The Gab1 scaffold regulates RTK-dependent dorsal ruffle formation through the adaptor Nck

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Summary

The polarised distribution of signals downstream from receptor tyrosine kinases (RTKs) regulates fundamental cellular processes that control cell migration, growth and morphogenesis. It is poorly understood how RTKs are involved in the localised signalling and actin remodelling required for these processes. Here, we show that the Gab1 scaffold is essential for the formation of a class of polarised actin microdomain, namely dorsal ruffles, downstream from the Met, EGF and PDGF RTKs. Gab1 associates constitutively with the actin-nucleating factor N-WASP. Following RTK activation, Gab1 recruits Nck, an activator of N-WASP, into a signalling complex localised to dorsal ruffles. Formation of dorsal ruffles requires interaction between Gab1 and Nck, and also requires functional N-WASP. Epithelial cells expressing Gab1ΔNck (Y407F) exhibit decreased Met-dependent Rac activation, fail to induce dorsal ruffles, and have impaired cell migration and epithelial remodelling. These data show that a Gab1-Nck signalling complex interacts with several RTKs to promote polarised actin remodelling and downstream biological responses.

Key words: Gab1, RTK, Dorsal ruffles, Nck, N-WASP, Morphogenesis, Actin

Introduction

Cells use spatially restricted signalling complexes that modulate actin dynamics and cell shape to coordinate numerous fundamental cellular processes, including cell migration, invasion and morphogenesis. Many of these signals are initiated in response to ligand activation of receptor tyrosine kinases (RTKs). However, the spatial and temporal details of the mechanisms that link RTK activation to downstream actin remodelling are poorly understood.

Hepatocyte growth factor (HGF) and its cognate RTK, Met, are potent activators of epithelial cell dispersal, morphogenesis and invasive growth (Birchmeier et al., 2003). This biological activity is principally due to the recruitment and phosphorylation of the scaffold protein Gab1 to the activated Met receptor (Peschard and Park, 2007). In vitro, stimulation of colonies of epithelial cells with HGF promotes breakdown of cell-cell junctions and actin remodelling, coupled with changes in cell shape and enhanced cell-matrix adhesion required for cell dispersal (Ridley et al., 1995; Royal et al., 2000; Royal and Park, 1995). Met-dependent tyrosine phosphorylation of Gab1 creates binding sites, promoting recruitment of several signalling proteins, including the p85 subunit of phosphatidylinositol 3-kinase (PI3K), the Crk adaptor protein, PLCγ and the tyrosine phosphatase Shp2 (Gual et al., 2000; Lamorte et al., 2002b; Maroun et al., 1999b; Maroun et al., 2000). Gab1-associated PI3K-dependent activation of Akt and its downstream pathways, is required for cell migration and escape from apoptosis (Birchmeier et al., 2003). Shp2 catalytic activity and recruitment to Gab1 are both essential for the sustained activation of MAPK required for invasive growth (Lamorte et al., 2002b; Peschard and Park, 2007). Met activation is also linked to pathways that modulate the actin cytoskeleton, cell adhesion and migration (Peschard and Park, 2007). These are coordinated through Gab1, where recruitment of Crk to Gab1 couples Met signalling to activation of Rap1 and Rac (Lamorte et al., 2002b; Rodrigues et al., 2005), and Gab1-Pak4 interaction is required for the localisation of Pak4 to lamellipodia and cell invasion (Paliouras et al., 2009). In epithelial colonies, Gab1 localises to lamellipodia with downstream proteins, such as Shp2, for prolonged periods (up to 15 minutes) following HGF stimulation (Frigault et al., 2008; Kallin et al., 2004; Maroun et al., 1999a). Gab1 contains a pleckstrin-homology (PH) domain, which tethers Gab1 to membranes through interactions with PIP_3 (Maroun et al., 1999b). Recruitment of Gab1 complexes to the membrane is required for Met-dependent processes, including cell scatter and epithelial morphogenesis (Gual et al., 2000; Lamorte et al., 2002a; Maroun et al., 1999a; Maroun et al., 2000; Paliouras et al., 2009).

In addition to Met, Gab1 is recruited to and phosphorylated by the activated epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) RTKs (Rakhit et al., 2000; Rodrigues et al., 2000), as well as other RTKs and cytokine receptors (Gu and Neel, 2003). Gab1 is recruited indirectly through the adaptor Grb2 to the Met, EGF and PDGF RTKs (Kallin et al., 2004; Nguyen et al., 1997; Rodrigues et al., 2000), but also binds directly to Met through its Met-binding motif (Lock et al., 2002; Weidner et al., 1996). Phenotypes of *Gab1*-null embryos (Itoh et al., 2000; Sachs et al., 2000), phenocopy mice lacking either HGF or PDGF-bb growth factors, as well as the EGF or Met RTKs, underscoring the importance of Gab1 in signalling by these RTKs in vivo.

Activation of the Met, EGF or PDGF RTKs in vitro leads to rapid reorganisation of the actin cytoskeleton, which is required for epithelial and endothelial remodelling and cell migration (Kedrin et al., 2007; Ridley et al., 1992). Activation of these RTKs by growth factors promotes rapid morphological changes in the plasma membrane, including formation of F-actin-rich membrane protrusions known as membrane ruffles (Chhabra and Higgs,

2007). Whereas related structures known as lamellipodia form broad cellular projections that adhere to the substratum, membrane ruffles form non-adherent, sheet-like protrusions (Chhabra and Higgs, 2007). Two distinctive forms of membrane-ruffling events have been reported: peripheral ruffles and circular dorsal ruffles or waves (Abercrombie et al., 1970). Dorsal ruffles are not thought to develop from peripheral ruffles (Araki et al., 2000). Although peripheral ruffles can occur continuously upon growth-factor stimulation (Ridley et al., 1992; Suetsugu et al., 2003), dorsal ruffles form transiently within the first 20 minutes of stimulation (Buccione et al., 2004), suggesting that dorsal and peripheral ruffles are differentially regulated and possess distinct physiological functions.

Several regulators of dorsal ruffle formation have been characterised; however, the mechanisms by which RTKs coordinate these structures are poorly defined. Dorsal ruffles form in response to stimulation by growth factors such as HGF, EGF, PDGF-bb and vascular endothelial growth factor (VEGF) (Buccione et al., 2004; Dowrick et al., 1993; Wu et al., 2003) and require regulators of actin polymerisation (Buccione et al., 2004) and Rac activity (Krueger et al., 2003; Lanzetti et al., 2004; Palamidessi et al., 2008; Ridley et al., 1992; Suetsugu et al., 2003; Wu et al., 2003). Formation of dorsal ruffles requires localisation of activated Rac to the plasma membrane; for instance, the small GTPase Rab5 is required for HGF-induced dorsal ruffles, as it trafficks activated Rac to the plasma membrane (Lanzetti et al., 2004; Palamidessi et al., 2008), indicating that regulators of Rac localisation to the membrane are crucial for dorsal ruffle formation. Cortactin, N-WASP and Wave-1 complexes, which are activators of Arp2/3-mediated actin polymerisation, have also been demonstrated to have a role in dorsal ruffle formation (Krueger et al., 2003; Legg et al., 2007; Suetsugu et al., 2003). Hence, although regulators of actin polymerisation appear to be common to dorsal ruffles and other membrane protrusions, the elements that couple these components to RTK signalling and localise them to the plasma membrane in the context of dorsal ruffles remain to be identified.

Nck adaptor proteins (Nck1 and Nck2), regulate actin polymerisation by recruiting N-WASP and WAVE complexes (Buday et al., 2002), and have been linked to induction of dorsal ruffles in fibroblasts by PDGF-bb (Rivera et al., 2006; Ruusala et al., 2008). These proteins contain three SH3 domains followed by a C-terminal SH2 domain (Chen et al., 1998). Through their SH2 domain, Nck proteins couple plasma-membrane-localised phosphotyrosine signals to regulators of the actin cytoskeleton, including Pak1, N-WASP and WAVE, which bind to the SH3 domains of Nck (Buday et al., 2002). Although Nck proteins have been proposed to bind directly to the EGF and PDGFβ RTKs (Chen et al., 2000; Hake et al., 2008; Nishimura et al., 1993), recent data suggests that RTKs might not be the major substrate of the Nck SH2 domain in vivo (Rivera et al., 2006).

How RTKs coordinate common signalling pathways to promote dorsal ruffle formation has not been defined. Here, we identify the Gab1 scaffold protein as an essential and common requirement for dorsal ruffle formation downstream from the Met, EGF and PDGFβ receptors in both epithelial colonies and fibroblasts. We demonstrate that Gab1 mediates formation of dorsal ruffles through a novel

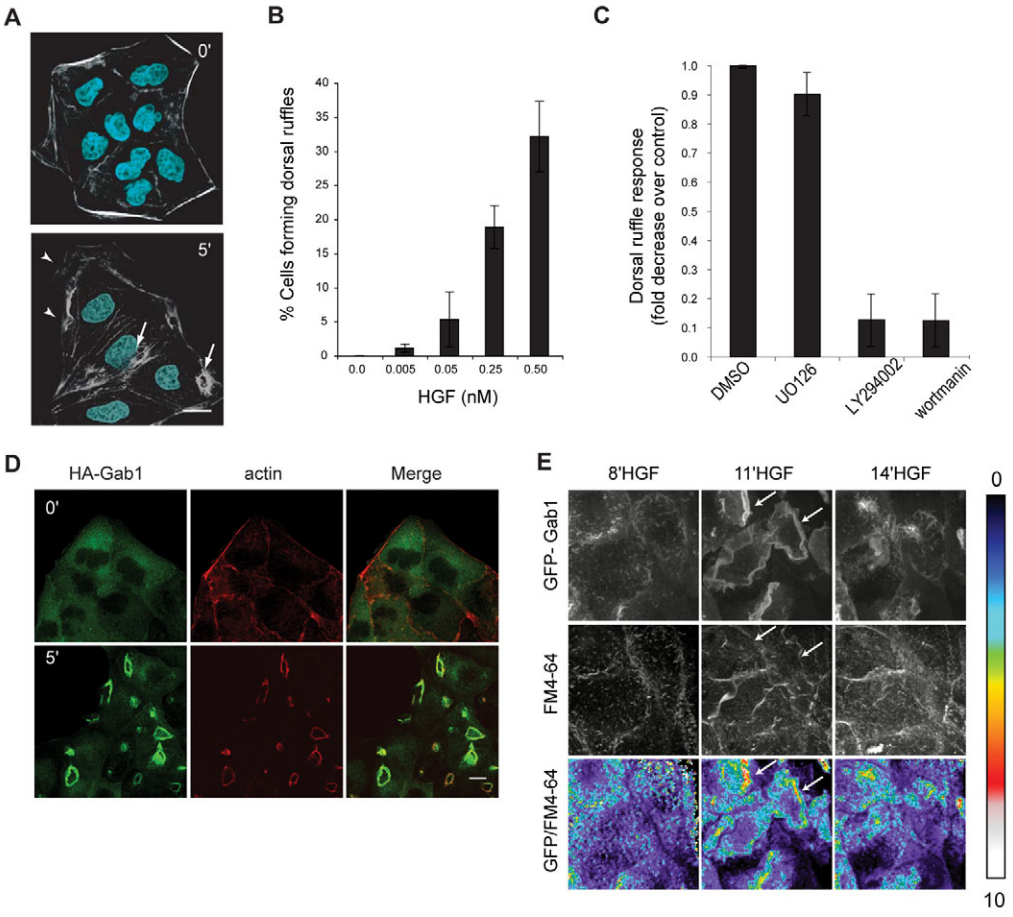

Fig. 1. Gab1 is recruited to and enriched in HGF-induced dorsal ruffles in MDCK cells. (**A**) MDCK cells before (0′) or after HGF stimulation (5′) were fixed and stained for F-actin and DAPI. Arrows indicate dorsal ruffles; arrowheads, peripheral ruffles. (**B**) MDCK cells were stimulated with the indicated concentrations of HGF, fixed and stained for F-actin. Depicted is the mean percentage of cells that form dorsal ruffles ± s.d. from three independent experiments. (**C**) MDCK cells were pretreated with DMSO or the indicated inhibitors for 30 minutes, stimulated with 0.5 nM HGF for 5 minutes, fixed and stained for F-actin and DAPI. Dorsal ruffle response is presented as the mean fold decrease compared with DMSO-treated cells ± s.e.m. from three independent experiments. (**D**) MDCK-HA-Gab1 cells stimulated or not with 0.5 nM HGF were fixed and stained for HA (red) and F-actin (green). (**E**) MDCK-GFP-Gab1 cells were stimulated with HGF and the membrane dye FM4-64 and live-imaged by SDCM. Representative time points from the movie are shown as a fourfold enlargement. Arrows represent areas of Gab1 enrichment within the dorsal ruffle membrane. Scale bars: 10 μm.

interaction with Nck adaptor proteins, which requires N-WASP, and establish that this is a common molecular mechanism for several RTKs. Additionally, we provide evidence that Gab1-mediated dorsal ruffles are necessary for biological responses such as epithelial migration and morphogenesis.

Results

HGF induces dorsal ruffles in epithelial cell colonies

Mechanisms regulating the induction of dorsal ruffle formation have been predominantly characterised in single cells such as fibroblasts, or in tumour cells (Krueger et al., 2003; Lanzetti et al., 2004; Orth et al., 2006; Suetsugu et al., 2003). To investigate a more physiologically relevant environment, we used polarised sheets of epithelial Madin Darby canine kidney (MDCK) cells as a model system to study dorsal ruffle formation in response to HGF. HGF potently induces dorsal ruffles in colonies of MDCK cells (Dowrick et al., 1993) (Fig. 1A,B). These occur throughout the colony, as visualised by concentric actin-rich rings on the apical cell surface, in a dose-dependent manner (Fig. 1A,B, see arrows). Since induction of dorsal ruffles by PDGF-bb in fibroblasts requires PI3K (Dharmawardhane et al., 2000; Scaife et al., 2003), we investigated a requirement for PI3K in response to HGF. Pretreatment of MDCK cells with PI3K inhibitors (LY294002 and wortmannin) prevented HGF-induced dorsal ruffle formation, whereas pretreatment with the MEK inhibitor UO126 had no effect (Fig. 1C), demonstrating that in a similar manner to PDGF-bb, the induction of dorsal ruffles by HGF in epithelial cell colonies requires PI3K activity.

Gab1 localises to dorsal ruffles and is required for their formation

Gab1 is required for Met-dependent lamellipodia formation, cell dispersal and epithelial morphogenesis (Frigault et al., 2008; Maroun et al., 1999a; Maroun et al., 2003; Weidner et al., 1996). Using MDCK cell lines stably expressing GFP- or HA-tagged Gab1 (MDCK-GFP-Gab1 and MDCK-HA-Gab1) (Frigault et al., 2008; Maroun et al., 2000), we found that Gab1 was rapidly localised to dorsal ruffles (within 2 minutes) in response to HGF (Fig. 1D; supplementary material Movie 1). Live-cell imaging of MDCK-GFP-Gab1 cells demonstrated that each dorsal ruffle persisted for approximately 8 minutes and occurred in cells throughout the colony (supplementary material Movie 1). Hence, dorsal ruffle formation was independent of the ability of cells to form lamellipodia (Fig. 1A, arrowhead), which only occur in cells at the edge of a colony.

Gab1 is specifically enriched in dorsal ruffles following HGF stimulation in MDCK cells, as established using a general membrane dye (FM4-64) and ratiometric analysis (Fig. 1E, arrows; colour bar represents relative GFP-Gab1 enrichment over FM4-64 dye). To determine whether Gab1 localisation to dorsal ruffles reflects a role for Gab1 in their formation, we examined the effects of siRNA-based depletion of Gab1 and Gab1 overexpression in MDCK cells. Using three independent siRNA duplexes that decreased Gab1 protein levels, we observed a statistically significant decrease in the formation of dorsal ruffles in response to HGF (Fig. 2A, supplementary material S1A,B). Up to 75% fewer dorsal ruffles were observed, with an average knockdown of 62% in Gab1 protein levels (Figs 2A, supplementary material S1A,B). By contrast, overexpression of Gab1 in MDCK cells more than doubled the number of cells with dorsal ruffles in response to HGF (25% of MDCK cells form dorsal ruffles compared with 60% of MDCK GFP-Gab1 cells; Fig. 2B; supplementary material Fig. S1C). Notably, in HeLa cells, which do not readily form dorsal ruffles in response to HGF, transient overexpression of Gab1 promoted the formation of dorsal ruffles in response to HGF (Fig. 2C). Thus, Gab1 expression is necessary to promote HGF-induced dorsal ruffles.

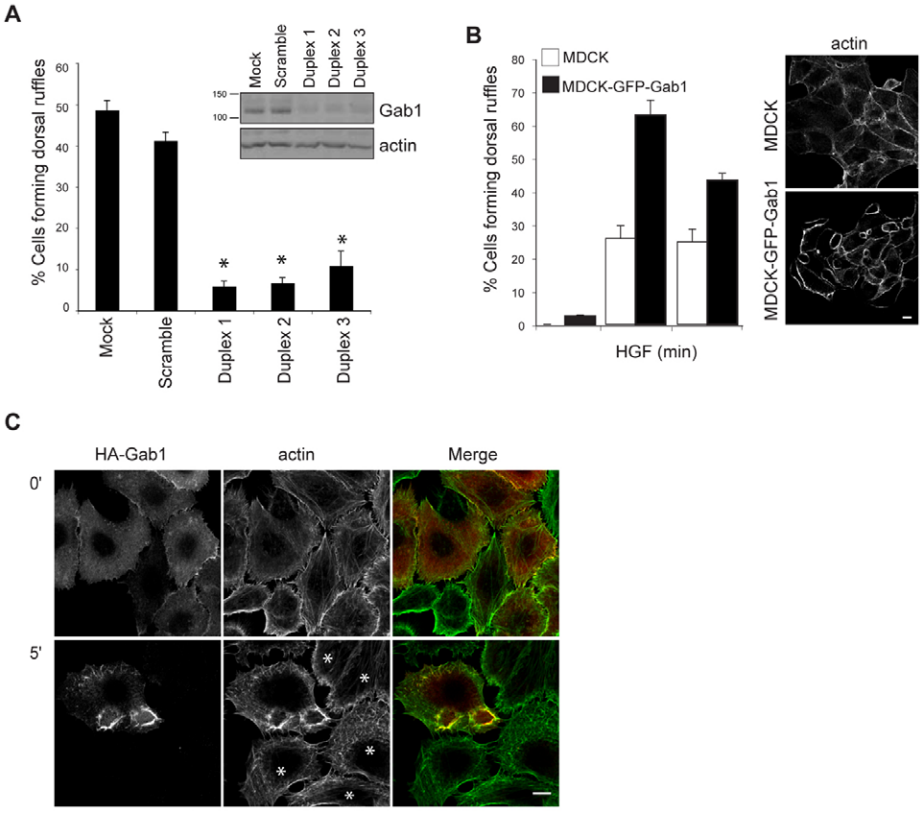

Fig. 2. Gab1 is required for and enhances HGF-induced dorsal ruffles in MDCK epithelial cells. (**A**) MDCK cells transfected with control or four independent siRNA duplexes against *Gab1* (Gab1 protein levels 96 hours after transfection are depicted in the inset) were stimulated with 0.5 nM HGF, fixed and stained for F-actin. Graph represents the mean percentage of cells that form dorsal ruffles ± s.e.m. from three independent experiments. Paired Student's *t*-tests demonstrate no significant difference between cells transfected with mock and scrambled siRNA; **P*<0.001. (**B**) MDCK or MDCK-GFP-Gab1 cells were stimulated with 0.5 nM HGF for 5 or 10 minutes, fixed and stained for F-actin. Data are expressed as the mean percentage of total cells forming dorsal ruffles ± s.e.m. from three independent experiments. Representative confocal images are shown. (**C**) HeLa cells transfected with HA-Gab1 with (5′) or without (0′) 0.5 nM HGF stimulation were fixed and stained for HA (red) and F-actin (green). Asterisks denote non-transfected cells. Scale bars: 10 μm.

Gab1 is required for dorsal ruffle formation downstream from EGF and PDGF receptors

Gab1 is phosphorylated following activation of the PDGFβ and EGF receptors (Rakhit et al., 2000; Rodrigues et al., 2000), both of which induce dorsal ruffle formation in fibroblasts (Buccione et al., 2004). To determine whether Gab1 is required for dorsal ruffle formation downstream from other RTKs, we used mouse embryonic fibroblast (MEF) cell lines established from *Gab1*-knockout embryos (*Gab1*$^{-/-}$ cells) (Holgado-Madruga and Wong, 2003). Stimulation of 6BGab1$^{+/+}$ cells with either PDGF-bb or EGF, promoted dorsal ruffle formation, as visualised by actin and cortactin staining (35% and 40%, respectively; Fig. 3A). By contrast, *Gab1*$^{-/-}$ cells (clones 7C and 2B, supplementary material Fig. S1D) exhibited an obvious decrease in dorsal ruffle formation in response to PDGF-bb (4% in 7C clones and 3% in 2B clones) and EGF (6% in 7C and 4% in 2B) (Fig. 3A,B). Re-expression of GFP-Gab1 in the *Gab1*$^{-/-}$ cells (7C-GFP-Gab1 and 2B-GFP-Gab1) was sufficient to rescue both EGF- and PDGF-bb-induced dorsal ruffle formation in both cell lines to ~60% (Fig. 3C,D). Furthermore, in HeLa cells, which do not form dorsal ruffles in response to EGF, overexpression of Gab1 was sufficient to promote these protrusions (supplementary material Fig. S1E). Together, these data demonstrate that Gab1 is essential for dorsal ruffle formation in both fibroblast and epithelial cells downstream from multiple RTKs.

Crk binding sites in Gab1 are required for Gab1-mediated dorsal ruffles

We next sought to elucidate the molecular basis for Gab1-dependent dorsal ruffle formation. To this end, we assayed dorsal ruffle formation in response to HGF using stable MDCK cell lines expressing a panel of Gab1 mutants specifically uncoupled from Crk, p85 and Shp2 (Fig. 4A; supplementary material Fig. S2B) (Lamorte et al., 2002b; Maroun et al., 1999b; Maroun et al., 2000) or through transient expression in HeLa cells. Assays for EGF- or PDGF-bb-induced dorsal ruffles were performed in 2B *Gab1*$^{-/-}$ fibroblasts expressing Gab1 mutants. Cell lines expressing a Gab1ΔCrk mutant showed significantly decreased dorsal ruffle formation in response to all RTKs. In response to HGF, less than 10% of MDCK cells expressing Gab1ΔCrk formed dorsal ruffles when compared with 40% of 2B-GFP-Gab1 cells (Fig. 4B,C; supplementary material Fig. S2A). Similarly, in response to EGF and PDGF-bb, 20% and 10%, respectively, of cells expressing Gab1ΔCrk formed dorsal ruffles compared with 60% of 2B-GFP-Gab1 cells (Fig. 4D). The PH domain of Gab1 is essential for Gab1-mediated dorsal ruffles (Fig. S2A,D). By contrast, recruitment of Shp2 was dispensable for dorsal ruffle formation downstream from all three RTKs. Recruitment of p85 to Gab1 was only required downstream from the EGF and Met RTKs in the absence of serum (Fig. 4D, supplementary material Fig. S2C). In the presence of serum, loss of p85 recruitment to Gab1 was dispensable for HGF-induced dorsal ruffles (Fig. 4B,C). These results demonstrate a crucial role for both membrane localisation provided by the Gab1 PH domain and the integrity of the Crk-binding sites for dorsal ruffle formation by all three RTKs.

Nck adaptor molecules bind to Y407 in Gab1

Crk is recruited to Gab1 via any of six Crk-SH2 domain consensus motifs (YxxP) found in Gab1 (Fig. 5A). Substitution of all six

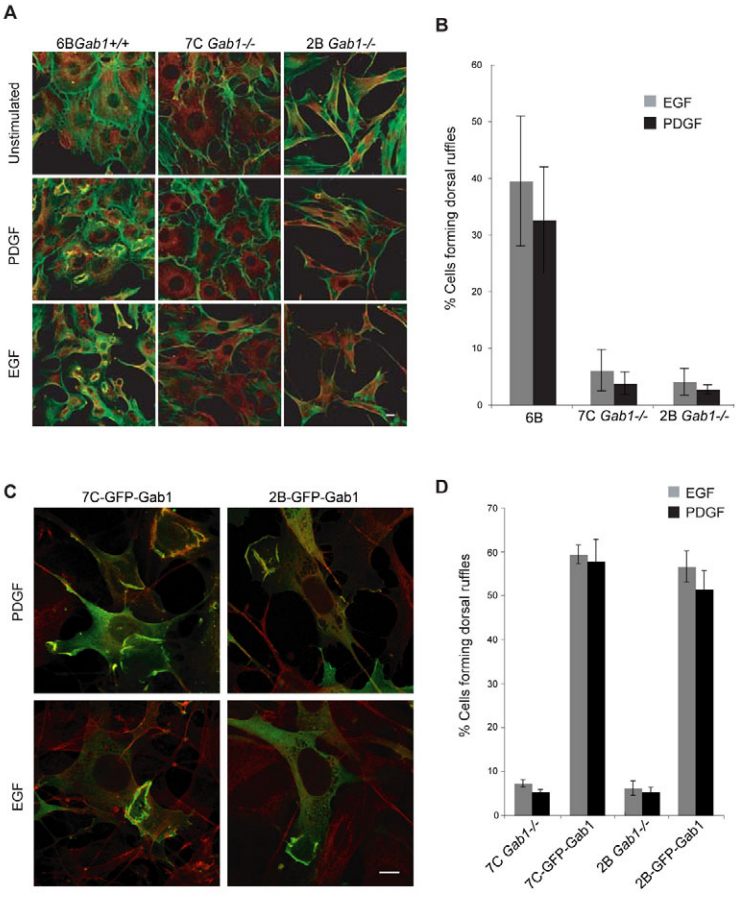

Fig. 3. Gab1 is required for EGF- and PDGF-bb-induced dorsal ruffles in fibroblast cells. (**A**) *Gab1*$^{+/+}$ cells (6B) and two clones of *Gab1*$^{-/-}$ cells (7C and 2B) were serum starved for 24 hours, stimulated for 5 minutes with 5 nM EGF or 11 nM PDGF-bb, fixed and stained for cortactin (red) and F-actin (green). (**B**) The mean percentage of cells that form dorsal ruffles ± s.d. from three independent experiments. (**C**) *Gab1*$^{-/-}$ cells (7C and 2B) were infected with a retrovirus expressing GFP-Gab1 (7C-GFP-Gab1, 2B-GFP-Gab1), serum starved for 24 hours, stimulated for 5 minutes with the indicated ligand and stained for F-actin (red) and (**D**) the percentage of cells forming dorsal ruffles ± s.d. from three independent experiments is shown. Scale bars: 10 μm.

tyrosine residues for phenylalanine (as in Gab1ΔCrk) is necessary to uncouple Crk from Gab1 (Lamorte et al., 2000) (Fig. 5A). To address the role for Crk, CrkII was either overexpressed or depleted through targeted siRNA in MDCK cells and dorsal ruffle formation assayed. Surprisingly, overexpression of CrkII in MDCK cells significantly reduced HGF-induced dorsal ruffles (Fig. 4E). Moreover, depletion of CrkII protein through RNAi of MDCK-GFP-Gab1 cells did not decrease dorsal ruffle formation (supplementary material Fig. S3A-B). These results indicate that Crk adaptor proteins are dispensable for dorsal ruffle formation in response to HGF, and raise the possibility that the Crk-binding sites on Gab1 recruit other molecules.

Sequence analysis of Gab1 revealed that three of the six tyrosine residues (Y242, Y307 and Y407) are contained within consensus phosphopeptide-binding motifs for the SH2 domains of the Nck1 and Nck2 adaptor molecules [Y(D/E)X(V/P)] (Frese et al., 2006) (Fig. 5A). MEF cells derived from Nck1/2 double-knockout embryos fail to form dorsal ruffles in response to PDGF-bb (Rivera et al., 2004; Ruusala et al., 2008), and although the mechanism through which Nck contributes to dorsal ruffles was not determined, this supports a possible role for Nck in this process. To examine whether Nck is recruited to Gab1 and if this is required for dorsal ruffle formation downstream from RTKs, we first established that in MDCK-HA-Gab1 cells, endogenous Nck proteins co-immunoprecipitate with Gab1 in response to HGF (Fig. 5B). In transient assays, Nck1 and 2 also co-immunoprecipitated with phosphorylated wt Gab1, but not Gab1ΔCrk (Fig. 5C), suggesting an interaction that is dependent on the Nck SH2 domain and tyrosine residues in Gab1. Site-directed mutagenesis of combinations of the six Crk-binding tyrosines in Gab1, demonstrated that a Gab1Y407F mutant failed to co-immunoprecipitate with Nck1 or Nck2, whereas a Gab1 mutant lacking the other putative Nck-binding sites (Gab1Y242-307F) retained the ability to co-immunoprecipitate with Nck1 and Nck2 (Fig. 5D and data not shown). Significantly, the

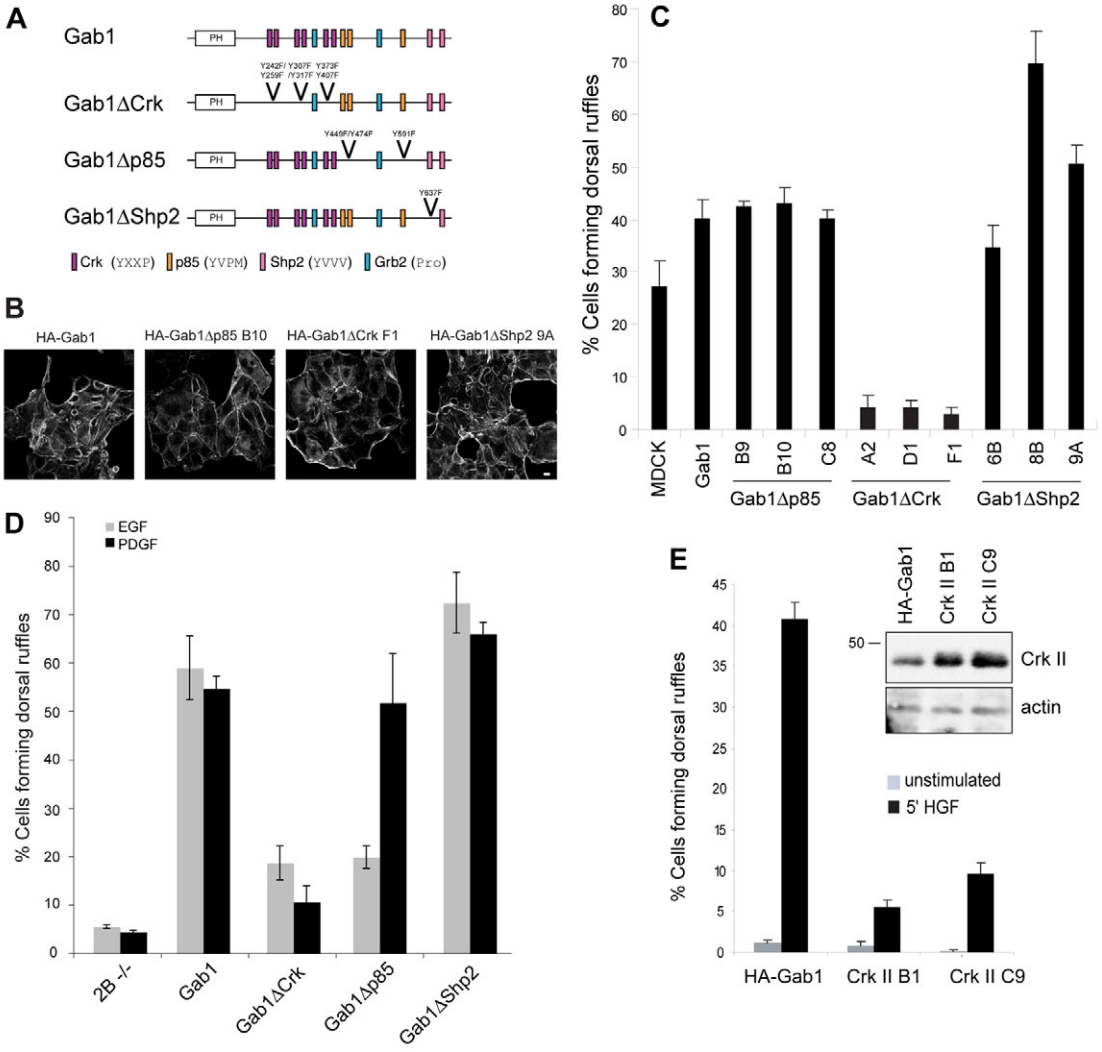

Fig. 4. RTK-mediated dorsal ruffles require the YxxP consensus motifs in Gab1. (**A**) Schematic depiction of tyrosine-to-phenylalanine substitutions that uncouple Gab1 from the indicated proteins. (**B**) WT MDCK cells, or lines stably expressing the indicated HA-Gab1 constructs, were stimulated with 0.5 nM HGF, fixed and stained for F-actin. (**C**) Mean percentage of total cells forming dorsal ruffles ± s.e.m. from three independent experiments. (**D**) 2B *Gab1*$^{-/-}$ cells infected with retroviruses expressing GFP-Gab1 or Gab1 mutants were serum starved for 24 hours, stimulated with 5 nM EGF or 11 nM PDGF-bb for 5 minutes, fixed and stained for F-actin. Data are expressed as the mean percentage of total cells forming dorsal ruffles ± s.e.m. from three independent experiments. (**E**) Two MDCK-CrkII cell lines (B1 and C9) were left untreated or stimulated with 0.5 nM HGF, fixed and stained for F-actin. Data are expressed as the mean percentage of total cells forming dorsal ruffles ± s.e.m. from three independent experiments. Inset depicts protein levels of CrkII in the cell lines used. Scale bar: 10 μm.

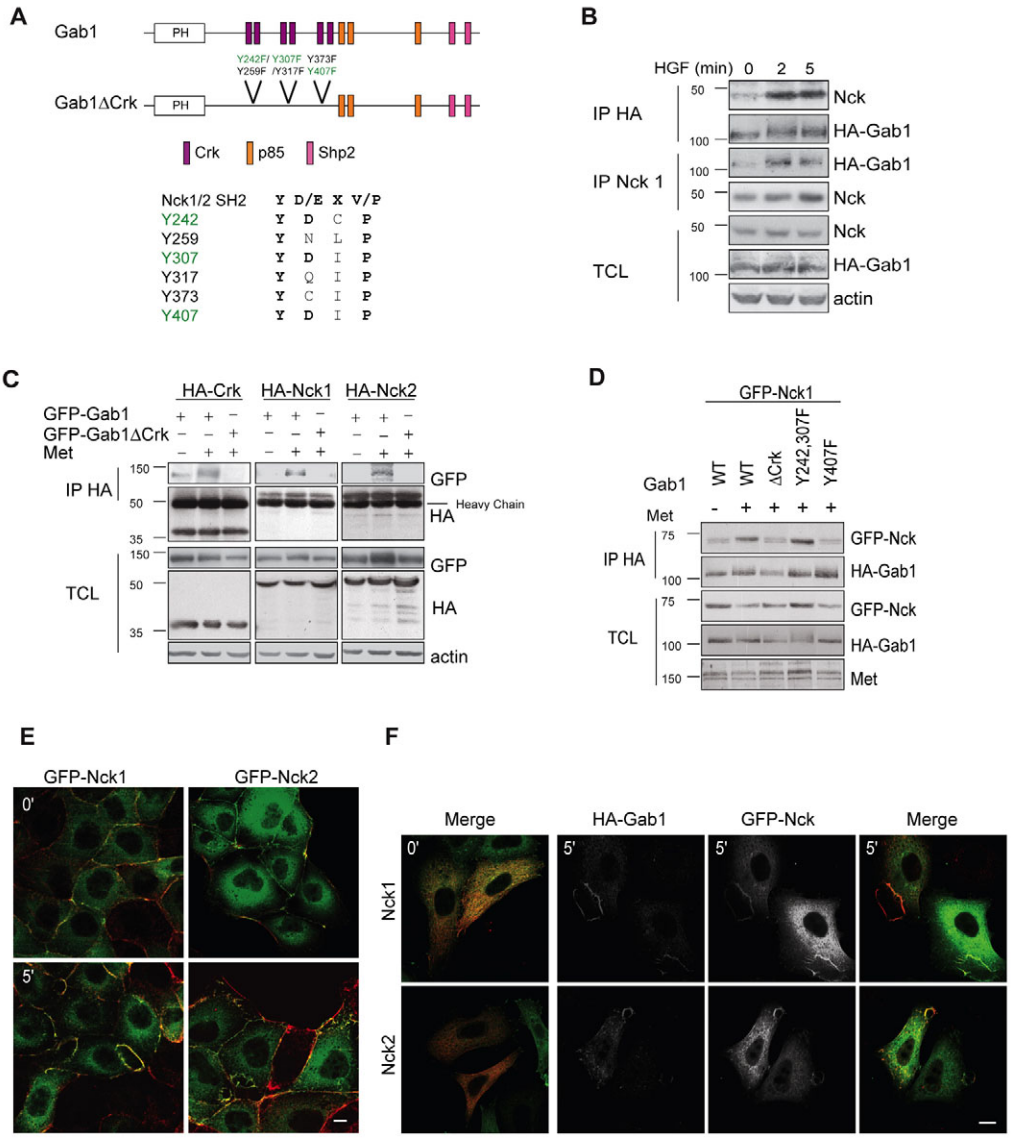

Fig. 5. Nck adaptor proteins interact with Y407 of Gab1. (**A**) Y242, Y307 and Y407 fit the minimum consensus binding motif for the Nck1 and Nck2 SH2 domains. (**B**) MDCK-HA-Gab1 cells were stimulated with 0.5 nM HGF for the indicated times, after which cell lysates were immunoprecipitated as indicated, resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted as indicated. (**C**,**D**) HEK293 cells were transiently transfected with the indicated constructs, lysates were immunoprecipitated with anti-HA antibody, resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted as indicated. (**E**) MDCK cells were transfected with GFP-Nck1 or GFP-Nck2, stimulated with 0.5 nM HGF, fixed and stained for F-actin. (**F**) HeLa cells were transfected with HA-Gab1 and GFP-Nck1 or 2 and stimulated with 0.5 nM HGF. Cells were fixed and stained for HA (red) and F-actin (green). Scale bars: 10 μm.

Gab1Y407F protein retained the ability to bind CrkI and CrkII in response to HGF (supplementary material Fig. S3C). A direct interaction of Nck with Gab1 is supported by the results of a far western blot, in which the SH2 domain of Nck1 bound directly to Gab1 (but only under conditions where Gab1 was tyrosine phosphorylated) and did not bind to a Gab1ΔCrk mutant (supplementary material Fig. S3D). Also in far western assays, the SH2 domain of Nck1 binds Y242, Y307 and Y407, and the degree of direct binding was dependent on the number of tyrosines available (supplementary material Fig. S3D). This is consistent with each of these phosphotyrosine residues representing a consensus binding site for the Nck SH2 domain. Hence, these data reveal a novel interaction between Gab1 and Nck adaptor proteins, which is dependent on Y407 of Gab1 in vivo.

A Nck-Gab1 interaction is required for RTK mediated dorsal ruffles

To characterise the significance of Gab1-Nck interactions for dorsal ruffle formation, we examined the subcellular localisation of Nck and the ability of the GabY407F mutant (hereafter Gab1ΔNck) to rescue dorsal ruffle formation. GFP-Nck1 and Nck2 localised to dorsal ruffles in response to HGF when expressed in MDCK and HeLa cells, supporting the formation of a Gab1-Nck complex in dorsal ruffles (Fig. 5E,F). Following either transient expression in HeLa cells (Fig. 6A), or stable expression in MDCK cells (Fig. 6B; supplementary material Fig. S4A), the Gab1ΔNck mutant, which retains five Crk binding sites, failed to enhance dorsal ruffle formation in response to HGF, whereas the Gab1Y242/307F mutant, which retains the ability to recruit Nck1 and Nck2 in vivo (Fig. 5D), enhanced dorsal ruffle formation in response to HGF to a similar level as wt Gab1 (Fig. 6A,B). Similar rescue experiments in 2B *Gab1*$^{-/-}$ fibroblasts demonstrated a dependence on Y407 in Gab1 for EGF- and PDGF-induced dorsal ruffles, where only 20% of 2B *Gab1*$^{-/-}$ cells expressing Gab1ΔNck formed dorsal ruffles compared with 70% of 2B-GFP-Gab1 cells (Fig. 6C). These data demonstrate that the inability of the Gab1ΔCrk mutant to promote dorsal ruffle formation downstream from the Met, EGF and PDGFβ RTKs is predominantly due to loss of Y407 and inability to recruit Nck1 and Nck2. In support of this, depletion of Nck1 in 2B GFP-Gab1 cells, significantly reduced dorsal ruffles induced by EGF or PDGF-bb by 70% compared with levels in cells treated with control siRNA (four

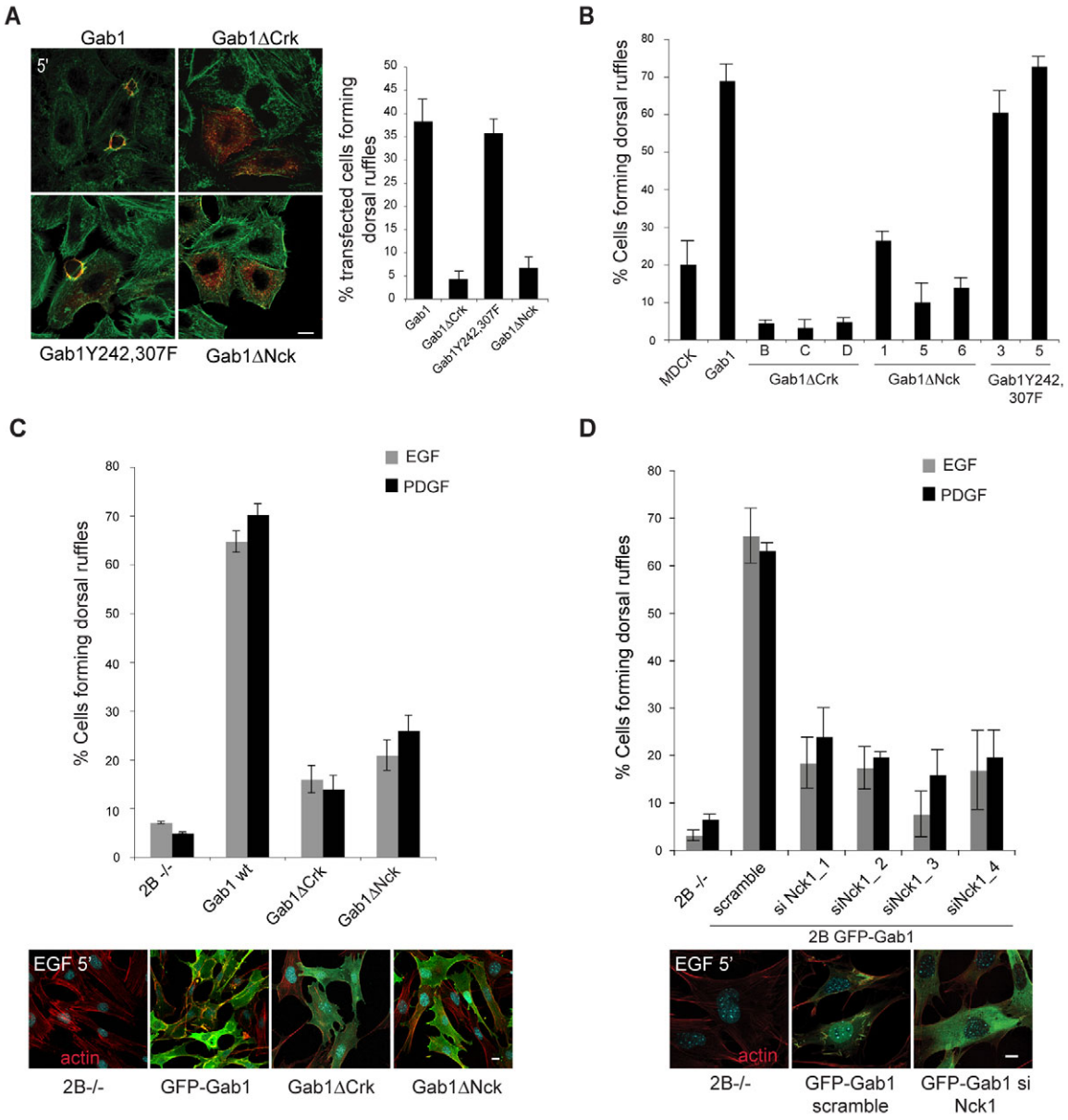

Fig. 6. Gab1-Nck interaction is required for growth factor-induced dorsal ruffles. (**A**) HeLa cells transfected with the indicated HA-Gab1 constructs were stimulated with 0.5 nM HGF, fixed and stained for HA (red) and F-actin (green). Data are expressed as mean percentage of the total transfected cells forming dorsal ruffles ± s.d. from three independent experiments. (**B**) MDCK-GFP-Gab1, GFP-Gab1ΔCrk (clones B, C, D), GFP-Gab1ΔNck (clones 1, 5, 6) and GFP-Gab1Y242,307F (clones 3 and 5) cells were stimulated with 0.5 nM HGF, fixed and stained for F-actin. Data are expressed as the mean percentage of total cells ± s.d. forming dorsal ruffles from three independent experiments. (**C**) 2B *Gab1*$^{-/-}$ cells infected or not with retrovirus expressing the indicated Gab1 constructs were serum-starved for 24 hours, stimulated with 5 nM EGF or 11 nM PDGF-bb, fixed and stained for F-actin. Data are expressed as the mean percentage of total cells ± s.d. forming dorsal ruffles from three independent experiments. (**D**) 2B-GFP-Gab1 cells were treated with control or four independent siRNA duplexes against Nck1 and treated as in C. Scale bars: 10 μm.

independent siRNAs; Fig. 6D; supplementary material Fig. S4B). Hence, the ability of Gab1 to stimulate dorsal ruffle formation is dependent on recruitment of Nck proteins via Y407.

Loss of Nck recruitment to Gab1 leads to reduced activation of Rac

The role of Nck proteins in the formation of actin-rich membrane protrusions involves the ability of these adaptors to activate the small GTPase Rac (Ruusala et al., 2008; ten Klooster et al., 2006; Yoshii et al., 1999), which is required for HGF and PDGF-bb induced dorsal ruffles (Krueger et al., 2003; Lanzetti et al., 2004; Palamidessi et al., 2008; Suetsugu et al., 2003). To determine whether a Gab1-Nck interaction contributes to HGF-dependent activation of Rac, endogenous Rac activity was measured in lysates from MDCK cells stimulated with HGF using pull-down assays with the PBD domain of Pak1 (GST-PBD, p21-binding domain) (Fig. 7A). Analysis of four independent experiments demonstrated that overexpression of Gab1 induced a 2.5-fold increase in HGF-dependent Rac activation

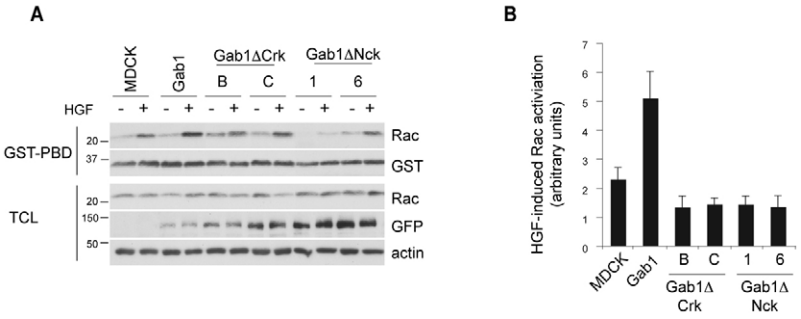

Fig. 7. Gab1 induces Rac activation through Nck. (**A**) Wild-type MDCK cells and cells stably expressing the indicated GFP-Gab1 constructs were stimulated or not with 0.5 nM HGF for 5 minutes. Lysates were incubated with GST-PBD coupled to glutathione-Sepharose beads to pull down activated Rac; precipitates were resolved by SDS-PAGE, transferred to nitrocellulose and probed as indicated. (**B**) Data are expressed as the mean HGF-induced Rac activation for each cell line assayed ± s.d. from three independent experiments.

over MDCK cells (Fig. 7A,B). Significantly, the HGF-induced Rac activation in MDCK cells stably expressing either a Gab1ΔCrk or a Gab1ΔNck mutant was not elevated, and was consistently lower than that in control MDCK cells (Fig. 7A,B). Hence, a Gab1-Nck complex is required for HGF-dependent Rac activation.

N-WASP is required for HGF-induced dorsal ruffles and associates with Gab1

The formation of dorsal ruffles in fibroblast cells also requires N-WASP-dependent Arp2/3 actin polymerisation (Legg et al., 2007). To examine whether Gab1-dependent dorsal ruffle formation also involves N-WASP, fibroblast cells were either subjected to knockdown of N-WASP by siRNA or cell lines were pretreated with the N-WASP inhibitor Wiskostatin (Peterson et al., 2004). Significantly, three independent siRNA duplexes targeting N-WASP, resulted in approximately a 75% reduction in both EGF and PDGF-bb-induced dorsal ruffles (Fig. 8A, supplementary material Fig. S5B). Consistent with this, pretreatment with Wiskostatin, also abrogated HGF-induced dorsal ruffle formation in MDCK-GFP-Gab1 cells in a concentration-dependent manner (Fig. 8B, supplementary material Fig. S5A), and had the same effect on PDGF-bb-induced dorsal ruffles in fibroblast cells, as previously reported (data not shown) (Legg et al., 2007). Altogether, these data demonstrate a requirement for N-WASP in Gab1-mediated RTK-dependent dorsal ruffles.

The SH3 domains of Nck proteins bind to a proline-rich domain within N-WASP, relieving auto-inhibition of N-WASP (Eden et al., 2002; Rivero-Lezcano et al., 1995; Rohatgi et al., 2001). We therefore tested whether N-WASP could be recruited to Gab1 in an Nck-dependent manner. Intriguingly, we found that in fibroblasts, endogenous N-WASP co-immunoprecipitated with Gab1 and that this was independent of stimulation with EGF, which promotes tyrosine phosphorylation of Gab1 (Fig. 8C). In addition, following transient co-transfection, N-WASP co-immunoprecipitated with wt Gab1, the Gab1ΔNck mutant, as well as other Gab1 mutants that uncouple Crk, Shp2, p85 or Grb2 recruitment to similar levels (Fig. 8D; supplementary material Fig. S5C,D). This association was not enhanced by tyrosine phosphorylation of Gab1, as induced through co-expression of Met, which is required for recruitment of Nck proteins to Gab1 (Fig. 8D; supplementary material Fig. 5C,D). Under basal conditions, Gab1 and N-WASP proteins localised to the cytosol (Fig. 8E, supplementary material Fig. S5E). However, upon activation of Met, Gab1 and N-WASP translocated to the membrane (Fig. 8E; supplementary material Fig. S5E), at which time Gab1 was phosphorylated by Met and recruited Nck (Fig. 5). These data support a role for Gab1 as a scaffold that promotes a RTK-dependent interaction between Nck and N-WASP at the plasma membrane (Fig. 10a). This would allow Nck to bind to and activate N-WASP-dependent Arp2/3 actin polymerisation (Rohatgi et al., 2001), which provides a mechanism to promote localised RTK-dependent actin polymerisation, leading to dorsal ruffle formation.

Gab1-Nck interactions are required for HGF-induced tubulogenesis

Since the formation of dorsal ruffles has been associated with membrane remodelling, we examined whether interactions between Nck and Gab1 were required for HGF-stimulated Gab1-dependent cytoskeletal changes during branching tubulogenesis. The HGF-Met axis is a regulator of kidney morphogenesis in vivo (Ishibe et al., 2009), and is a potent inducer of this process in vitro in MDCK cells, where HGF induces the formation of branching tubules (Montesano et al., 1991). This HGF-dependent program requires Gab1 (Maroun et al., 1999a; Weidner et al., 1996).

To determine whether Nck recruitment to Gab1 was required for cell migration and tubulogenesis, we used MDCK cells overexpressing a chimeric CSF-Met mutant receptor (CSF-MetΔGrb2) that is only able to promote a morphogenic program upon overexpression of wt Gab1 (Lamorte et al., 2002b; Lock et al., 2002; Maroun et al., 1999a; Maroun et al., 2000; Paliouras et al., 2009). In response to CSF-MetΔGrb2 activation, three independent clones of MDCK cells expressing the Gab1ΔNck mutant were significantly impaired in their ability to scatter (Fig. 9A) or form branching tubules (Fig. 9B). By contrast, cells expressing wt Gab1 scattered and formed branching tubules in a similar manner to MDCK cells stimulated with HGF (Fig. 9B). These data demonstrate that the integrity of Y407, and hence Nck recruitment, is required for Gab1-dependent cell migration and tubulogenesis, thus coupling the ability of epithelial cells to form dorsal ruffles with their ability to undergo a morphogenic program.

Discussion

In this study, we identify a novel role for the Gab1 scaffold protein in the formation of dorsal ruffles downstream from several RTKs. Using a combination of genetics, cell biology and biochemistry, we provide mechanistic understanding that Gab1-dependent signals required for dorsal ruffle formation involve the actin-nucleation protein N-WASP, as well as a direct interaction between Y407 in Gab1 with the SH2 domain of Nck adaptor proteins. Disruption of the Gab1-Nck interaction abolishes growth-factor-induced dorsal ruffles, and renders epithelial MDCK cells incompetent to undergo actin remodelling required for an invasive morphogenic program. We propose that in response to RTK activation, the recruitment of Nck to a Gab1-N-WASP complex promotes activation of N-WASP and provides a mechanism for spatially restricted actin remodelling downstream from several RTKs.

Growth-factor-induced dorsal ruffles have been implicated in diverse cellular processes, including macropinocytosis (Dowrick et

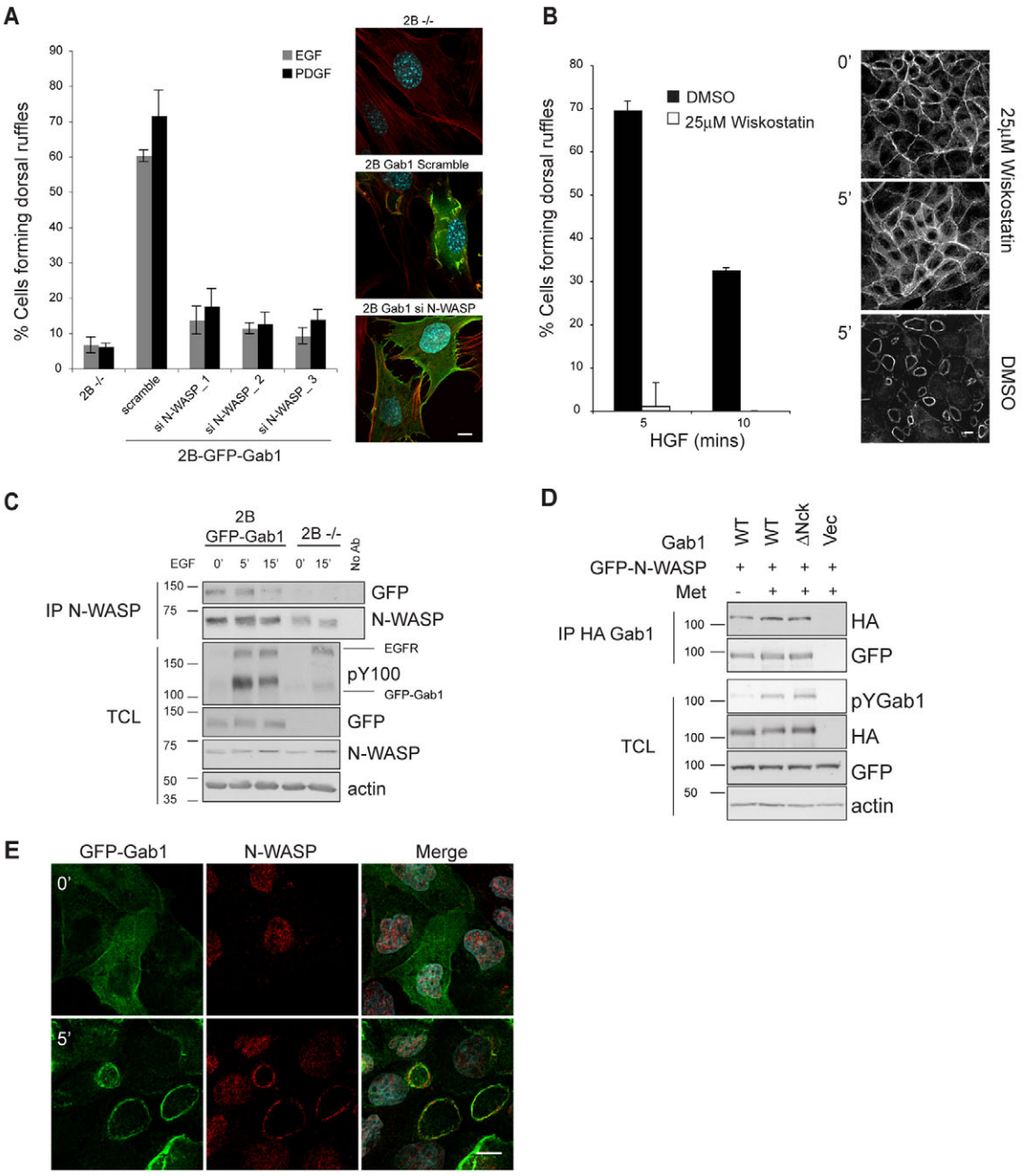

Fig. 8. Gab1-mediated dorsal ruffles requires N-WASP (**A**) 2B *Gab1*$^{-/-}$ cells rescued with GFP-Gab1 were treated with control siRNA or three independent siRNA duplexes to knockdown N-WASP, serum starved for 24 hours then stimulated with 5 nM EGF or 11 nM PDGF for 5 minutes, fixed and stained for F-actin. The mean percentage of cells forming dorsal ruffles ± s.d. from three independent experiments is shown with representative images of the actin cytoskeleton in each condition. (**B**) MDCK-GFP-Gab1 cells were pretreated with 25 μM Wiskostatin or DMSO (control) and stimulated with 0.5 nM HGF in the presence of inhibitor for 5 or 10 minutes. Cells were then fixed and stained for F-actin. Data are expressed as the mean percentage of total cells forming dorsal ruffles from three independent experiments. (**C**) Endogenous N-WASP was immunoprecipitated from lysates of 2B *Gab1*$^{-/-}$ cells rescued with GFP-Gab1 stimulated or not with 5 nM EGF for the indicated time points, resolved by SDS-PAGE, transferred to nitrocellulose and probed as indicated. Lysates from 2B *Gab1*$^{-/-}$ cells were used as a control. (**D**) Lysates from HEK293 cells transfected with the indicated constructs were immunoprecipitated with anti-HA antibody, resolved by SDS-PAGE, transferred to nitrocellulose and probed as indicated. (**E**) MDCK-GFP-Gab1 were stimulated or not with 0.5 nM HGF for 5 minutes, fixed and stained for endogenous N-WASP. Scale bars: 10 μm.

al., 1993), receptor internalisation (Orth et al., 2006) and actin remodelling involved in cell migration (Krueger et al., 2003; Legg et al., 2007; Ruusala et al., 2008; Suetsugu et al., 2003). Several signalling pathways have been implicated in the formation of dorsal ruffles downstream from RTKs, including PI3K (Dharmawardhane et al., 2000; Scaife and Langdon, 2000) and Rac (Palamidessi et al., 2008) in addition to the actin-binding and actin-nucleating proteins, cortactin and N-WASP (Krueger et al., 2003; Legg et al.,

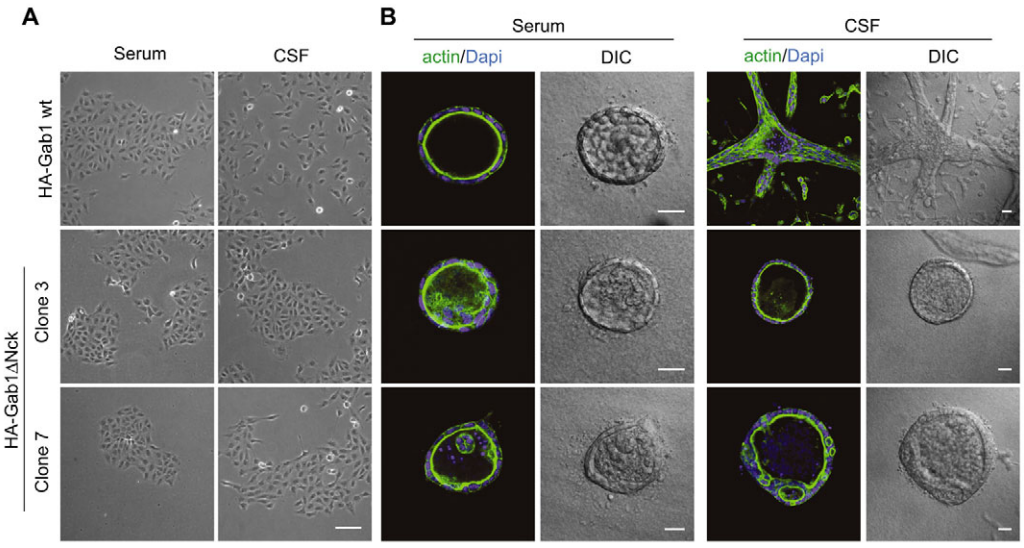

Fig. 9. The Gab1-Nck interaction is required for Met-dependent epithelial cell migration and morphogenesis. (**A**) MDCK CSF-MetΔGrb2 cells stably expressing wt HA-Gab1 or HA-Gab1ΔNck were stimulated with 25 ng/ml CSF-1 for 12 hours, fixed and phase images (10×) were taken. Representative images are shown for two out of three clones from three independent experiments. Scale bar: 100 µm. (**B**) Cells as in A were seeded into a 3D collagen matrix, allowed to form cysts (2 weeks), stimulated with CSF-1 (50 ng/ml) for 1 week, fixed and stained for F-actin (green) and DAPI. Representative images from three independent experiments are shown. Scale bars: 20 µm.

2007). Several RTKs induce the formation of dorsal ruffles, including Met, EGF, PDGFβ and VEGF RTKs (Buccione et al., 2004; Dowrick et al., 1993; Krueger et al., 2003; Orth et al., 2006; Wu et al., 2003). Gab1 is recruited to each of these RTKs, where it participates in the activation of downstream signalling pathways, including PI3K and Rac (Dance et al., 2006; Gu and Neel, 2003; Jackson et al., 2004; Laramee et al., 2007; Maroun et al., 1999a; Rakhit et al., 2000; Rodrigues et al., 2000). Several lines of evidence support a requirement for Gab1 in RTK-induced dorsal ruffles: using siRNA to knockdown Gab1 and *Gab1*$^{-/-}$ MEF cells, we show that Gab1 is required for dorsal ruffle formation downstream from the Met, EGF and PDGFβ RTKs (Figs 2 and 3). Overexpression of Gab1, also substantially enhances the formation of dorsal ruffles in response to RTK activation in both sheets of MDCK epithelial cells, as well in HeLa cells (Fig. 2B,C).

From structure-function studies we have identified Nck adaptor molecules as well as N-WASP, as Gab1-interacting proteins required for formation of RTK-induced, Gab1-dependent dorsal ruffles (Figs 2, 3, 6, 8). The specific uncoupling of Nck from Y407 in Gab1 abrogates the ability of Gab1 to rescue EGF and PDGF-bb-induced dorsal ruffles in *Gab1*$^{-/-}$ cells (Fig. 6C) and Gab1-dependent HGF-induced dorsal ruffles in MDCK cells (Fig. 6B). Consistent with this, Nck proteins are required for PDGF-bb-induced dorsal ruffles in fibroblasts (Rivera et al., 2006; Ruusala et al., 2008). Although Nck proteins have been identified in a complex with activated EGF and PDGFβ RTKs (Chen et al., 2000; Hake et al., 2008; Ruusala et al., 2008), the mechanism of Nck recruitment to a PDGFβ RTK complex does not appear direct, and the p130Cas scaffold was identified as a predominant Nck phosphotyrosine-binding protein in NIH-3T3 cells in response to PDGF-bb (Rivera et al., 2006).

Three consensus-binding sites (YD/EXP) for the Nck1 and Nck2 SH2 domains were identified in Gab1 by sequence prediction (Y242, Y307, Y407) and confirmed as Nck1 SH2-domain binding sites, by far-western analyses (supplementary material Fig. S3D). However, only substitution of Y407, for phenylalanine, specifically uncouples recruitment of Nck1 and Nck2 to Gab1 following Met activation (Fig. 5D) and abrogates dorsal ruffle formation (Fig. 6A,B). This apparent heterogeneity in Nck binding, might, in part, reflect preferential tyrosine phosphorylation of Y407 by RTKs in vivo, whereas Y242 and Y307 are preferentially phosphorylated by activated Src (Chan et al., 2009). Moreover, Y242, Y307, Y407 represent only three of the six binding sites for Crk proteins on Gab1 (Lamorte et al., 2000) (Fig. 5A) and following overexpression, CrkII can compete with Nck for binding to Gab1 (Fig. 4E; supplementary material Fig. S4C,D), possibly restricting the preferential recruitment of Nck to Y407 (Fig. 4E). A similar recruitment of Nck and Crk adaptor proteins to overlapping sites is observed on p130Cas, following activation of the PDGFβ RTK (Rivera et al., 2006), although the binding preferences for each adaptor have not been resolved by structure-function studies. Interestingly, p130Cas-null fibroblasts show attenuated dorsal ruffle formation in response to PDGF-bb and recent evidence supports a role for both Nck and Crk adaptor proteins downstream from p130Cas (Antoku and Mayer, 2009; Rivera et al., 2006). However, in the case of Gab1, loss of the single Nck-binding site, which does not prevent Crk recruitment (supplementary material Fig. S4C), is sufficient to abrogate Gab1-enhanced dorsal ruffle formation in MDCK and fibroblast cells (Fig. 6B,C; supplementary material Fig. S3C). Furthermore, knockdown of CrkI or CrkII by siRNA does not ablate HGF-induced dorsal ruffles in epithelial cells and overexpression of Crk proteins abrogates their formation (Fig. 4E). Hence, we conclude that recruitment of Crk to Gab1 is not essential for Met, EGF or PDGFβ RTK-induced dorsal ruffle formation (Fig. 6A-C). Although both Gab1 and p130Cas are tyrosine phosphorylated downstream from all three RTKs, only the Gab1 scaffold is recruited to these RTKs at the plasma membrane following activation (Lock et al., 2003; Rakhit et al., 2000; Rodrigues et al., 2000; Weidner et al., 1996). Hence, Gab1 would therefore represent a mechanism whereby Nck molecules are recruited to and engage with several RTK signalling complexes at the plasma membrane.

Gab1 also participates in the activation of downstream signalling pathways, including PI3K and Rac pathways (Dance et al., 2006; Gu and Neel, 2003; Jackson et al., 2004; Laramee et al., 2007; Maroun et al., 1999a; Rakhit et al., 2000; Rodrigues et al., 2000). Activation of both PI3K and Rac is required for dorsal ruffle formation downstream from PDGF (Vidali et al., 2006) and the Met RTK (Palamidessi et al., 2008). A role for Crk adaptor proteins in Rac activation downstream from the Met RTK has been established (Lamorte et al., 2003; Lamorte et al., 2002b; Watanabe et al., 2006).

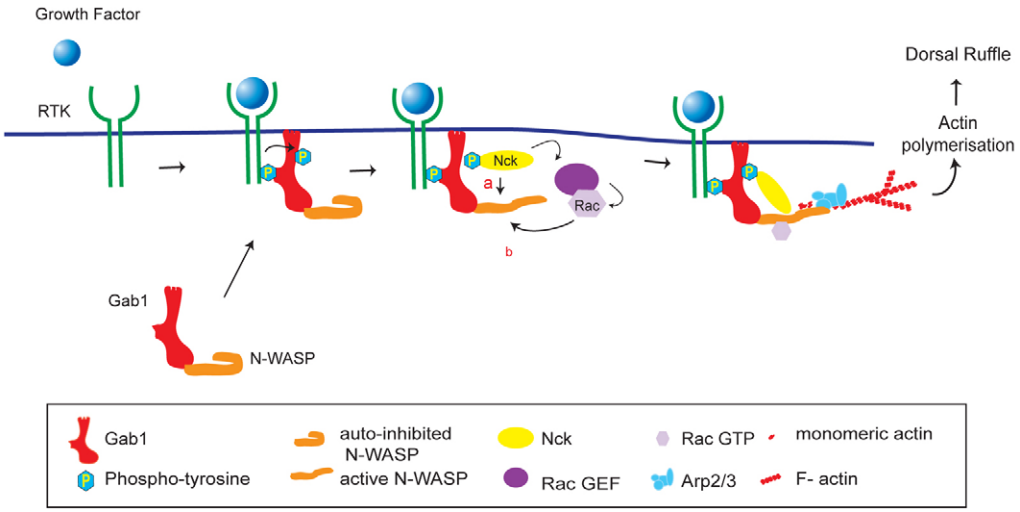

Fig. 10. Model for Gab1-mediated dorsal ruffle formation. Growth-factor-induced RTK activation results in recruitment of a Gab1-N-WASP complex to the membrane. Gab1 is subsequently phosphorylated at Y407, creating a docking site for Nck proteins. The ensuing complex promotes Nck-dependent activation of N-WASP by relieving the auto-inhibited conformation of N-WASP through either or both of binding of Nck-SH3 domains to N-WASP (a) and activation of Rac (b), which can subsequently bind to and activate N-WASP. Locally activated N-WASP recruits the Apr2/3 complex, resulting in actin polymerisation and the formation of a dorsal ruffle.

However, MDCK cells expressing the Gab1ΔNck mutant, that retains robust Crk binding, show a significant decrease in HGF-induced Rac activation (>60%), which is not further diminished by removal of Crk (Fig. 7A-B), implicating a Gab1-Nck interaction in Met-dependent Rac activation. Nck proteins can lead to activation of Rac by indirect recruitment of the Rac GEFs α-PIX and β-PIX via Pak1 (ten Klooster et al., 2006; Yoshii et al., 1999), or in the case of Nck2, by binding directly to the Rac GEF Dock180 (Tu et al., 2001). Interestingly, Rac-GTP can activate N-WASP in vitro (Tomasevic et al., 2007); hence, Gab1-Nck-dependent Rac activation might act as a mechanism for activation of N-WASP (Fig. 10). However, uncoupling Nck from Gab1 does not completely abrogate HGF-induced Rac activation when compared with wt MDCK cells (Fig. 7), indicating that alternative mechanisms for Rac activation probably exist. PI3K activity also contributes to Gab1-dependent Rac activation (Royal et al., 2000), possibly through recruitment of Rac GEFs, such as Dock180, to PIP_3 at the plasma membrane (Cote et al., 2005). Moreover, Gab1-dependent recruitment of the p85 subunit of PI3K is also required for formation of EGF- and HGF-induced dorsal ruffles under serum-free conditions (Fig. 4D; supplementary material Fig. S2C). Although PI3K is required for Rac activation downstream from the PDGFβR (Tomas et al., 2001), recruitment of p85 to Gab1 downstream from the PDGFβR is dispensable (Fig. 4D). This is consistent with the PDGFβR directly and robustly recruiting p85 (Kashishian et al., 1992), and supports the finding that the tyrosines at the p85 binding site in Gab1 are not highly phosphorylated downstream from PDGF-bb (Kallin et al., 2004). Indeed, PDGFβRs with mutations in the p85 binding sites are unable to induce dorsal ruffles (Wennstrom et al., 1994). The ability of Gab1-p85 interactions to promote localised activation of PI3K (Maroun et al., 1999a; Rodrigues et al., 2000) would also lead to further accumulation of PIP_3, and hence positively regulate localisation of Gab1 molecules in the dorsal membrane. Consistent with this, a Gab1 protein lacking the PH domain, although recruited to and phosphorylated by Met, fails to induce dorsal ruffles (Fig. S2A,D).

The ability of Nck adaptors to engage regulators of the actin cytoskeleton probably represents an essential function for Nck in recruitment to the Gab1 complex. One of these includes the actin -nucleating protein N-WASP (Buday et al., 2002). We have demonstrated a requirement for N-WASP in HGF-, EGF- and PDGF-bb-induced dorsal ruffles, either following siRNA knockdown or through use of the N-WASP inhibitor Wiskostatin, (Fig. 8A-B), consistent with previous studies for the PDGFβ RTK (Legg et al., 2007). Nck proteins can bind directly to N-WASP in an SH3-dependent manner and relieve N-WASP auto-inhibition (Rohatgi et al., 2001) and as such, are potent activators of N-WASP-mediated Arp2/3 actin polymerisation in vitro (Tomasevic et al., 2007). In contrast to a model whereby Nck acts to recruit N-WASP to Gab1, we identify a constitutive association between Gab1 and endogenous N-WASP, which is not detectably increased following HGF stimulation (Fig. 8D). The enhanced formation of dorsal ruffles observed upon overexpression of Gab1 in MDCK cells might be explained by increased local clustering of Nck and N-WASP molecules, which is associated with robust actin polymerisation (Blasutig et al., 2008; Chen et al., 2000; Rivera et al., 2004). Together, these data support a model whereby upon activation of RTKs, recruitment of Nck through its SH2 domain to Y407 on Gab1, facilitates the interaction between Nck SH3 domains and N-WASP, relieving the auto-inhibition of N-WASP (Fig. 10). Localised activation of N-WASP at the plasma membrane would subsequently promote localised actin polymerisation and the induction of a dorsal ruffle. Consistent with this, Gab1 and N-WASP localise predominantly to the cytoplasm in unstimulated cells, but are recruited to the plasma membrane, specifically within the dorsal ruffle, following HGF stimulation (Fig. 8E; supplementary material Fig. S5E).

A biological function for dorsal ruffles has remained elusive. In support of a role for dorsal ruffle formation in cell migration and morphogenesis, through rapid reorganisation of the actin cytoskeleton, the Gab1ΔNck mutant abrogated Met-dependent cell migration and branching tubulogenesis in MDCK cells (Fig. 9A,B).

Furthermore, the inhibition of dorsal ruffle formation with the anion transporter inhibitor SITS also inhibits scatter of epithelial colonies in response to HGF (Dowrick et al., 1993). The ability to form dorsal ruffles has also been correlated with the amoeboid-to-mesenchymal mode of cell motility in a 3D matrix (Palamidessi et al., 2008). Since dorsal ruffles occur rapidly and transiently upon HGF stimulation, these data imply that dorsal ruffles are an early, prerequisite event in the signalling pathways, leading to a morphogenic response.

Our findings describe a novel role for the Gab1 scaffold protein in growth-factor-induced formation of dorsal ruffles, and implicates Gab1 as a key scaffold that integrates RTKs with signals that lead to actin polymerisation involving Nck and N-WASP. Although Nck proteins are well-established regulators of the actin cytoskeleton, how these proteins couple to activated RTKs and localise to spatially restricted plasma membrane microdomains, was not mechanistically understood. The identification of a Gab1-Nck complex now provides a direct and common mechanism whereby activated RTKs recruit the spatially restricted signals required for actin nucleation and dorsal ruffle formation.

Materials and Methods

Reagents and antibodies

The anti-Met antibody 147 has previously been described (Maroun et al., 1999a; Rodrigues et al., 1991). Commercial antibodies were purchased as follows: Gab1, Upstate Biotechnology; actin, CrkL, N-WASP and polyclonal myc, Santa Cruz Biotechnology, pY627 Gab1, Cell Signaling; GFP, phalloidin Alexa Fluor 488 and 546, and Alexa Fluor 488- and 555-conjugated secondary antibodies, Molecular Probes; HA.11 monoclonal, Covance; CrkI/II and pan-Nck, BD Biosciences. The Nck1 and Nck2 rabbit polyclonal antibodies were a generous gift from Louise Larose (Lussier and Larose, 1997). *Gab1* siRNA duplexes (CAGAUGUCUUGGAA-UACUA; CGAACAUUUCCAGAAGGAA; GAGCGAACUGAUUCACAAA), CrkI/II siRNA (AACCATGCTATCAAATGGCAA) and scrambled siRNA duplex (AllStars negative control 1027281) were purchased from Qiagen. siRNA duplexes against murine *Nck1* (siGENOME 042187 duplexes 01 to 04) and *Wasl* (N-WASP) (siGENOME 055607 duplexes 01 to 03) were purchased from Thermo Scientific. HGF and recombinant CSF-1 were a generous gift from Genentech. EGF was purchased from Roche Diagnostics; PDGF-bb and Wiskostation from Calbiochem; U0126 inhibitor from Promega; LY294002 from BIOMOL Research Labs; and wortmannin was from Sigma.

Plasmids and mutagenesis

The following constructs were described previously: pcDNA1.1 pcDNA1.1-HA-Gab1, pcDNA1.1-HA-Gab1ΔPH, C2eGFP-Gab1 (Maroun et al., 1999b). pRK5-HA-Nck1 and Nck2 (Chen et al., 1998) and eGFP-Nck1-C1 and eGFP-Nck2-C1 constructs (Latreille and Larose, 2006) were kindly provided by Louise Larose (McGill University, Montreal, Canada). GFP-N-WASP was a generous gift from Mark McNiven (Mayo Cancer Center, Rochester, MI). GST-PBD was generously provided by Nathalie Lamarche (McGill University, Montreal, Canada). GFP-Gab1-C2 constructs were subcloned into the *Xho*I and *Eco*RI sites of pMSCV-puro (Clontech) using the *Nhe*I and *Eco*RI sites. Site-directed mutagenesis in *Gab1* was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene). HA and GFP-Gab1 mutants were generated using the following primers: Gab1Y407F, 5′-CGGAA-AGATGCTAGCTCTCAAGATTGCTTTGATATTCCACGGACC-3′; Gab1Y407R, 5′-GGTCCGTGGAATATCAAAGCAATCTTGAGAGCTAGCATCTTTCCG-3′; Gab1Y242,307F, 5′-GCAACAAATGATGTTTGACTGCCCGCCGTGCCGGCT-GAC-3′ and 5′-CGCAGATGAGACATGTATCGATCAGTTTCGACATTCCGC-CAAC-3′.

Cell culture and transfections

Madin-Darby canine kidney (MDCK), HeLa and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MDCK cell lines expressing GFP-Gab1 (B1-3), GFP-Gab1ΔPH, HA-Gab1(7d6), HA-Gab1ΔCrk (A2, D1 and F1), HA-Gab1ΔShp2 (6B, 8B and 9A) and HA-Gab1Δp85 (B9, B10 and C8) have been described previously (Frigault et al., 2008; Lamorte et al., 2002; Maroun et al., 1999a; Maroun et al., 2000). All MDCK stable cell lines were established through transfection with Superfect (Qiagen) and maintained under antibiotic selection. Transient transfections of siRNA duplexes in MDCK cells were performed using Hiperfect (Qiagen). Cells underwent two rounds of transfection; experiments were performed 96 hours after initial transfection. For siRNA transfections in fibroblasts, cells were transfected in suspension using Hiperfect and 48 hours later, cells were serum starved for 24 hours and then stimulated with EGF and PDGF. Transient transfection of HeLa and HEK293 cells was performed using Lipofectamine Plus (Invitrogen) reagent. Wild-type and *Gab1*-null MEF cells were a generous gift from M. Holgado-Madruga (Holgado-Madruga and Wong, 2003). These were cultured under the same conditions as described above in the presence of 4 μg/ml G418.

Retroviral production and infection

Retroviruses were produced in VSVG-293 cells by co-transfection with vPak-VGV helper plasmid (Burns et al., 1993) and the retroviral expression vectors pMSCV-GFP-Gab1 and Gab1 mutants. The supernatant was collected 48 hours after transfection for 5 days, pooled and applied on cells at an equivalent titre with 8 μg/ml polybrene (Roche). Plates were centrifuged at 300 ***g*** for 30 minutes. Virus was removed 48 hours after infection and stably infected cells were established by selection with 2 μg/ml puromycin (Sigma). All resistant clones were pooled.

Far western blots

GST, GST-Nck1 full-length and GST-Nck1 SH2 constructs and their production have been described previously (Lussier and Larose, 1997). GFP-Gab1 constructs were immunoprecipitated with antibody against GFP, and resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were denatured and renatured, blocked as described (Wu et al., 2007) and probed overnight at 4°C with ^{32}P-labelled GST or ^{32}P-labelled GST-Nck (specific activity 2×10^8 c.p.m./ml, in binding buffer: 0.1% glycerol, 0.01 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Tween-20, 0.2% milk powder, 1 mM DTT) prepared according to the manufacturer's recommendations (pGEX2TK; Pharmacia). At the end of the incubation period, the membranes were extensively washed in Tris-buffered saline containing 0.1% Triton X-100 and exposed for autoradiography.

Growth factor stimulation and confocal immunofluorescence microscopy

MDCK and HeLa cells were seeded on glass coverslips (Bellco Glass, Vineland, NJ) and stimulated with 0.5 nM HGF or 5 nM EGF 48 hours later for 5 minutes. Wild-type and *Gab1*-null MEF (*Gab1*$^{-/-}$) cells were seeded on glass coverslips and 24 hours later were serum starved for 24 hours before stimulation with 5 nM EGF or 11 nM PDGF-bb for 5 minutes. Coverslips were washed twice with PBS and fixed with 2% paraformaldehyde (PFA, Fisher Scientific). Staining procedures and image analysis have previously been described (Abella et al., 2005). Confocal live-cell imaging was performed on a WaveFX spinning disk (Quorum Technologies, Guelph, ON) mounted on a motorised microscope (Leica, Wetzlar, Germany) using an EM-CCD camera (Hamamatsu, Shizuoka, Japan). Each time frame represents the maximum intensity projection from 25 *z*-planes acquired with 0.5 μm intervals using a 63× objective. Data was analysed using Volocity 4.1 and MetaMorph software. For ratiometric analysis, images were normalised and ratio images were calculated by division using MetaMorph Arithmetic. A multiplier was used to generate the ratio images with only integer values. The scale bar corresponds to the brightness of GFP relative to that of the membrane marker FM4-64.

Dorsal ruffle assays

Cells plated on coverslips were stimulated with 0.5 nM HGF, 5 nM EGF or 11 nM PDGF for 5 minutes, fixed in 2% PFA and stained with Phalloidin Alexa Fluor 488 or -545 and DAPI. Dorsal ruffles were counted in at least eight fields of view (40× objective) per experiment and represented as a percentage of the total number of cells counted. For inhibitor experiments, cells were pretreated with DMSO, UO126 (20 μM), LY29002 (20 μM) or wortmannin (0.2 μM) for 30 minutes or Wiskostatin (25 μM) or DMSO control for 15 minutes, stimulated with 0.5 nM HGF for 5 minutes.

Immunoprecipitation, western blotting and Rac assays

HeLa or MDCK cells were harvested in TGH lysis buffer and immunoprecipitation and western blot procedures were followed as previously described (Abella et al., 2005). Densitometric analysis of western blots was performed using NIH ImageJ software. Rac assays were performed using the p21-binding domain of Pak1 (GST-PBD) coupled to glutathione Sepharose beads. MDCK cells were plated 48 hours before stimulation with 0.5 nM HGF (5 minutes) in the presence of serum. Assays were performed as previously described (Picard et al., 2009). HGF-induced Rac activation was determined by normalising GST-PBD-associated Rac with the corresponding total endogenous Rac levels and then represented as fold induction over non-stimulated samples.

Collagen and scatter assays

MDCK were seeded in collagen as previously described (Paliouras et al., 2009). Subsequently, cells were washed three times with 1× phosphate-buffered saline (PBS), fixed with fresh 4% PFA for 20 minutes, and washed extensively in PBS. Slices of the collagen culture were incubated with Alexa Fluor 546 Phalloidin for 2 hours and a LSCM (Confocal LSM 510, Carl Zeiss) was used to acquire DIC, Alexa Fluor 488 and DAPI images. Scatter assays have previously been described (Paliouras et al., 2009).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/8/1306/DC1

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