoxically, EphA2 has both tumor-promoting and -suppressing roles. EphrinA ligands suppress the cell proliferation via activation of EphA2, whereas extracellular growth factors promote cell proliferation by an indirect activation of EphA2 through their tyrosine kinase receptor activation.[13,14] However, the molecular mechanisms by which EphA2 promotes the Ras-Erk pathway without the engagement of ephrinA are unknown. We report here that EphA2 phosphorylates Tyr542/580 of Shp2, subsequently recruiting Grb2 to activate Erk, and that Gab1 might function in Shp2T468M-dependent Erk activation besides or cooperatively phosphorylated Shp2/Grb2-mediated signaling. Moreover, we demonstrate the possible role for this Shp2-mediated Erk activation signal in cancer progression and in LEOPARD syndrome.

RESULTS

EphA2 is required for HGF-induced prolonged and/or enhanced activation of Erk

Shp2 is involved in the activation of the Ras-Erk pathway by growth factor signaling.[1,3] To confirm that Shp2 is required for the HGF-mediated activation of Erk in epithelial NMuMG cells, we examined the degree and duration of Erk activation of the cells stimulated with HGF. When NMuMG cells were stimulated with HGF, phosphorylation of Erk peaked at 15 min after the stimulation and continued for 180 min (Figures 1a and b). When those depleted of Shp2 were stimulated with HGF, the peak Erk phosphorylation was reduced (Figures 1a and b). In addition to the reduction of peak activation of Erk, the dephosphorylation of Erk after the peak was faster than the control cells, indicating the role for Shp2 in the enhancement and the prolongation of Erk phosphorylation in NMuMG cells upon HGF stimulation (Figures 1a and b). We wondered how Shp2 is involved in the regulation of Erk activation in the cells stimulated with HGF. Previously, phosphorylation of Tyr542 and Tyr580 is reported to be important for Shp2-mediated Erk activation,[7] although the kinases that phosphorylate these tyrosine residues have not been identified. Growth factor receptor simulation is thought to activate not only

their receptors but also other signaling pathway, including EphA2.[14] Therefore, we hypothesized that EphA2 might be responsible for HGF-induced Erk activation signal involving Shp2.

To test the requirement of EphA2 for HGF-induced prolonged and enhanced activation of Erk, we studied the effect of depletion of EphA2 on the HGF-induced Erk activation in NMuMG cells. Knockdown of EphA2 resulted in shortened Erk activation and reduced phosphorylation of Tyr542/580 of Shp2, which was required for recruitment of Grb2 (Figures 1c and d). Consistently, the association of Shp2 with Grb2 was reduced in the cells depleted of EphA2 (Figure 1c), suggesting that EphA2 is required for phosphorylation of Shp2 and Shp2-dependent prolonged and enhanced activation of Erk. To further investigate whether EphA2 is involved in Erk activation in other types of cells stimulated with growth factors, we examined the fibroblast growth factor 2-stimulated human umbilical vein endothelial cells and HGF-stimulated C6 glioma cells. In both cells, knockdown of EphA2 led to attenuation of Erk activation and reduction of Grb2 binding to phosphorylated Shp2 (Figures 1e–h). These results indicate the common signaling pathway that involves EphA2-regulated phosphorylation of Shp2 in Erk activation of the cells stimulated with the growth factors. To understand the consequence of prolonged Erk activation mediated by EphA2 and Shp2, we analyzed the proliferation of NMuMG cells stimulated with HGF. The cell number of those depleted of either EhpA2 or Shp2 cultured for a few days in the presence of HGF was half of the control cells, indicating the essential role of EphA2 and Shp2 in HGF-dependent cell proliferation (Figure 1i).

To further confirm the contribution of EphA2 to the Shp2-mediated Erk activation, we compared two cell lines: NMuMG cells and HEK 293T cells, both of which express similar amount of Met and Shp2, although NMuMG cells express EphA2 more than HEK293T cells (Figure 2a). The extent of Erk activation in NMuMG cells was much greater than that in HEK293T cells when those cells were stimulated with HGF (Figure 2a). Furthermore, HGF-dependent phosphorylation of Tyr542 was found in NMuMG cells but not in HEK293T cells. These results raise the possibility that the expression of EphA2 might determine the duration and amplitude of Erk activation, because expression of both Met and Shp2 are comparable between the two cell lines. To test this, we expressed EphA2 in HEK293T cells and examined the phosphorylation of Shp2, the association of Shp2 with Grb2 and the Erk activation upon HGF stimulation. Forced expression of EphA2 in the cells resulted in the phosphorylation of Tyr542/580 of Shp2 and the association of Shp2 with Grb2 (Figures 2b and c). Furthermore, the HGF-dependent Erk activation was significantly enhanced in the cells expressing EphA2 (Figure 2d), suggesting the contribution of EphA2 to growth factor-induced enhancement and prolongation of Erk activation.

Shp2 phosphorylation-dependent Erk activation irrespective of its phosphatase activity

Phosphatase-active mutant of Shp2 (N308D) and phosphatase-defective mutant of Shp2 (T468M) cause Noonan syndrome and LEOPARD syndrome, respectively (Figure 3a).[5,15] To examine the effect of EphA2 on the Erk activation in the cells expressing these Shp2 mutants, we expressed Shp2 mutants with EphA2. The cells expressing either wild-type Shp2 or mutant Shp2 (T468M or N308D) showed the similar degree of phosphorylation on Tyr542/580, whereas Erk activation was weaker in those expressing wild-type than those expressing mutant Shp2 (Figure 3b). Of note, there was no difference in the Erk activation between the cells expressing Shp2T468M and those expressing Shp2N308D (Figure 3b). These data suggest the phosphatase-independent mechanism underlying Shp2-mediated Erk activation.

There was no difference in the association of mutant Shp2 (T468M or N308D) and wild-type Shp2 with Grb2 (Figure 3c),

indicating that the association of Shp2 with Grb2 is not sufficient to induce Erk activation, because the activation of Erk in the cells expressing either Shp2T468M or Shp2N308D with EphA2 was greater than those expressing wild-type Shp2 with EphA2 (Figure 3b). However, the Erk activation in the cells expressing EphA2 and Shp2T468M was greater than those expressing EphA2
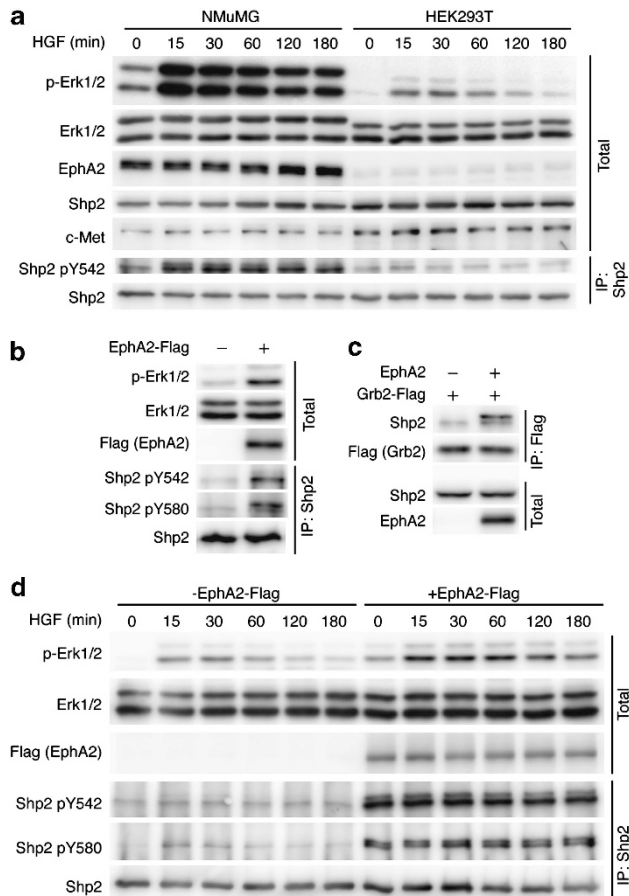
a

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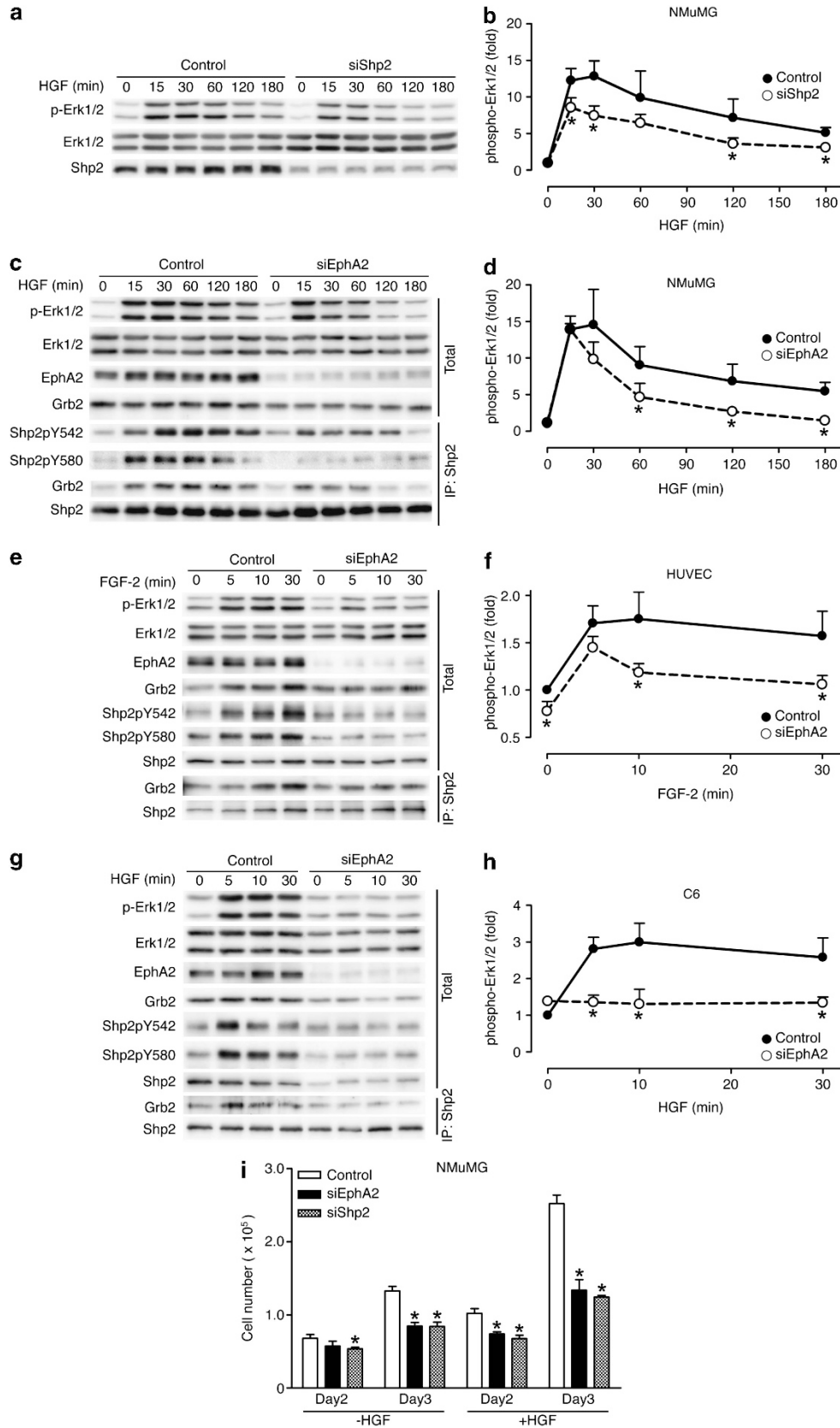
Figure 2. EphA2 can regulate Erk activation through the phosphorylation of Shp2 and the association of Shp2 with Grb2. (**a**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-Shp2 (IP: Shp2) prepared from NMuMG (left panel) and HEK293Tcells (right panel) stimulated with HGF (20 ng/ml) for the time indicated on the top. (**b**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-Shp2 (IP: Shp2) prepared from HEK293T cells tranfected with the plasmids indicated on the top. (**c**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-Flag (IP: Flag) prepared from HEK293T cells transfected with the plasmids indicated on the top. (**d**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-Shp2 (IP: Shp2) prepared from HEK293T cells transfected with plasmids indicated on the top and stimulated with HGF for the time indicated on the top. (**a–d**) Each result is the representative of at least three independent experiments.

and Shp2T468M with mutations on both Tyr542 and Tyr580 (Shp2T468M/2YF) (Figure 3d), indicating that phosphorylation of Tyr542/580 is required for EphA2 in Shp2-mediated Erk activation. Consistently, Shp2T468M/2YF mutant could not associate with Grb2 even in the cells expressing EphA2 (Figure 3e). Furthermore, we found that Grb2 with SH3 mutation (either P49L or G203R) but not that with SH2 mutation (R86K) could bind to Shp2T468M (Figure 3f), indicating that the association of Shp2 with Grb2 is mediated by the phosphorylation of Tyr542 and Tyr580 of Shp2 and the SH2 domain of Grb2. Overexpression of a dominant-negative form of Grb2, Grb2P49L, significantly reduced EphA2-dependent Shp2T468-mediated Erk phosphorylation (Figure 3g and Supplementary Figure S1), although it did not completely block it, suggesting the contribution of Grb2 to this Erk activation signal and implying the alternative signaling independent of Grb2.

To understand the molecular mechanism underlying the difference in the degree of Erk activation between the cells expressing wild type and those expressing Shp2T468M, we examined the role of Gab1 in Shp2-mediated Erk activation, because the complex of Gab1 and Shp2 is reported to be essential for growth factor-induced Erk activation. Tyr627 of Gab1 is essential for the binding of Shp2 to Gab1.[16] We found that Tyr627 was less phosphorylated in the cells expressing wild-type Shp2 than those expressing either Shp2T468M or Shp2N308D (Figure 3h). Furthermore, a dominant-negative mutant Gab1 (Gab1Y627F) incapable of being phosphorylated inhibited the EphA2-dependent Shp2T468M-mediated Erk phosphorylation, although phosphorylation of Tyr542/580 of Shp2T468M was preserved (Figure 3i). Collectively, these data suggest that phosphorylation of Tyr542/580, which recruits Grb2, is required but not sufficient for EphA2-dependent Shp2T468M-mediated Erk activation.

Requirement of tyrosine phosphorylation of Shp2 and dispensability of phosphatase activity of Shp2 for the Erk activation in the cells expressing Shp2 and EphA2

To test both the requirement of phosphorylation of Tyr542/580 and the dispensability of phosphatase activity of Shp2 in the cells stimulated with HGF, we examined the Erk activation in HEK293 cells expressing Shp2T468M and in those expressing Shp2T468M/2YF or wild-type Shp2 when stimulated with HGF. Even in the cells without forced expression of EphA2, the cells expressing Shp2T468M exhibited greater Erk activation than those expressing either Shp2T468M/2YF or wild-type Shp2 in response to HGF (Figure 4a), although the Erk activation in the cells expressing Shp2T468M/2YF was greater than that in the cells expressing wild-type Shp2 (Figure 4c). Increase in phosphorylation of Tyr542/580 even in the cells expressing Shp2T468M and wild-type Shp2 was subtle compared with cells without forced expression of Shp2

Figure 1. Contribution of EphA2-dependent phosphorylation of Shp2 to the growth factor-induced Erk activation. (**a**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from the NMuMG cells pretreated with short interfering RNAs (siRNAs) indicated on the top and stimulated with HGF for the time indicated on the top. (**b**) Quantitative analyses of Erk phosphorylation by HGF in cells treated with control siRNA (control, closed circle) or siRNA against Shp2 (siShp2, open circle). Fold induction after the HGF stimulation was plotted as the value of the intensity of phosphorylated (p)-Erk divided by that of Erk (0 min) was 1. Experiments were performed at least three times. Data are represented as mean with s.d. *$P < 0.05$ between the cells treated with control and those treated with siShp2. (**c**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-Shp2 (IP: Shp2) prepared from NMuMG cells pretreated with sRNAs indicated on the top and stimulated with HGF for the time indicated on the top. (**d**) Phosphorylation of Erk observed in (**c**) was quantified as described in the legend for (**b**). (**e**) and (**g**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates of human umbilical vein endothelial cells (HUVECs) stimulated with fibroblast growth factor-2 (FGF-2) (**e**) and those of C6 glioma cells stimulated with HGF (**g**) as described in the legend of (**b**). (**f**) and (**h**) Phosphorylation of Erk observed in (**e**) and (**g**) was quantified, respectively, as described in the legend of (**b**). (**i**) The number of NMuMG cells treated with either control siRNA (white), EphA2 siRNA (black) or Shp2 siRNA (gray) and cultured in the presence or absence of HGF was counted. The cells transfected for 24 h with siRNAs were replated at the density of 2×10^4 per well in the 12-well plate and were subjected to the calculation at the time after being replated as indicated at the bottom. Data are represented as mean of at least three independent experiments with s.d. *$P < 0.05$ between the cells treated with control and those treated with either EphA2 siRNA or Shp2 siRNA.
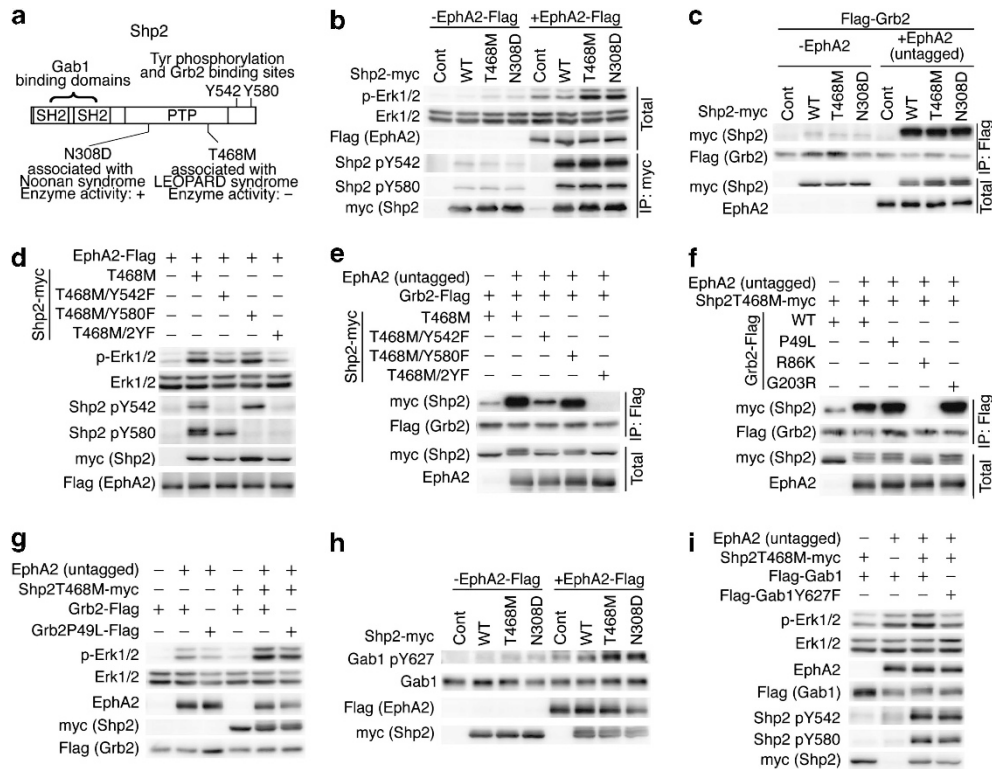
Figure 3. EphA2 and disease-associated Shp2 mutants synergistically enhance basal Erk activity in HEK293T cells. (**a**) Schematic illustration of the domain structure of Shp2 and the amino-acid residues that we examined in this study. SH2, Src homology 2 domain; PTP, protein tyrosine phosphatase domain. (**b**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-myc (IP: myc) prepared from HEK293T cells transfected with the plasmids indicated on the top. (**c**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-Flag (IP: Flag) prepared from HEK293T cells transfected with the plasmids indicated on the top. (**d**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from HEK293T cells transfected with the plasmids indicated on the top. (**e** and **f**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates (Total) or the immunoprecipitates with anti-Flag (IP: Flag) prepared from HEK293T cells transfected with the plasmids indicated on the top. (**g**–**i**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from HEK293T cells transfected with the plasmids indicated on the top. (**b**–**i**) Each result is the representative of the three independent experiments.

(Figure 4a). Notably, the Erk activation in the cells expressing Shp2T468M was decreased within 120 min (Figure 4c).

In clear contrast to the cells without forced expression of EphA2, the Erk activation in the cells expressing EphA2 with Shp2T468M was prolonged even 120 min after the stimulation with HGF (Figures 4b and d and Supplementary Figure S2). There was no EphA2-dependent enhancement or prolongation of Erk activation among the cells without forced expression of Shp2, those expressing Shp2T468M/2YF and those expressing wild-type Shp2 (Figures 4b and d). These data suggest that HGF-induced prolonged activation of Erk involving Shp2 does not need Shp2 phosphatase activity but tyrosine phosphorylation of Shp2.

We examined the Gab1 phosphorylation to understand the difference of Erk phosphorylation among Shp2T468M, Shp2T468M/2YF and wild-type Shp2 in HEK293T cells and those overexpressing EphA2. Tyr627 phosphorylation of Gab1 in the cells expressing Shp2T468M/2YF was weaker than those expressing Shp2T468M and stronger than those expressing wild-type Shp2 in both cells (Supplementary Figure S3), suggesting the role for Gab1 in Shp2T468M-mediated Erk activation.

Tyrosine kinase activity of EphA2 is required for phosphorylation of Shp2 and Erk activation by Shp2

To examine how EphA2 phosphorylates Shp2, we studied the effects of mutated EphA2 on Shp2T468M-dependent Erk

activation in HEK293T cells. We found that the Erk activation and the phosphorylation of Tyr542/580 of Shp2 was abolished in the cells expressing EphA2K646M, which lacks the kinase activity[17] (Figure 5a). Recently, the phosphorylation of Ser897 by Akt was reported to be important for cancer cell migration.[18] The replacement of Ser897 with Ala did not affect the Erk activation (Figure 5a). Besides these, EphA2 has multiple tyrosine phosphorylation sites (Tyr588, Tyr594 and Tyr772) important for its tyrosine kinase activity and for recruiting SH2 domain-containing molecules.[19] The cells expressing either EphA2Y588F or Y772F exhibited less Shp2T468M-dependent Erk activation and phosphorylation of Shp2T468M than those expressing wild-type EphA2 (Figure 5a). These data suggest that the kinase activity of EphA2 is essential for Shp2 phosphorylation and that the additional molecules downstream of EphA2 might modify the phosphorylation of Shp2. To further confirm the importance of tyrosine kinase activity, we examined the effect of Dasatinib, a potent inhibitor for EphA2, on the activation of Erk and on the phosphorylation of Shp2.[20] Treatment of the cells expressing Shp2T468M and EphA2 with Dasatinib resulted in a rapid decrease in Erk phosphorylation as well as in the phosphorylation of Shp2 and EphA2 (Figures 5b and c). We finally examined whether EphA2 directly phosphorylates Shp2 in vitro using purified EphA2 and Shp2. EphA2 phosphorylated Tyr542 and Tyr580 of Shp2 (Figure 5d). These results indicate the importance of tyrosine kinase activity of EphA2 in the phosphorylation of Shp2.
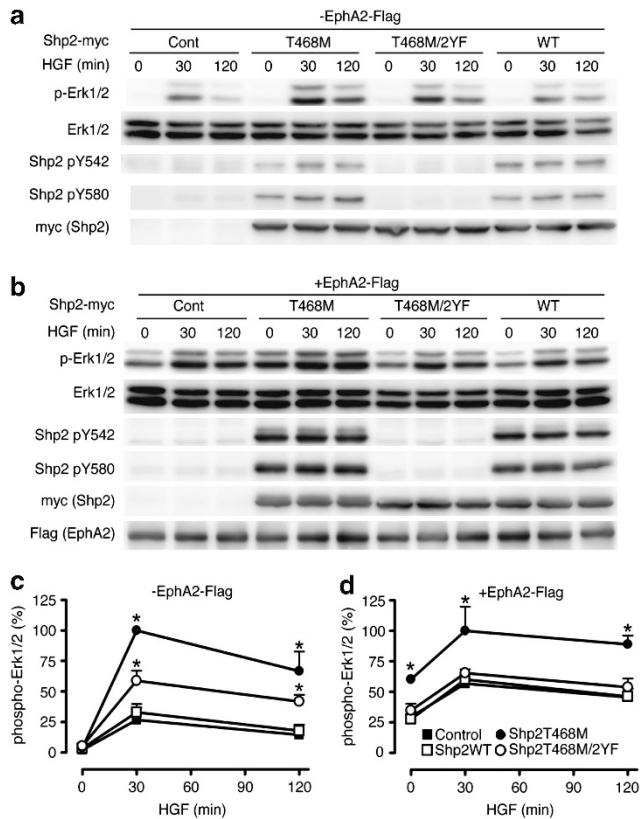
Figure 4. Phosphatase-independent and EphA2-dependent Erk activation in Shp2-mediated signal. (a and b) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from HEK293T cells transfected with the plasmids indicated on the top and stimulated with HGF for the time indicated on the top. (c and d) Quantitative analyses of phosphorylation of Erk by HGF in cells expressing empty vector (closed square), Shp2WT-myc, (open square), Shp2T468M-myc (closed circle) or Shp2T468M/2YF (open circle). The relative phosphorylation of Erk was calculated as the value of the intensity of phosphorylated (p)-Erk divided by that of Erk at 30 min in cells transfected with Shp2T468M was 100%. Experiments were performed at least three times. Data are represented as mean with s.d. *$P < 0.05$ among the four groups.

Figure 5. Tyrosine kinase activity of EphA2 is required for phosphorylation of Shp2 and Erk activation by Shp2. (a) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from HEK293T cells transfected with the plasmids indicated on the top. A representative result of three independent experiments. (b) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from HEK293T cells transfected with the plasmids indicated on the top and treated with Dasatinib (100 nM) or dimethylsulfoxide (DMSO) for the time indicated on the top. (c) Quantitative analysis of (b). The relative Erk phosphorylation after the treatment with Dasatinib was calculated as the intensity of p-Erk divided by that of Erk from the untreated cells at the 0 min was 100%. Data are represented as mean with s.d. *$P < 0.05$ between the cells treated with DMSO and those treated with Dasatinib. (d) Immunoblot analyses with the antibodies indicated at the left using GST-Shp2 incubated with GST-EphA2 for the time indicated on the top.

Phosphorylation of Shp2 and Erk in breast cancer-expressing EphA2

We then tried to test whether the signal, EphA2-mediated phosphorylation of Shp2 and subsequent Erk activation, is involved in diseases. EphA2 is often overexpressed in a variety of malignant cancers, including breast, lung, prostate and colon cancers.[11] We, therefore, hypothesized that the phosphorylation of Shp2 is enhanced by EphA2 in cancer. To test this hypothesis, we performed immunohistochemical analyses of human breast cancer tissues to investigate the relevance of EphA2 expression to the phosphorylation of Shp2. In normal mammary glands, neither EphA2 nor phosphorylation of Shp2 at Tyr580 was detected (Figure 6a). However, in the ductal carcinoma in situ and in the invasive breast cancer, the expression of EphA2 and the phosphorylation of Shp2 at Tyr580 (Shp2Y580) were detected in the serially immunostained sections (Figures 6b and c). In six of the nine cases tested (Supplementary Table 1), we found increases in EphA2 expression as well as increases in the Shp2Y580 phosphorylation, suggesting that the increased expression of
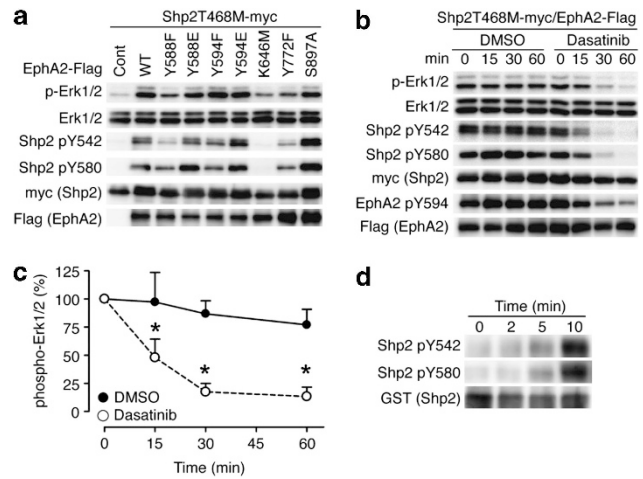
EphA2 in mammary cancer cells leads to the phosphorylation of Shp2.

We further investigated the phosphorylation of Erk in those cancer tissues to understand the Erk activation induced by EphA2 and Shp2 as we analyzed using cultured cells. The cancer tissues double positive for EphA2 and phospho-Shp2Y580 (six among nine tested in this study) were also phospho-Erk positive (Figure 6 and Supplementary Figure S4).

EphA2b is involved in the heart defects induced by Shp2T468M in zebrafish

The phenotypes of the patients with LEOPARD syndrome, such as cardiac defects, short trunk and increased distance between two eyes, can be reproduced in the zebrafish when zebrafish mutant Shp2 mRNAs corresponding to human LEOPARD syndrome mutant mRNAs are injected into the embryos.[21,22] By using the zebrafish model of LEOPARD syndrome, we first tested the significance of phosphorylation of Tyr542 and Tyr580 of Shp2 and second examined the involvement of EphA2 in the abnormal phenotype. The percentage of cardiac defects of the embryos injected with Shp2T468M/2YF mRNAs was less than those injected with Shp2T468M mRNAs, but more than those injected with wild-type Shp2 mRNAs and those uninjected (Figures 7a and b). These data indicate the importance of Tyr542 and Tyr580 for the cardiac abnormality and suggest the involvement Grb2-independent signaling, probably the Gab1-mediated signal as we found in the cultured cells (Figures 3h and i). Consistent with the results with cultured cells, the embryos injected with Shp2T468M mRNAs exhibited stronger Erk activation than those injected with wild-type Shp2 mRNAs, those injected with Shp2T468M/2YF mRNAs and those uninjected (Figure 7c).
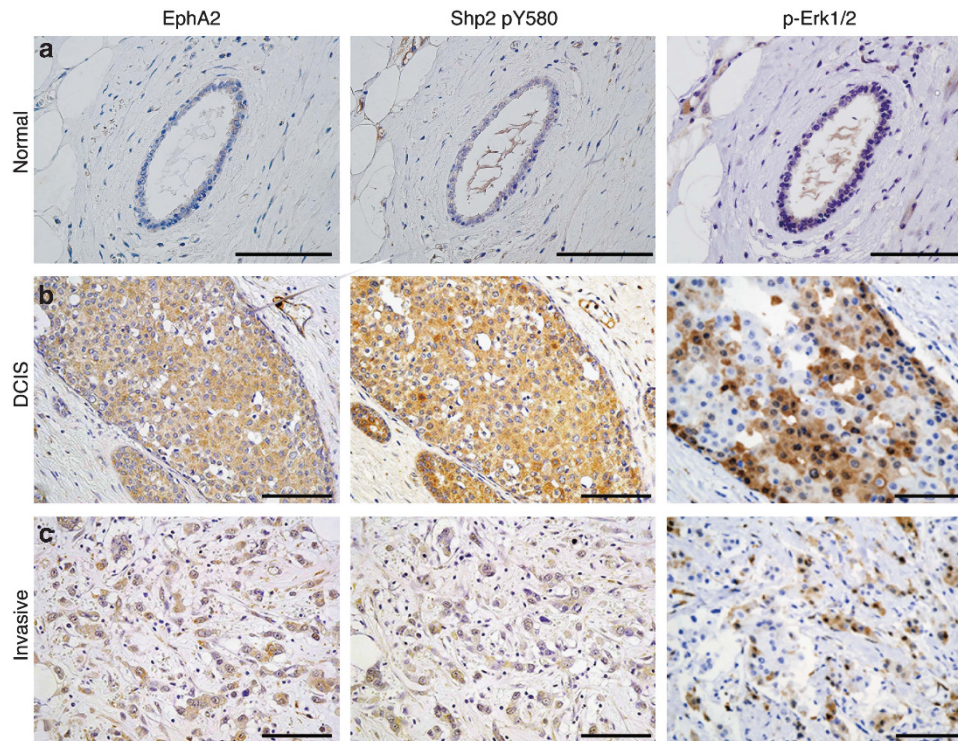
Figure 6. Phosphorylation of Shp2 and Erk1/2 in breast cancer expressing EphA2. Immunohistochemical analyses with the antibodies indicated on the top using specimens obtained from normal breast tissue (**a**), ductal carcinoma in situ (DCIS) (**b**) and invasive ductal carcinoma (**c**). Hematoxylin was used for counterstaining. Bar, 100 μm. Positive immunoreactivity is shown as brown color because of detection using 3,3'-diaminobenzidine (DAB) as described in the Materials and methods section.

To test the contribution of EphA2 to the defects found in zebrafish LEOPARD syndrome model, we knocked down the expression of EphA2. In zebrafish, two orthologs of human EphA2 (hereafter designated as EphA2a and EphA2b) are expressed.[23] Knockdown of EphA2a and EphA2b by morpholino oligos were confirmed by real-time reverse transcription–polymerase chain reaction (Figures 7d and e and Supplementary Figure S5). The heart defects found in the embryos injected with Shp2T468M mRNAs was suppressed by knockdown of EphA2b, but not by that of EphA2a (Figure 7f), suggesting the involvement of EphA2b in LEOPARD syndrome-like phenotypes in zebrafish. We then examined the expression of EphA2 in zebrafish hearts. In situ hybridization assay to detect EphA2 mRNAs revealed that neither EphA2a nor EphA2b mRNAs was expressed in the heart of normal embryos on the day 3 post-fertilization (Figure 7g). However, in the embryos injected with Shp2T468M mRNAs, both EphA2a and A2b mRNAs were detected in the heart (Figure 7h, Supplementary Figure S6 and Supplementary Table 2), suggesting that Shp2T468M promotes EphA2 expression.

We tested this possibility by examining whether Shp2T468M increases the expression of EphA2 in mammalian cultured cells. When Shp2T468M was overexpressed in NMuMG cells, the expression of EphA2 was increased (Figures 7i and j). Consistently, EphA2 mRNA was increased in NMuMG expressing Shp2T468M (Supplementary Figure S7a). This increase was significantly but not completely suppressed in the cells overexpressing Shp2T468M/2YF (Figures 7i and j). Similar to EphA2 expression, basal Erk activity and phosphorylation of Gab1 at Tyr627 was augmented by Shp2T468M and, to a lesser extent, by Shp2T468M/2YF (Figure 7i), suggesting the contribution of Shp2T468M-dependent Erk activation involving Gab1-mediated signal to the promotion of EphA2 expression. Because EphA2 is a direct transcriptional target of the Ras-Erk pathway,[24] we tested the effects of MEK inhibitor, U0126,

on the induction of EphA2 protein by Shp2T468M. The increase in EphA2 and EphA2 mRNA by Shp2T468M was significantly suppressed by U0126 (Figures 7k and l and Supplementary Figure S7b). These results indicate that Shp2T468M enhances the expression of EphA2 via Erk-mediated signaling.

DISCUSSION

We identified EphA2 as a tyrosine kinase that phosphorylates Tyr542 and Tyr580 of Shp2 to enhance and prolong the Erk activation downstream of receptor tyrosine kinases in the cells stimulated with growth factors. Furthermore, we demonstrated the involvement of the Erk phosphorylation by EphA2-mediated phosphorylation of Shp2 in cancer progression and in an Shp2-associated disease, LEOPARD syndrome.

Shp2 is a positive regulator of Erk downstream of growth factor receptor signaling.[1–3] It remains controversial whether the phosphatase activity is required for Shp2-dependent Erk activation. Sprouty and the phosphorylation sites to which p120RasGAP binds are dephosphorylated by Shp2; therefore, Shp2 positively regulates Erk, because both tyrosine-phosphorylated Sprouty and p120RasGAP binding to phosphorylated tyrosines inactivate Ras-Erk signal pathway.[2,8,25,26] Shp2 functions not only as a phosphatase but also as a scaffold molecule to provide Grb2 binding sites by becoming phosphorylated at Tyr542/580 in its carboxy terminus. Therefore, the binding of Shp2 with Grb2 results in Erk activation in a manner independent of phosphatase activity of Shp2. It is important to clarify how Tyr542/580 are phosphorylated upon growth factor stimulation. In this study, we found that EphA2 directly phosphorylated these tyrosine residues (Figure 5d). The prolonged and enhanced Erk activation in the cells stimulated with growth factors were reduced in cells depleted of EphA2 (Figures 1c, e and g).
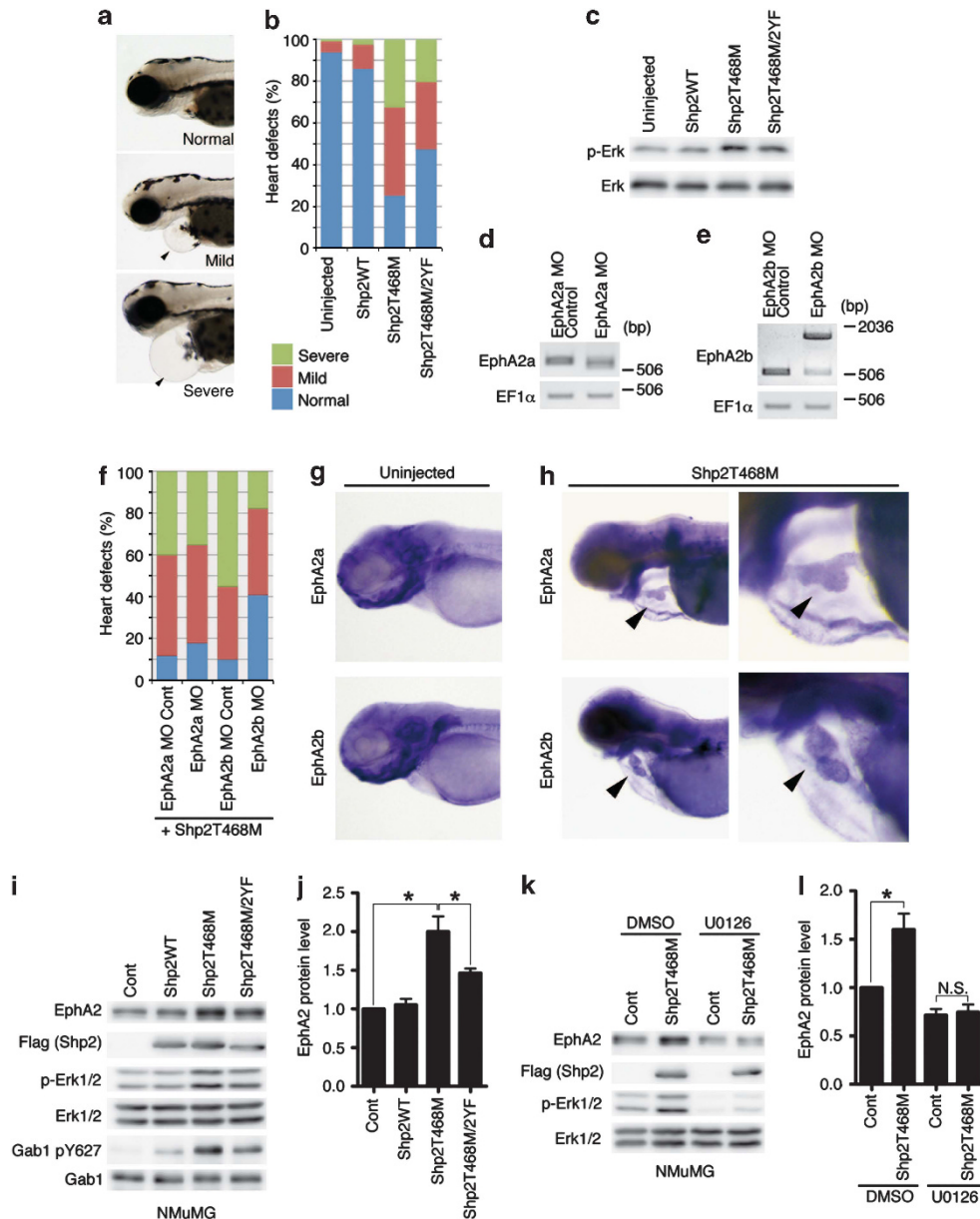
Figure 7. Shp2T468M associated with LEOPARD syndrome induces heart defects in zebrafish and promotes EphA2. (**a**) Heart defects in 3 days post-fertilization (dpf) zebrafish embryos after injection with Shp2T468M (200 pg per embryo) at one-cell stage embryos. Arrowheads indicate the cardiac edema. (**b**) Proportion of the phenotypes observed in the 3 dpf embryos injected with mRNAs ($n = 100–120$ per injection) indicated at the bottom was calculated according to the number of the normal or abnormal phenotypes of the fish among the total embryos subjected to the observation. The grade of abnormal cardiac edema was scored by the degree of the swelling. (**c**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from the zebrafish embryos ($n = 10$) 8 h after injection with the mRNAs indicated on the top. (**d** and **e**) Reverse transcription–polymerase chain reaction (RT–PCR) analysis using primers for EphA2a, EphA2b or EF-1α and total RNA prepared from the zebrafish embryos 8 h after injection with morpholino oligos against EphA2a (4.2 ng per embryo) or EphA2b (8.4 ng per embryo) into one-cell stage embryos. (**f**) Proportion of the phenotypes of the 3 dpf embryos injected with Shp2T468M mRNA and the morpholino oligos as indicated on the bottom ($n = 100–120$ per injection) at one-cell stage embryos was analyzed as in (**b**). (**g**) Lateral views of 3 dpf embryos hybridized with probes indicated on the left. (**h**) Lateral views and their close-up images (right) of 3 dpf embryos injected with Shp2T468M mRNA at the one-cell stage and hybridized with probes indicated on the left. Arrowheads indicate the hearts. (**i**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from NMuMG cells infected with the retrovirus vectors indicated on the top. (**j**) The result of quantitaive analyses of (**i**). The relative expression of EphA2 was calculated as the value of the expression of EphA2 induced by the infection with control virus was 1. The mean value with s.d. of four independent experiments was indicated. *$P < 0.05$ among the four groups. (**k**) Immunoblot analyses with the antibodies indicated at the left using the cell lysates prepared from NMuMG cells infected with retrovirus vectors indicated on the top and treated with U0126 (20 μM) or dimethylsulfoxide (DMSO) for 24 h indicated on the top. (**l**) Expression of EphA2 observed in (**k**) was quantified as described in (**j**).

Simultaneous reduction of phosphorylation of Tyr542/580 was observed in those cells. Although ZAP70 is reported as a potential kinase that can phosphorylate Tyr542/580 in immune cells,[9] its expression in epithelial, endothelial and glial cells is unclear.

Therefore, EphA2 seems to be responsible for the phosphorylation of Tyr542/580 in NMuMG epithelial cells, HUVECs and C6 glioma cells (Figures 1c, e and g). These results suggest that EphA2-dependent phosphorylation of Shp2 contributes to the

enhancement and prolongation of growth factor-induced Erk activation, although we cannot exclude the involvement of phosphatase activity of Shp2.

Mutations of *PTPN11* gene encoding Shp2 lead to the diseases that belong to the enhanced Ras-Erk signaling diseases.[4,5] When EphA2 was coexpressed, both phosphatase-active mutant Shp2 (Shp2N308D) and phosphatase-defective mutant Shp2 (Shp2T468M) induced more Erk activation than wild-type Shp2 (Figure 3b). These results suggest that Shp2 can induce Erk activation partly irrespective of its phosphatase activity. The binding of Shp2T468M to Grb2 requires the phosphorylation of Tyr542/580, concurring with the importance of phosphorylation of these residues for Shp2-mediated Erk activation.

Gab1 might function besides or together with Grb2 in mutant Shp2-induced Erk activation. Although wild-type Shp2 and mutant Shp2 (T468M and N308D) equally bound to Grb2, Erk was more phosphorylated in the cells expressing mutants than in those expressing wild-type Shp2 (Figure 3b). Binding of Gab1 to Shp2 is essential for HGF-dependent Erk activation.[16,27] Shp2T468M/2YF could induce weak phosphorylation of Erk when expressed with EphA2 than wild-type Shp2. Overexpression of a dominant-negative form of Grb2 could not completely inhibit the Erk activation in the cells expressing Shp2T468M and EphA2. These data suggest that phosphorylation of Tyr542/580 of Shp2 for Grb2 binding is not sufficient for full Erk activation by Shp2T468M.

In addition to the phosphatase activity, unfolding of Shp2 seems to be important to activate Erk as reviewed previously.[2] In the inactive Shp2 folding, the first SH2 domain of Shp2 is thought to bind to the phosphatase domain of Shp2 to block the association of the substrate with phosphatase domain. However, when Tyr542/580 are phosphorylated by unfolding, the former and the latter binds to the first SH2 and the second SH2 to allow the phosphatase domain to recognize the substrate by the active folding.[28,29] The cells expressing EphA2 with wild-type Shp2 did not show the activation of Erk, whereas those expressing EphA2 with either Shp2T468M or Shp2N308D showed the substantial Erk activation (Figure 3b). Thus, unfolding might be important for the activation mediated by EphA2. Activation of growth factor receptors induces the phosphorylation of adaptor scaffolding molecules for Shp2 binding to unfold Shp2. The significance of unfolding of Shp2 is also suggested in stomach cancer; CagA protein of *Helicobacter pylori* binds to Shp2 to activate Erk signaling by unfolding Shp2.[30] Therefore, without appropriate unfolding, overexpressed EphA2 and wild-type Shp2 might be incapable of activating Erk. These data suggest the dual roles of phosphorylated Tyr542 and Tyr580: for the active intramolecular folding to allow the exposure of phosphatase domain to its substrate and for the association of Shp2 with Grb2.

Our results indicate that Shp2T468M enhances Erk activation in zebrafish embryos at 8 hours postfertilization (hpf) (Figure 7c). However, it has been reported that another LEOPARD-associated mutant of Shp2, zShp2T462A, inhibits Erk activation in zebrafish embryos at 24 hpf.[22] It is still controversial whether the Erk is constitutively activated. In the induced pluripotent stem-derived cardiomyocytes, from the patient with LEOPARD syndrome, show an increase in activation of Erk.[6] The Erk activation might vary during development of the embryos expressing Shp2T468M. Further study is required to examine whether LEOPARD-associated mutants of Shp2 always function as a positive regulator for Erk activation.

The possible role of EphA2 in the phosphorylation of Shp2 was demonstrated in the breast cancers. The increase in EphA2 expression paralleled the phosphorylation of Tyr580 of Shp2 in the breast cancers (Figure 6). Therefore, Shp2 phosphorylated by increased EphA2 might recruit Grb2 to activate Ras-Erk signaling.[2,7,8] Consistently, phospho-Erk was detected in EphA2 and phospho-Shp2Y580 double-positive cancer specimens. The increased expression of EphA2 has been reported in other cancers.[11] In addition, EphA2 is suggested to activate Erk signaling without binding to ephrinA upon growth factor stimulation.[12,14] Thus, Shp2-dependent Erk activation signal involving Grb2 pathway may be hyperactivated in the malignant cancers that overexpress EphA2 to promote cancer cell proliferation. There are several points that should be explored: (1) How is EphA2 activated to phosphorylate Shp2 in the cells stimulated with growth factors? (2) Is the binding of ephrinA with EphA2 required for EphA2 activation? (3) How does Shp2T468M promote the expression of EphA2? However, at least the contribution of EphA2 to the Shp2-mediated Erk activation found in this study might account for the cell proliferation and invasion mediated by Erk in EphA2-expressing cancers.

In conclusion, EphA2 directly phosphorylates Tyr542/Tyr580 of Shp2 to recruit Grb2, thereby inducing Ras-Erk signal pathway in growth factor-induced signaling and cancers with the enhanced expression of EphA2. In the patients with LEOPARD syndrome, Gab1 might function to activate Erk besides or together with Grb2 (Supplementary Figure S8).

MATERIALS AND METHODS

EphA2 kinase assay
GST-Shp2 (amino acids (aa) 224–593) fusion protein was expressed and purified from *Escherichia coli* using glutathione sepharose. GST-EphA2 (aa 572–976) fusion protein was obtained from Carnabiosciences (Kobe, Japan). The recombinant GST-Shp2 (1 μg) and GST-EphA2 (40 ng) proteins were incubated in the kinase buffer (50 mм Tris-HCl (pH 7.4), 150 mм NaCl, 10 mм $MgCl_2$ and 1 mм ATP) for the time indicated. The reaction was stopped by adding EDTA to 20 mм. Phosphorylation of Shp2 was examined by immunoblotting using anti-Shp2 pY580 and anti-Shp2 pY542.

Immunohistochemistry
The studies involving human tissues from breast cancer patients were approved by the Medical Ethics Committee of Hokkaido University. In all cases, informed consent was obtained for the use of resected specimens. Paraffin-embedded sections were deparaffinized and rehydrated. Antigens were retrieved by boiling sections in citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase was blocked with 3% H_2O_2. Sections were blocked with 5% normal goat serum and were incubated with rabbit polyclonal anti-EphA2 (1:500), anti-Shp2 pY580 (1:50) or anti-phospho-Erk1/2 (1:50) diluted with Can Get Signal immunostain solution A (Toyobo, Osaka, Japan) at room temperature for 1–2 h. After extensive washing in TBST, sections were detected with biotinylated goat anti-rabbit secondary antibody for 30 min followed by amplification with Vectastain ABC kit (Vector Laboratory, Burlingame, CA, USA), and were visualized with 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin. Endothelial cells were used as positive internal controls for EphA2, phosphorylated Shp2 and phospho-Erk. Two board-certified pathologists independently assessed the immunostained slides. Any difference in the immunoreactivity of the cells was resolved by consensus. Immunoreactivity of the cells was assessed for staining intensity, which was scored on a scale of 0 to 3$^+$, with 0 being absence of staining, 1$^+$ being weakly, 2$^+$ being moderate and 3$^+$ being strongly immunoreactive.

Injection and *in situ* hybridization of zebrafish
Shp2 mRNAs were synthesized using mMESSAGEmMACHINE kit (Ambion, Austin, TX, USA). Morpholino oligos and synthetic mRNAs were injected into the blastomere of one-cell stage embryos. Whole-mount *in situ* hybridization was performed according to the conventional method. The experiments using zebrafish was approved by the animal committee of National Cerebral and Cardiovascular Center and was performed according to the guideline for the animal use provided by the committee.

Statistical analysis
We analyzed the difference in values among multiple groups by one-way analysis of variance followed by Bonferroni's post-test and that of the two groups by two-tailed *t*-test using Prism (GraphPad Software, San Diego, CA, USA). The values are expressed as means with s.d. *P*-values < 0.05 were considered statistically significant.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)