The EMBO Journal (2005) 24, 3235–3246 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
www.embojournal.org

THE EMBO JOURNAL

Phosphorylation of EEA1 by p38 MAP kinase regulates μ opioid receptor endocytosis

Gaëtane Macé[1,2,4], Marta Miaczynska[3,5], Marino Zerial[3] and Angel R Nebreda[1,2,*]

[1]European Molecular Biology Laboratory, Heidelberg, Germany, [2]CNIO (Spanish National Cancer Center), Madrid, Spain and [3]Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany

Morphine analgesic properties and side effects such as tolerance are mediated by the μ opioid receptor (MOR) whose endocytosis is considered of primary importance for opioid pharmacological effects. Here, we show that p38 mitogen-activated protein kinase (MAPK) activation is required for MOR endocytosis and sufficient to trigger its constitutive internalization in the absence of agonist. Further studies established a functional link between p38 MAPK and the small GTPase Rab5, a key regulator of endocytosis. Expression of an activated mutant of Rab5 stimulated endocytosis of MOR ligand-independently in wild-type but not in p38α−/− cells. We found that p38α can phosphorylate the Rab5 effectors EEA1 and Rabenosyn-5 on Thr-1392 and Ser-215, respectively, and these phosphorylation events regulate the recruitment of EEA1 and Rabenosyn-5 to membranes. Moreover, phosphomimetic mutation of Thr-1392 in EEA1 can bypass the requirement for p38α in MOR endocytosis. Our results highlight a novel mechanism whereby p38 MAPK regulates receptor endocytosis under physiological conditions via phosphorylation of Rab5 effectors.

The EMBO Journal (2005) **24,** 3235–3246. doi:10.1038/sj.emboj.7600799; Published online 1 September 2005
Subject Categories: membranes & transport; signal transduction
Keywords: EEA1; endocytosis; MAP kinase; Rab5; signalling

Introduction

Despite its powerful analgesic properties, morphine is almost only administrated in cases of intensive pain because of the development of adverse side effects, including respiratory depression, tolerance and dependence. Morphine acts through a seven-transmembrane G protein-coupled receptor (GPCR), called μ opioid receptor (MOR) (Wang *et al*, 1994). Studies using opioid receptor knockout mice have demonstrated that MOR mediates all morphine biological effects, both beneficial and adverse (Matthes *et al*, 1996; Kieffer, 1999).

Agonist stimulation of MOR induces the activation of Gi/o proteins, including signals responsible for the analgesic effect, and can also trigger receptor internalization (Sternini *et al*, 1996; Law *et al*, 2000), a process requiring G-protein-receptor kinase(s) (GRK)-mediated phosphorylation of the receptor and recruitment of β-arrestin2 protein. The receptor is endocytosed via clathrin-coated vesicles and transported to early endosomes (Keith *et al*, 1998; Ferguson, 2001). Two mechanisms have been envisaged to explain a possible role of MOR endocytosis in tolerance to morphine, defined as the decrease in drug efficacy with time (reviewed by Kieffer and Evans, 2002). Tolerance could be caused by a reduction in the number of MORs at the plasma membrane. Alternatively, MOR endocytosis could be a protective process against the development of tolerance, which would be consistent with the failure of morphine to trigger MOR endocytosis despite potently inducing tolerance *in vivo* (Finn and Whistler, 2001; He *et al*, 2002). The regulation of MOR endocytosis is, therefore, of primary importance for the understanding of opioid pharmacological effects.

Endocytic trafficking of plasma membrane receptors is regulated by monomeric small GTPases of the Rab family (Zerial and McBride, 2001). Within this family, Rab5 is involved in endocytosis of several receptors, including GPCRs (reviewed by Seachrist and Ferguson, 2003). Rab5 coordinates multiple processes, such as the formation of clathrin-coated vesicles, their fusion with early endosomes and homotypic early endosome fusion, as well as motility of endosomes along microtubules (Zerial and McBride, 2001). As for other Rab GTPases, the activity of Rab5 is regulated via two overlapping cycles. First, Rab5 shuttles between the cytosol and the membrane, chaperoned by Rab GDP dissociation inhibitor (GDI) (Sasaki *et al*, 1990; Pfeffer *et al*, 1995). Specific delivery to the membrane is catalyzed by GDI displacement factors that promote the dissociation of the Rab–GDI complex (Sivars *et al*, 2003). Second, upon membrane delivery, Rab5 oscillates between the GDP-bound (inactive) and GTP-bound (active) forms (Rybin *et al*, 1996) regulated by GDP/GTP exchange factors (GEF) (Horiuchi *et al*, 1997) and GTPase-activating proteins (GAP) (Lanzetti *et al*, 2000).

The fraction of GTP-bound Rab5 on the membrane is thus rate limiting for endosome dynamics (Rybin *et al*, 1996). This activity is exerted through the interaction with effector proteins, including Rabaptin-5, phosphatidylinositol 3-kinases (PI3-K; VPS34/p150 and p85α/p110β), EEA1 and Rabenosyn-5 (Zerial and McBride, 2001). EEA1, one of the best-characterized Rab5 effectors, is involved in tethering/docking and fusion of early endosomes (Mills *et al*, 1998; Simonsen *et al*, 1998; Christoforidis *et al*, 1999b). EEA1 displays a complex modular architecture consisting of an N-terminal C_2H_2 zinc-finger, which includes a Rab5-binding site, four heptad repeats and a C-terminal region containing

*Corresponding author. CNIO (Spanish National Cancer Center), Melchor Fernández Almagro 3, 28029 Madrid, Spain.
Tel.: +34 91 7328038; Fax: +34 91 7328033;
E-mail: anebreda@cnio.es
[4]Present address: Institut Gustave Roussy-CNRS UPR2169, 94805 Villejuif Cedex, France
[5]Present address: International Institute of Molecular and Cell Biology, Ks. Trojdena 4, 02-109 Warsaw, Poland

Received: 9 March 2005; accepted: 8 August 2005; published online: 1 September 2005

a calmodulin-binding motif (IQ), a second Rab5-binding site and the C-terminal FYVE domain that specifically binds to phosphatidylinositol 3-phosphate (PI(3)P) (Mu *et al*, 1995; Patki *et al*, 1997; Simonsen *et al*, 1998). Rab5 plays a dual role in the recruitment of EEA1, by direct interaction with the N- and C-terminal binding sites as well as by association with VPS34, the PI3-K that generates PI(3)P (Christoforidis *et al*, 1999b). The mechanism of membrane recruitment of EEA1 is shared by Rabenosyn-5, which also contains a Rab5-binding domain and PI(3)P-binding FYVE finger (Nielsen *et al*, 2000). EEA1 and Rabenosyn-5 are both necessary and play complementary roles in Rab5-dependent endosome tethering and fusion (Nielsen *et al*, 2000).

Other recently established regulators of endocytosis are the p38 mitogen-activated protein kinases (MAPK), a family of Ser-Thr kinases that can regulate numerous cellular responses (reviewed by Nebreda and Porras, 2000). The first evidence for a regulatory role of this signalling pathway in endocytosis was provided by the finding that, in the stress-induced response, p38α can phosphorylate Rab GDI, enhancing its activity in retrieving Rab5 from the membrane, with the consequent loss of EEA1 from early endosomes (Cavalli *et al*, 2001). Endocytosis of AMPA receptors as well as phagolysosome biogenesis has been shown to be modulated by p38α (Fratti *et al*, 2003; Huang *et al*, 2004). In this study, we discovered a new mechanistic role for p38α MAPK in endocytosis, centered on the regulation of membrane recruitment of Rab5 effectors.

Results

MOR endocytosis requires p38α MAPK

As long-term exposure to morphine has been reported to correlate with p38 MAPK activation (Ma *et al*, 2001; Singhal *et al*, 2002), we investigated the function of this signalling pathway in MOR endocytosis. We established an HEK293 cell line expressing GFP-tagged MOR. These cells were stimulated by Damgo ([Tyr-DAla-Gly-MePhe-Gly-ol]enkephalin), a specific agonist of MOR, and activation of p38 MAPKs was examined by Western blotting using phospho-specific antibodies. In agreement with the absence of detectable endogenous MOR in these cells (data not shown), we found that Damgo was only able to induce p38 MAPK phosphorylation in HEK293 cells expressing GFP-MOR (Figure 1A). In contrast, p38 phosphorylation induced by UV treatment was independent of GFP-MOR expression. By immunoprecipitation followed by *in vitro* kinase assay, we confirmed that Damgo induced p38α MAPK activation (Figure 1B). The activation of p38α by Damgo was rapid and transient, with the kinase activity peaking after 5 min of stimulation. Quantitative assessment indicated that p38α activity levels upon Damgo stimulation were 8–16% of those obtained in UV-treated cells.

MOR endocytosis was investigated in GFP-MOR-expressing HEK293 cells by biotinylation of intact cells after Damgo stimulation, followed by the purification of biotinylated membrane proteins using avidin beads. In this assay, we detected a decrease in the amount of GFP-MOR associated with plasma membrane after 30 min of Damgo stimulation (Figure 1C), which was not due to degradation of the MOR receptor (Supplementary Figure S1). MOR downregulation was confirmed by radioligand binding assays using ^{3}H-labelled Damgo, which showed an ~40% decrease in Damgo-specific plasma membrane binding sites after 30 min of stimulation (see below).

Given the Damgo-induced p38 MAPK activation, we investigated the requirement for p38 MAPK activity in MOR internalization. First, the p38α and p38β inhibitor SB203580 strongly impaired the internalization of GFP-MOR in Damgo-stimulated cells (Figure 1C). Second, we used mouse embryonic fibroblasts (MEFs) from p38α−/− mice, which lack the most abundant p38 MAPK family member (Adams *et al*, 2000). In agreement with previous studies (Nitsche and Pintar, 2003), endogenous MOR was expressed in wild-type (wt) MEFs and only slightly reduced in p38α−/− MEFs (Figure 1F), as detected by Western blotting and radioligand binding assays (Figure 1D, compare lanes 1 and 4). MEFs (wt and p38α−/−) were incubated with Damgo and plasma membrane expression of MOR was quantified in radioligand binding assays. Damgo stimulation decreased by about 70% the number of surface Damgo-binding sites in wt MEFs. In contrast, Damgo failed to induce any detectable endocytosis of endogenous MOR in p38α−/− MEFs, whereas expression of exogenous p38α in these cells restored endocytosis to about the same level as in wt cells (Figure 1D). Using primary MEFs derived from three different sets of littermate embryos, we confirmed that Damgo induced MOR internalization in wt but not in p38α−/− cells (data not shown). Altogether, the results demonstrate that Damgo-induced MOR endocytosis requires p38α MAPK.

Specific activation of p38 MAPKs induces constitutive MOR endocytosis

Having shown the requirement for p38 MAPK, we next investigated whether its activation was sufficient to induce MOR endocytosis. HEK293 cells expressing GFP-MOR were transfected to coexpress MKK6DD, a constitutively active form of the p38 MAPK activator MKK6 (Alonso *et al*, 2000). MKK6DD expression resulted in higher levels of p38 MAPK phosphorylation both in untreated and Damgo-stimulated cells, without affecting MOR expression (Figure 1E). Interestingly, MKK6DD-expressing cells showed reduced levels of GFP-MOR plasma membrane expression in the absence of Damgo stimulation (Figure 1C). Moreover, stimulation with Damgo for 30 min resulted in the almost complete loss of GFP-MOR plasma membrane in MKK6DD-expressing cells (Figure 1C). By radioligand binding assays, we confirmed that expression of MKK6DD reduced by 40–70% the number of Damgo-binding sites on the plasma membrane in wt MEFs (Figure 1D) and increased Damgo-induced MOR endocytosis from 50 to 90% in GFP-MOR-expressing HEK293 cells (Figure 2A, lanes 2 and 5). The level of p38 MAPK phosphorylation in HEK293 cells transfected with MKK6DD varied between experiments, but there was good correlation between MKK6DD-induced p38 MAPK phosphorylation and MOR endocytosis (Figure 1C and E versus Figure 2A and B).

To confirm the specificity of the above effect, we prevented the stimulatory activity of p38 MAPK using chemical inhibitors. Treatment of MKK6DD-expressing cells with SB203580 (Figure 1C) or PD169316 (data not shown) significantly increased the amount of GFP-MOR at the plasma membrane. Furthermore, expression of MKK6DD (Figure 1G) was unable to induce MOR endocytosis in p38α−/− MEFs (Figure 1D,

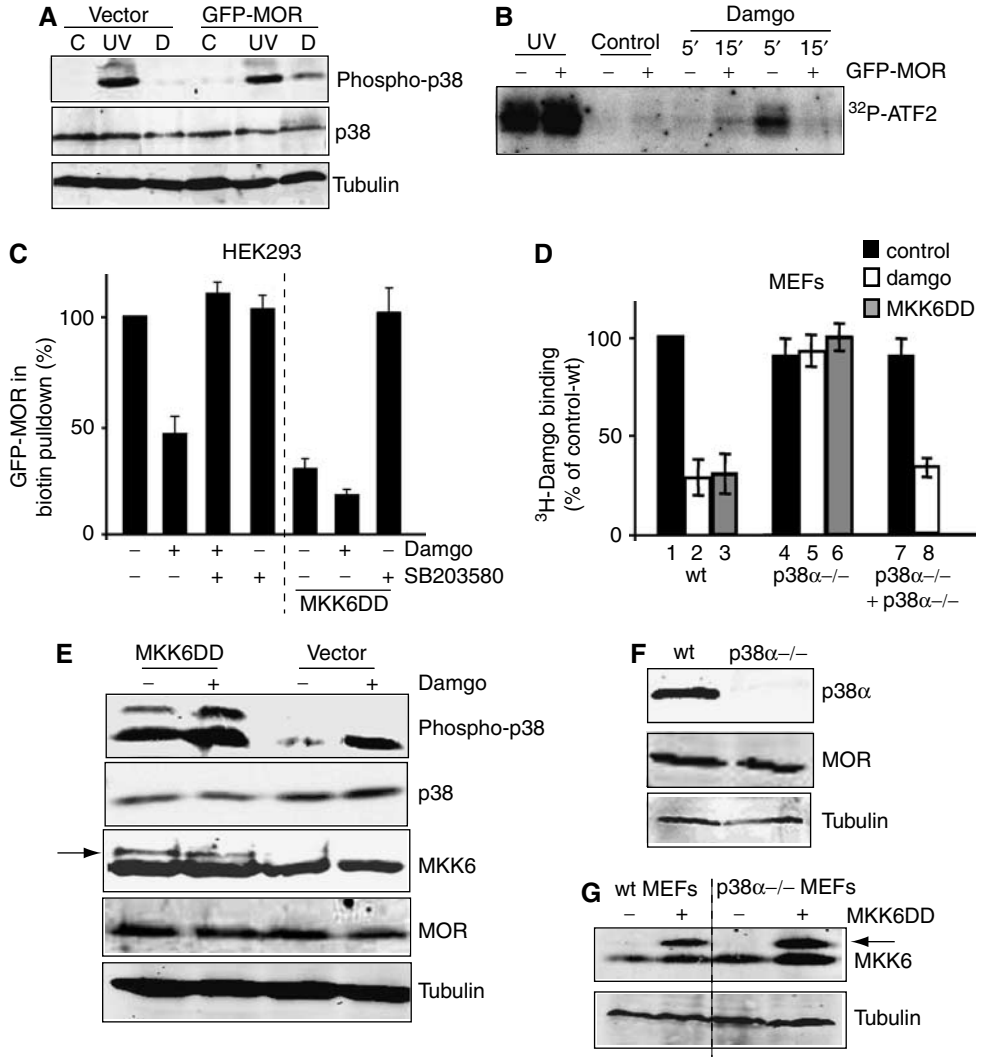

Figure 1 p38 MAPK activation is necessary and sufficient for MOR endocytosis. (**A**) Western blots of lysates from HEK293 cells transfected with GFP-MOR or the vector, either unstimulated (C) or after stimulation with Damgo for 5 min (D) or UV. (**B**) Kinase activity in p38α immunoprecipitates from HEK293 cells transfected or not with GFP-MOR (+ and −, respectively) and either left untreated (Control) or stimulated with UV or Damgo. (**C**) Plasma membrane-associated GFP-MOR in HEK293 cells, either expressing MKK6DD or incubated with Damgo for 30 min in the presence or absence of SB203580, as indicated. GFP-MOR was detected by avidin pulldown of biotinylated membrane proteins followed by Western blot using a GFP antibody. The histogram represents the quantification of the GFP-MOR band (pulldown versus total cell lysate) using the Odyssey Imaging system. The experiment was repeated three times. (**D**) Damgo binding assays in wt or p38α−/− MEFs and p38α−/− MEFs expressing exogenous p38α, either unstimulated, after 30 min Damgo stimulation or transfected with MKK6DD. Results are expressed as the percentage of binding in wt MEFs and represent the mean ± s.e.m. of six experiments. (**E**) Western blots of lysates from HEK293 cells expressing GFP-MOR alone (vector) or together with MKK6DD before (−) and after (+) 5 min of Damgo stimulation. The arrow indicates the overexpressed MKK6DD. (**F**) Western blots of lysates from wt and p38α−/− immortalized MEFs. (**G**) Western blots of lysates from wt and p38α−/− MEFs either transfected or not with MKK6DD (indicated by an arrow).

lane 6). These observations indicate that the constitutive endocytosis of MOR induced by MKK6DD is dependent on p38α activity.

Previous studies have reported that morphine is not able to trigger MOR endocytosis (Whistler and von Zastrow, 1998) and we obtained the same result in HEK293 cells expressing GFP-MOR (Figure 2A). We found that, in contrast to Damgo, short-term exposure to morphine was not able to induce p38 MAPK activation (Figure 2B). We next investigated whether constitutive activation of p38 MAPKs could overcome the inability of morphine to trigger MOR endocytosis. Interestingly, morphine-induced MOR endocytosis was stimulated by the activation of p38 MAPKs upon expression of MKK6DD. Quantification by radioligand binding assays showed that upon morphine stimulation up to 75% of GFP-MOR was endocytosed after 30 min in cells expressing MKK6DD (Figure 2A, lane 6). We next extended our results to the neuroblastoma cell line SH-SY5Y, which can differentiate into cells with a neuronal phenotype and express endogenous MOR. In these neuronal cells, p38 MAPK activation was required for Damgo-induced MOR endocytosis (Figure 2C). Moreover, treatment of SH-SY5Y cells with 50 μM H_2O_2, a stress that moderately activates p38 MAPK (Supplementary Figure S2), increased the rate of Damgo-induced MOR endocytosis and enabled also MOR endocytosis upon morphine treatment (Figure 2C). Taken together, our results show that constitutive activation of p38 MAPKs is sufficient to trigger MOR endocytosis, even in the absence of agonist stimulation or in the presence of morphine, which normally fails to induce MOR internalization.

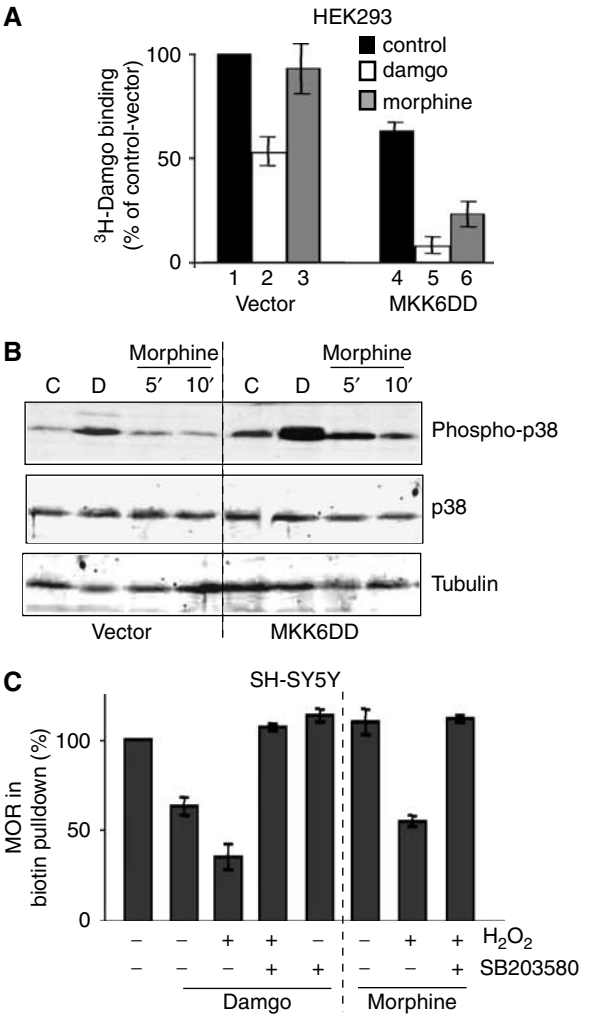

Figure 2 Specific activation of p38 MAPK stimulates morphine-induced MOR endocytosis. (**A**) Damgo binding assays in HEK293 cells expressing GFP-MOR alone (Vector) or together with MKK6DD, either unstimulated (Control) or after 30 min Damgo or morphine stimulation. The results are expressed as the percentage of binding in the untreated cells expressing GFP-MOR. The experiment was repeated three times. (**B**) Western blots of lysates from HEK293 cells expressing GFP-MOR alone or together with MKK6DD, nonstimulated (C) and stimulated with Damgo for 5 min (D) or with morphine for the indicated times. (**C**) Plasma membrane-associated levels of MOR in SH-SY5Y cells treated with Damgo or morphine, either alone or together with H_2O_2 in the presence or absence of SB203580, as indicated. MOR was visualized as in Figure 1C, but using a MOR antibody for the Western blot. The experiment was repeated twice.

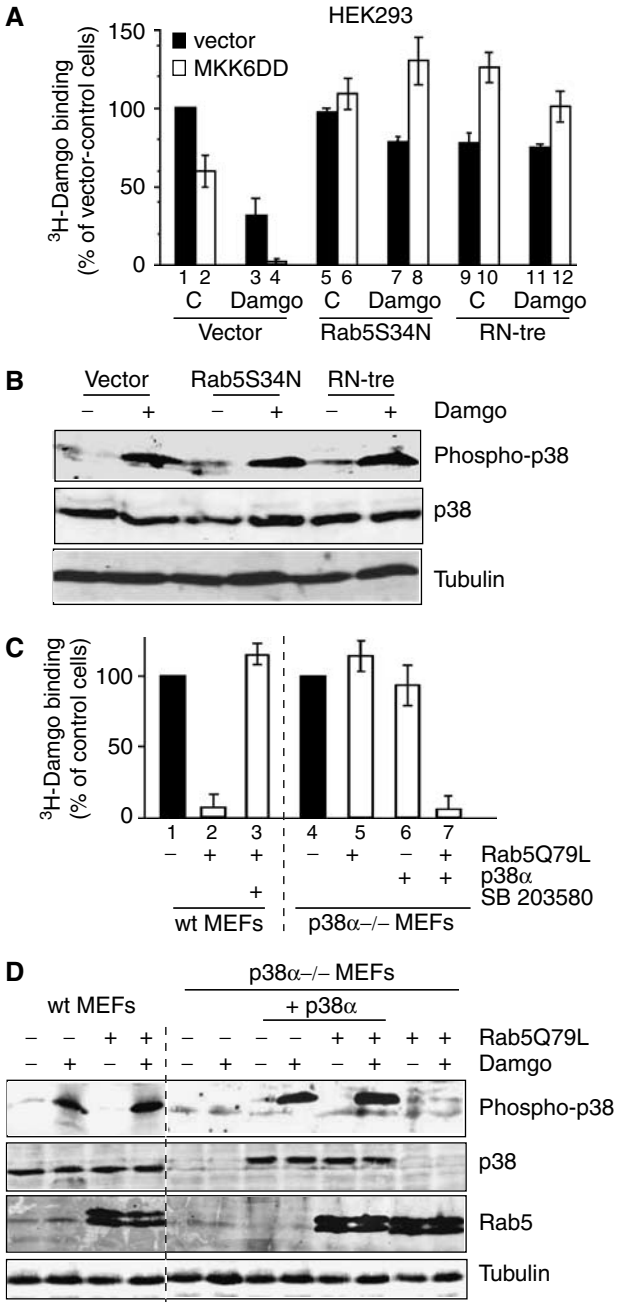

Figure 3 Rab5 is required for MOR endocytosis. (**A**) Damgo binding assays in HEK293 cells expressing GFP-MOR alone or together with MKK6DD and transfected with Rab5S34N or RN-tre before and after Damgo stimulation. The values are normalized to those of untreated GFP-MOR cells and are expressed as the mean ± s.e.m. of three experiments. (**B**) Western blots of lysates from HEK293 cells expressing GFP-MOR alone (Vector) or together with the indicated proteins, before (−) and after (+) Damgo stimulation for 5 min. (**C**) Damgo binding assays of wt and p38α−/− MEFs transfected with Rab5Q79L and myc-tagged p38α or the vectors alone and then incubated with SB203580, as indicated. The results are expressed as the percentage of binding in the corresponding control-vector cells. The experiment was repeated three times. (**D**) Western blots of lysates from wt and p38α−/− MEFs transfected with Rab5Q79L, myc-tagged p38α or the vectors alone, before and after Damgo stimulation for 5 min, as indicated.

Rab5 is involved in Damgo induced-MOR endocytosis

The small GTPase Rab5 is a key regulator of transport in the early endocytic pathway (Zerial and McBride, 2001) and previous work has shown that a dominant-negative Rab5 mutant (Rab5-N133I) restores membrane expression of a MOR mutant, which is normally not detected at the plasma membrane (Li *et al*, 2001). To determine whether p38 MAPK requires Rab5 activity in the regulation of MOR endocytosis, we transiently transfected HEK293 cells expressing GFP-MOR, either alone or together with MKK6DD, with the dominant-negative mutant Rab5S34N or with RN-tre, a Rab5 GAP (Lanzetti *et al*, 2000). We found that either Rab5S34N or RN-tre markedly inhibited the constitutive (triggered by MKK6DD) as well as the Damgo-induced GFP-MOR internalization (Figure 3A, compare lane 2 with lanes 6 and 10, and lanes 3 and 4 with lanes 7 and 8, and 11 and 12),

without affecting p38 MAPK activation (Figure 3B). These results implicated Rab5 in the stimulation of MOR internalization by p38α MAPK.

Can the constitutively active mutant Rab5Q79L bypass the requirement for p38 MAPK in Damgo-induced MOR internalization? The effect of Rab5Q79L on MOR endocytosis was compared in wt and p38α−/− MEFs (Figure 3C). Expression of Rab5Q79L significantly decreased the level of plasma membrane-associated MOR only in wt but not in p38α−/− cells (Figure 3C, compare lanes 2 and 5). The stimulation of MOR internalization observed in wt cells was abrogated upon treatment with SB203580 (Figure 3C, lane 3). Moreover, transfection of p38α into p38α−/− cells was able to rescue the ability of Rab5Q79L to induce constitutive (Damgo-independent) MOR endocytosis (Figure 3C, lane 7). Importantly, we did not observe any changes in the levels of p38 MAPK phosphorylation upon Rab5Q79L expression, either alone or in combination with Damgo (Figure 3D). Thus, the failure of Rab5Q79L to trigger MOR endocytosis in p38α−/− cells indicates an essential requirement for p38α in Rab5Q79L activity.

Regulation of EEA1 activity by p38α MAPK phosphorylation

Stress-induced activation of p38α can result in the phosphorylation of Rab GDI, enhancing its activity in retrieving Rab5 from the membrane (Cavalli *et al*, 2001). However, the phosphorylation of Rab GDI by p38 MAPK and the consequent acceleration in the cycle of Rab5 membrane association/dissociation cannot, at least single-handedly, explain the effects of p38 MAPK on MOR endocytosis. The observation that increased levels of Rab5:GTP cannot promote endocytosis in p38α−/− cells argues that downstream effectors or regulators of Rab5 may be phosphorylated by p38 MAPK. To test this hypothesis, we screened for substrates of p38α among the Rab5 effectors and regulators, including the Rabaptin-5–Rabex-5 complex, EEA1, Rabenosyn-5 and the GAP RN-tre (TrH domain) (Zerial and McBride, 2001). Indeed, we found that the C-terminus of EEA1 (residues 1257–1411) and the full-length Rabenosyn-5 are both phosphorylated by p38α MAPK (Figure 4A and E). To identify the p38α phosphorylation site on EEA1, we first mutated several Ser and Thr residues located in the C-terminus to Ala. Mutation of Thr-1392, which is a canonical Ser/Thr-Pro MAPK phosphorylation site, to Ala abolished p38α-mediated phosphorylation of the EEA1 C-terminus (Figure 4A), whereas analogous mutations of Ser-1394 and Ser-1395 did not affect phosphorylation by p38α (data not shown). Using a phospho-threonine antibody that recognizes EEA1 phosphorylated on Thr-1392 (Figure 4A, right panel), we confirmed the phosphorylation of endogenous EEA1 upon activation of p38 MAPKs by MKK6DD both in HEK293 cells and in SH-SY5Y neuronal cells (Figure 4B). Importantly, Damgo stimulation also induced the phosphorylation of endogenous EEA1, which was mediated by p38 MAPK, as indicated by the inhibitory effect of SB203580 in HEK293 cells expressing GFP-MOR and in the neuronal cell line SH-SY5Y (Figure 4C). However, EEA1 phosphorylation was not observed upon morphine stimulation (Figure 4C, left panel), providing a good correlation between p38 MAPK activation, EEA1 phosphorylation and MOR endocytosis. Interestingly, the C-terminus of EEA1 could efficiently be phosphorylated

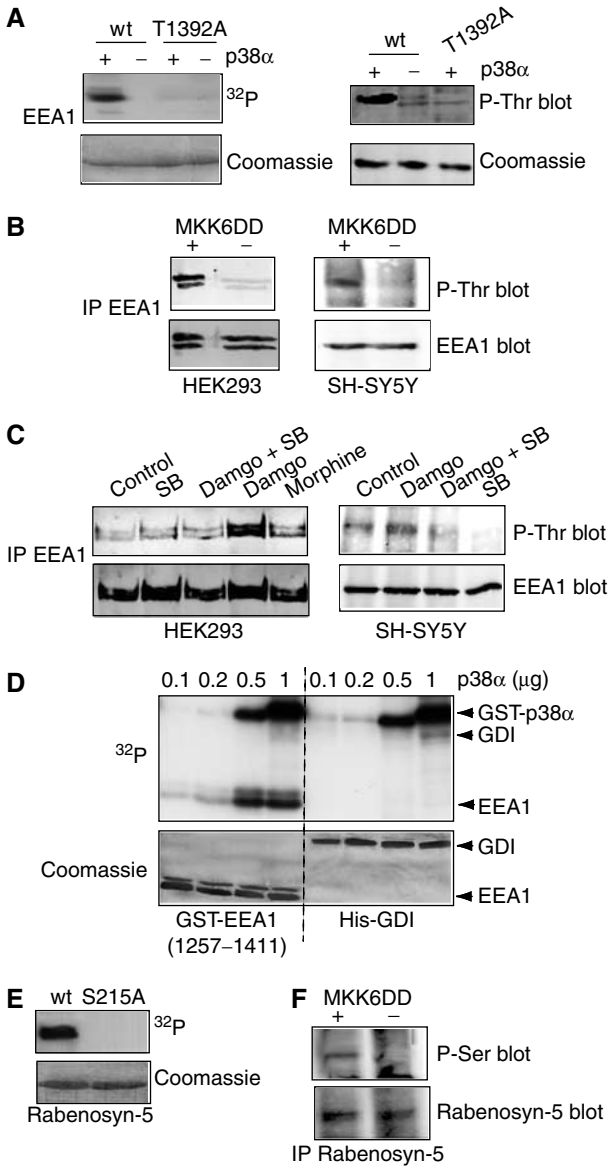

Figure 4 p38α MAPK phosphorylates EEA1 and Rabenosyn-5. (**A**) *In vitro* kinase assay using MKK6DD-activated p38α (+) or MKK6DD alone (−) and the GST-fused C-terminus of EEA1 (amino acids 1257–1411) wt or with the mutation T1392A (10 μg each) as substrates (left panels). Western blot using a phospho-Thr antibody of GST-EEA1 (1257–1411) wt or T1392A (1 μg) after phosphorylation with MKK6DD-activated p38α or MKK6DD alone (right panels). (**B**) Endogenous EEA1 was immunoprecipitated from MKK6DD-expressing HEK293 cells (left panel) or SH-SY5Y cells (right panel) and blotted with antibodies against EEA1 or phospho-Thr. (**C**) HEK293 or SH-SY5Y cells were treated as indicated and endogenous EEA1 was immunoprecipitated and blotted with antibodies against EEA1 or phospho-Thr. (**D**) *In vitro* kinase assay using the indicated amounts of MKK6DD-activated GST-p38α and either 10 μg GST-EEA1 (1257–1411) or 15 μg His-GDI. (**E**) *In vitro* kinase assay using MKK6DD-activated p38α and the GST-Rabenosyn-5 wt or with the mutation S215A (10 μg each). (**F**) Endogenous Rabenosyn-5 was immunoprecipitated from SH-SY5Y cells expressing MKK6DD and blotted with antibodies against Rabenosyn-5 or phospho-Ser.

in vitro by 10-fold lower amounts of p38α than required for phosphorylating Rab GDI (Figure 4D).

The C-terminal fragment of EEA1 contains the PI(3)P-binding FYVE domain (Stenmark *et al*, 2002). Based on the

EEA1 crystal structure (Dumas *et al*, 2001), the p38 MAPK phosphorylation site lies within the FYVE domain dimer interface. The N-terminus of Rabenosyn-5 also includes an FYVE domain containing two p38 MAPK canonical phosphorylation sites (Nielsen *et al*, 2000; Stenmark *et al*, 2002). Consistently, mutation of Ser-215 to Ala abolished p38 MAPK-mediated phosphorylation of Rabenosyn-5 (Figure 4E), indicating that this is the major p38 MAPK phosphorylation site. Moreover, using a phospho-serine antibody, we confirmed the phosphorylation of endogenous Rabenosyn-5 upon activation of p38 MAPKs by MKK6DD in the neuronal cell line SH-SY5Y (Figure 4F).

Since the FYVE domain of EEA1 is required for homodimerization, endosomal membrane localization and, consequently, activity in endosome fusion (Stenmark *et al*, 1996; Callaghan *et al*, 1999), we first investigated whether Thr-1392 phosphorylation by p38α modulated the membrane recruitment of EEA1. The C-terminus of EEA1 either wt or containing the nonphosphorylatable mutation T1392A were *in vitro* translated and their ability to bind to isolated endosomal membranes was analyzed. Indeed, the EEA1 mutant T1392A was less efficiently recruited to endosomal membranes compared to wt EEA1 (Figure 5A). Quantitative analysis (Supplementary Figure S3) indicated that the recruitment of EEA1-T1392A was about 50% of the wt EEA1 value, both in the presence and absence of exogenous Rab5:GDI (the latter reflecting binding by the endogenous Rab5). The fact that EEA1-T1392A is still capable of membrane binding, albeit at reduced levels, is in agreement with multiple molecular interactions targeting EEA1 to endosomes (Simonsen *et al*, 1998; McBride *et al*, 1999; Lawe *et al*, 2003).

In order to confirm in intact cells the results obtained using purified endosomal membranes, we transiently expressed full-length wt EEA1 and the corresponding mutants T1392A or T1392D (to mimic p38α phosphorylation) in wt and p38α−/− MEFs and determined their localization by both immunofluorescence microscopy analysis (Figure 5B) and subcellular fractionation (Figure 5C). In agreement with the *in vitro* experiments, EEA1-T1392A expressed in either wt or p38α−/− MEFs was also less efficiently recruited to endosomal membranes than wt EEA1. Interestingly, the T1392D mutation rescued to a large extent the membrane localization of EEA1. These results suggest that phosphorylation of Thr-1392 is required for the efficient recruitment of EEA1 to endosomal membranes *in vivo*. Surprisingly, in p38α−/− cells expressing wt EEA1, we observed small clusters of endosomes corresponding to tightly packed and tethered individual endosomes (Figure 5B, panel 4, inset). We estimated a frequency of about 14 clusters per p38α−/− cell versus not more than 1 per wt cell. Such a phenotype is reminiscent of the effects induced by the lack of EEA1 function in the conversion from membrane tethering to fusion (Christoforidis *et al*, 1999a; Lawe *et al*, 2002). The T1392D mutation also partially restored EEA1 fusion activity, as assessed by a decreased number of clustered endosomes (Figure 5B, panel 6; about 6 clusters/cell). Altogether, our results suggest that p38α MAPK-mediated phosphorylation enhances both EEA1 membrane localization and fusion activity.

We then investigated whether the Damgo-induced EEA1 phosphorylation correlated with membrane recruitment. Interestingly, Damgo increased the membrane recruitment of EEA1 in wt but not p38α−/− cells, indicating that Damgo-induced EEA1 membrane localization is strictly p38α dependent (Figure 6A). Moreover, incubation with SB203580 for 1 h reduced by about 25% the levels of membrane-localized EEA1 in wt cells, suggesting that p38α contributes to the regulation of membrane recruitment of EEA1 also in nonstimulated cells. Importantly, membrane recruitment of both EEA1 and Rabenosyn-5 was also increased, in a p38 MAPK-dependent manner, upon Damgo treatment of SH-SY5Y neuronal cells (Figure 6B).

As mentioned above, we have detected the presence of membrane-bound EEA1 in p38α−/− cells by immunofluorescence and immunoblotting (Figure 5B and C). To address the possible contribution of p38β MAPK activity to membrane localization of EEA1, we used siRNAs to knock down both p38α and p38β in HeLa cells. Remarkably, the down-regulation of both p38 MAPKs resulted in a quantitative solubilization of endogenous EEA1 (Figure 6C). In a complementary experiment, HeLa cells were incubated with the p38α and p38β inhibitor SB203580 for 18 h. This long-term treatment resulted in a 50–75% solubilization of endogenous EEA1 and Rabenosyn-5 (Figure 6D and Supplementary Figure S4), confirming the involvement of the p38 MAPK family in membrane recruitment of these two Rab5 effectors.

To investigate the importance of EEA1 phosphorylation by p38α on Rab5-induced MOR endocytosis, we coexpressed Rab5Q79L together with different EEA1 mutants (Figure 7A). Expression in wt cells of EEA1 T1392A significantly inhibited Rab5Q79L-induced MOR endocytosis, whereas expression of wt EEA1 further potentiated it (Figure 7B). Importantly, the expression of EEA1T1392A also impaired Damgo-induced MOR endocytosis in wt cells, in the absence of Rab5Q79L expression (Figure 7C). These results confirm that phosphorylation on Thr-1392 affects EEA1 function. Strikingly, expression of EEA1 T1392D was able to bypass the requirement for p38α in Rab5Q79L-induced MOR endocytosis (Figure 7B). Moreover, overexpression of EEA1 T1392D alone (Figure 7D) was sufficient to induce some constitutive MOR endocytosis in p38α−/− cells, which was enhanced in the presence of either Damgo or morphine (Figure 7C). The ability of the EEA1 T1392D mutant to complement endogenous Rab5 activity in p38α−/− cells confirms an important role for EEA1 phosphorylation in the regulation of Rab5 function.

Discussion

In this study, we have established that p38 MAPKs play a key role in the modulation of MOR endocytosis. In the case of analgesic treatment, MOR activity upon chronic stimulation is the result of a balance between MOR signalling, which mediates the analgesic effect, and MOR endocytosis, which regulates tolerance (Finn and Whistler, 2001; He *et al*, 2002; Kieffer and Evans, 2002). Thus, through the stimulation of MOR endocytosis, p38 MAPK is a primary candidate regulator of tolerance to morphine. Since p38α−/− mice are embryonic lethal (Adams *et al*, 2000), the testing of this hypothesis should await the generation of conditional p38α knockout mice.

By investigating the molecular mechanisms that underlie MOR endocytosis, we have further uncovered an unexpected functional connection between p38 MAPK activity and the endocytic machinery regulated by the small GTPase

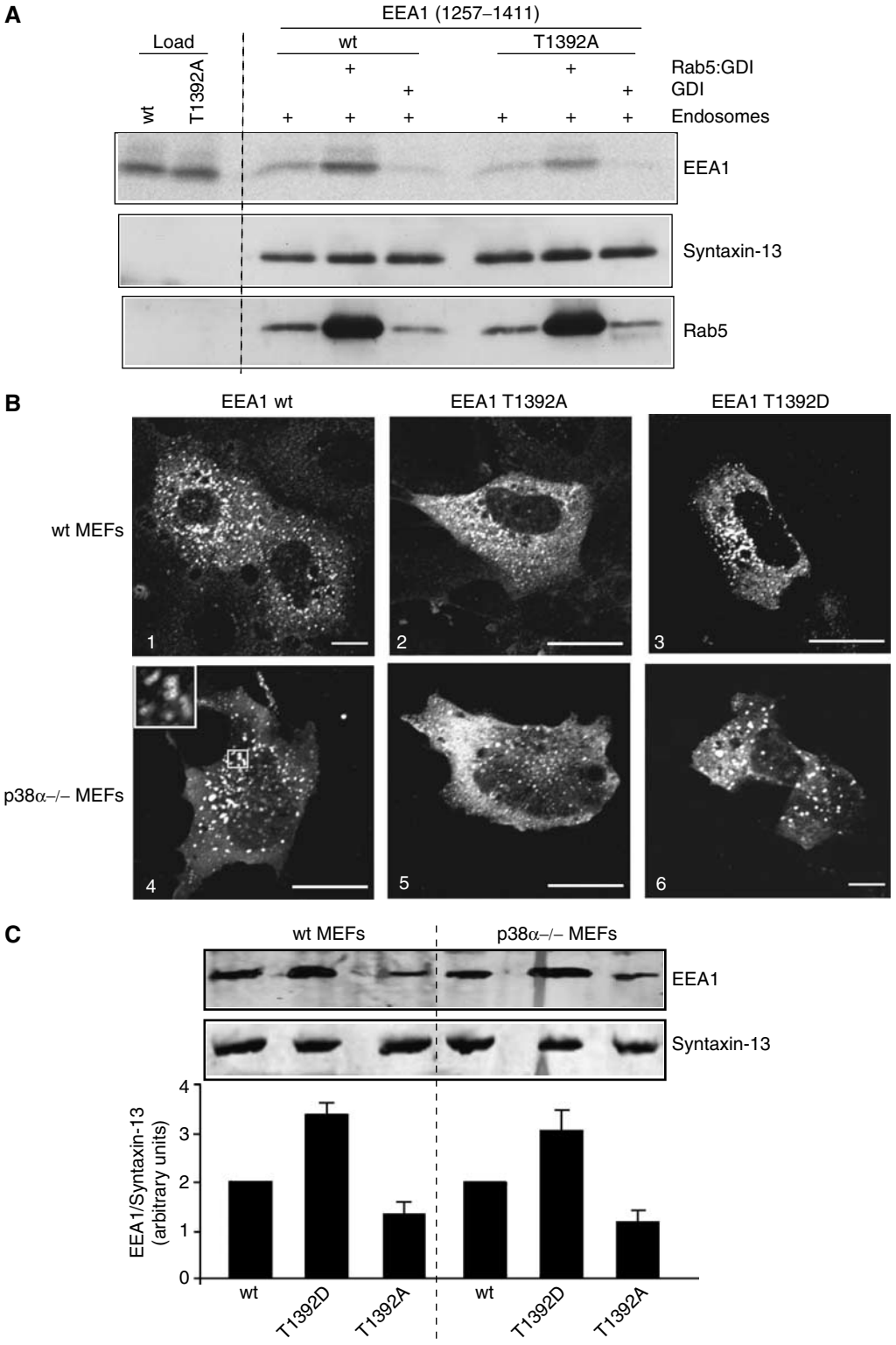

Figure 5 Phosphorylation of Thr-1392 regulates EEA1 subcellular localization. (**A**) *In vitro* recruitment of ^{35}S-labelled EEA1 (1257–1411) wt and T1392A to early endosomes in the absence or in the presence of Rab5:GDI complex or GDI alone. Proteins bound to endosomal membranes were detected by autoradiography and Western blot. Syntaxin-13 was used as a membrane marker. (**B**) MEFs (wt or p38α−/−) were transiently transfected with full-length EEA1 either wt, T1392A or T1392D, fixed and stained with antibodies to EEA1. Scale bar represents 20 μm. (**C**) Western blots of membrane preparations from wt or p38α−/− MEFs transiently transfected with full-length EEA1 either wt, T1392A or T1392D. The histogram represents the mean ± s.e.m. of two experiments.

Rab5. A role for p38 MAPKs in endocytosis has been previously proposed based on the ability of p38α to phosphorylate Rab GDI upon oxidative stress (Cavalli *et al*, 2001). Surprisingly, our results indicate that p38 MAPK activity is also required under normal growth conditions to regulate ligand-dependent receptor stimulation and endocytosis. Cells

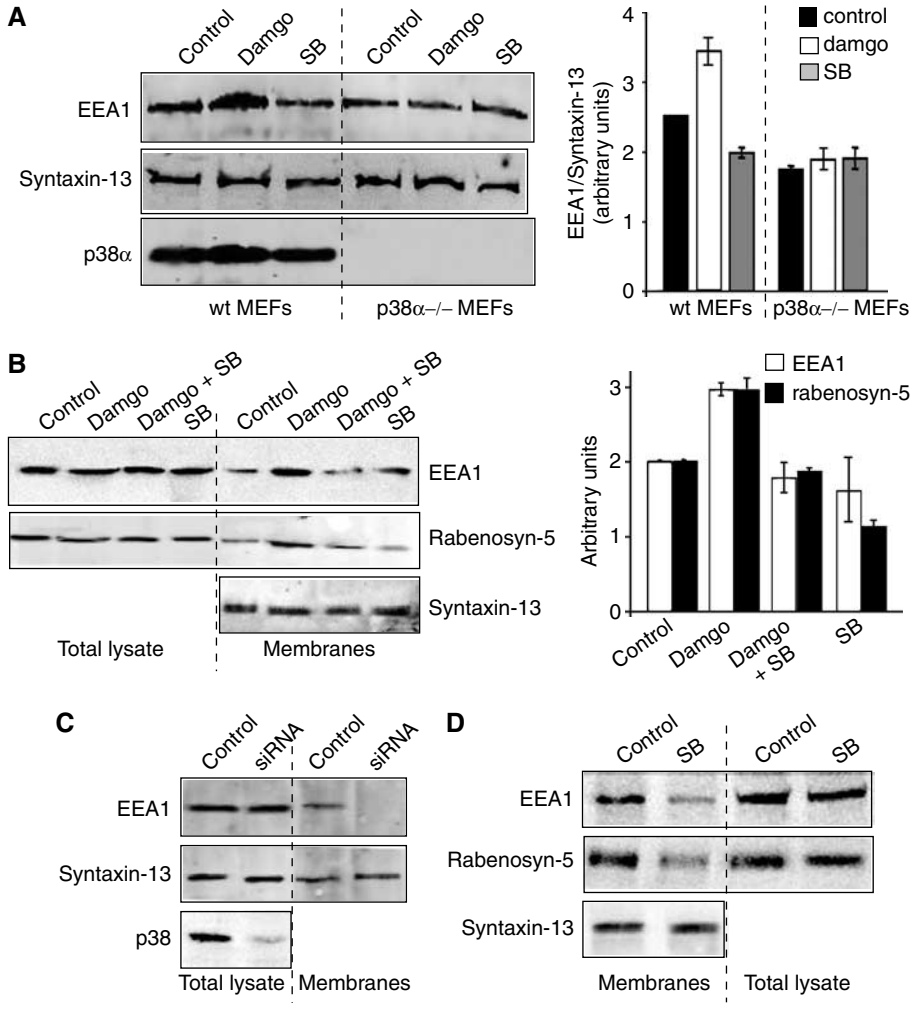

Figure 6 p38 MAPKs regulate EEA1 membrane localization. (**A**) Western blots of membrane preparations from wt or p38α−/− MEFs transfected with EEA1wt and treated with 1 μM Damgo or 10 μM SB203580 for 30 min and 1 h, respectively. The histogram represents the quantification of two experiments. (**B**) Western blots of total lysates and membrane preparations from SH-SY5Y cells treated with Damgo, SB203580 or both together as in (A). The histogram represents the quantification by densitometric analysis of the EEA1 and Rabenosyn-5 bands versus Syntaxin-13. The experiment was repeated three times. (**C, D**) Western blots of total lysates and membrane preparations from HeLa cells either transfected with luciferase siRNA (Control) and a mixture of p38α and p38β siRNAs (C) or incubated with 10 μM SB203580 for 18 h. The experiment was repeated twice.

respond to stress with very high levels of p38α activation. On the other hand, basal, low-level activity of p38α in nonstressed cells may have different functions, as postulated, for example, in the regulation of mRNA turnover (Ambrosino *et al*, 2003). We propose that phosphorylation of EEA1 by p38α operates under physiological conditions to regulate the balance between soluble and membrane-bound Rab5 effectors. This housekeeping p38α function, however, is counteracted by the stress-induced hyperactivation of p38α, which is likely to result in the phosphorylation of additional p38 MAPK substrates, including Rab GDI. Consistent with this idea, we found that EEA1 is a better *in vitro* substrate for p38α than GDI, and MOR stimulation induces significantly lower levels of p38α activity than UV treatment. These results suggest that p38 MAPKs may play different mechanistic roles in endocytosis under basal versus stress or other stimulatory conditions via quantitative changes in activity on a spectrum of various substrates.

A genome-wide screen has recently uncovered a wider role of protein kinases in the regulation of endocytosis (Pelkmans *et al*, 2005). Interestingly, silencing of several kinases by RNAi caused the accumulation of phosphorylated p38 MAPK on enlarged early endosomes. Our results provide a mechanistic explanation for the role of p38 MAPK on early endosomes, where it can phosphorylate effectors and regulators of the Rab5 machinery, thus modulating endocytosis depending on the level of activation.

It has been previously shown that the membrane recruitment of Rab5 effectors, such as EEA1, results from the additive contributions from multiple interactions (e.g. binding to Rab5:GTP, PI(3)P, Syntaxins, Ca^{2+}/calmodulin and oligomerization; Simonsen *et al*, 1998; McBride *et al*, 1999; Lawe *et al*, 2003). Our results here suggest that p38 MAPK-mediated phosphorylation is a new mechanism providing a regulatory function on the membrane recruitment and activity of Rab5 effectors, such as EEA1 and Rabenosyn-5. We showed that p38 MAPKs can phosphorylate EEA1 and Rabenosyn-5 on Thr-1392 and Ser-215, respectively. Interestingly, both phosphorylation sites are located within the FYVE finger, which by binding to PI(3)P plays an essential

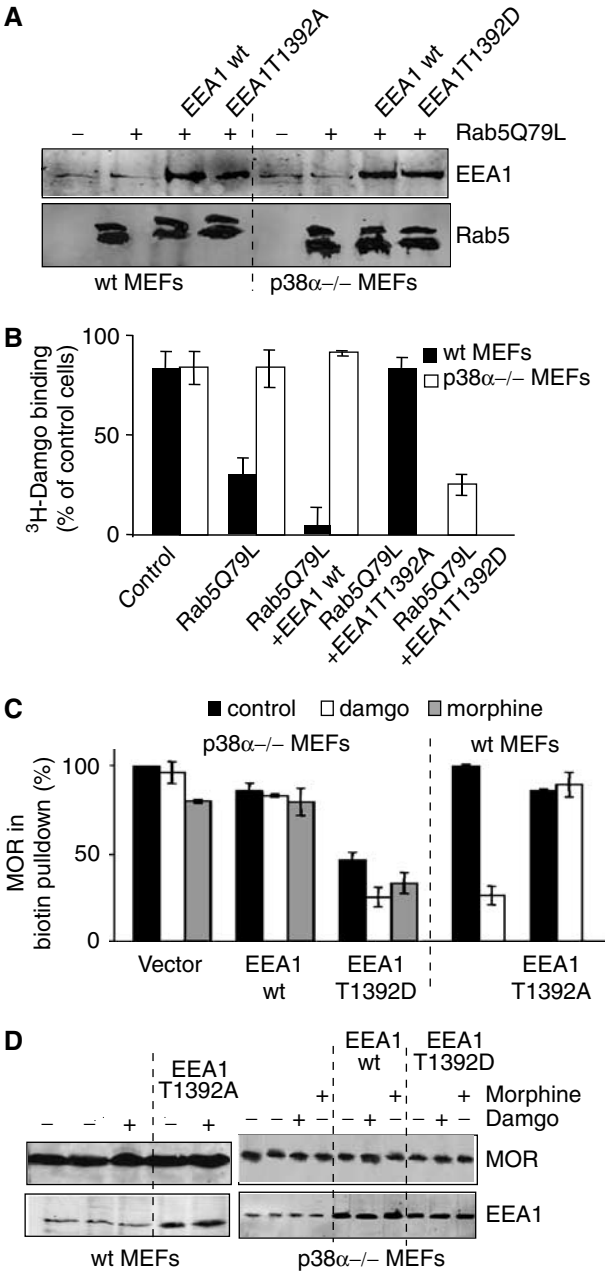

Figure 7 EEA1 phosphorylation is required for MOR endocytosis. (**A**) Western blots of total lysates from wt and p38α−/− MEFs transfected with Rab5Q79L and full-length wt EEA1, or the mutants T1392A and T1392D, as indicated. (**B**) Damgo binding assays in wt and p38α−/− MEFs transfected with Rab5Q79L alone or together with EEA1 wt or the indicated mutants. The results are expressed as the percentage of binding in cells transfected with the empty plasmids and represent the mean±s.e.m. of two experiments performed in triplicate. (**C**) Plasma membrane-associated levels of MOR in p38α−/− and wt cells transfected with either empty vector, EEA1 wt or the T1392D and T1392A mutants and treated with Damgo or Morphine, as indicated. MOR was visualized by avidin pulldown of biotinylated membrane proteins followed by Western blot using a MOR antibody. The histogram represents the quantification of the MOR band (pulldown versus total cell lysate) using the Odyssey Imaging system. The experiment was repeated twice. (**D**) Western blots of total lysates from wt and p38α−/− MEFs transfected with wt EEA1 or the mutants T1392A and T1392D and stimulated with Damgo or Morphine, as indicated.

role in the recruitment of the proteins to the early endosome membrane. In the crystal structure of the EEA1 C-terminus, Thr-1392 lies in a region that has been implicated as FYVE domain dimer interface (Dumas *et al*, 2001). Phosphorylation may thus be important for EEA1 binding to Rab5 or PI(3)P on the early endosome membrane, insertion of a hydrophobic loop into the membrane bilayer or electrostatic interactions (Hayakawa *et al*, 2004; Kutateladze *et al*, 2004). This is consistent with the implication of amino acids 1365–1368 and 1392–1397 in the stabilization of EEA1 membrane docking (Hayakawa *et al*, 2004).

In summary, our results document a new and unexpected role for p38 MAPKs in the regulation of receptor endocytosis. In showing that p38α can phosphorylate the Rab5 effectors EEA1 and Rabenosyn-5 and regulate their membrane binding, our data indicate that Rab5 effector activity is not only dependent on the so far established Rab5 regulatory cycles (membrane/cytosol and GTP/GDP) but can be also directly regulated by p38 MAPK phosphorylation. This is yet another example of coordinated regulation between signalling and membrane trafficking, expanding the repertoire of mechanisms that modulate receptor endocytosis.

Materials and methods

Cell culture and transfection

Primary MEFs were generated, cultured and immortalized by infection with SV40 large T-expressing retrovirus as previously described (Ambrosino *et al*, 2003; Porras *et al*, 2004). HEK293 cells and MEFs were grown in Dulbecco's minimal essential medium (Gibco-BRL) supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. Stably transfected HEK293-GFP-MOR cells were grown in medium supplemented with 1 mg/ml G-418 (Gibco-BRL) alone or together with 100 μg/ml of hygromycin (Gibco-BRL) for MKK6DD-expressing clones.

HEK293 cells were plated in 10 cm plates and 24 h later were transfected using the calcium phosphate method with either 10 μg of pcDNA3.1-GFP-MOR or the pcDNA3.1 vector (Invitrogene). The medium was changed 16 h post-transfection and the cells were incubated for another 48 h, before selection with 1 mg/ml of G-418. Single clones were screened for the expression of GFP-MOR by radioligand binding assay using ^{3}H-labelled Damgo (Amersham, Pharmacia). HEK293-GFP-MOR cells were cotransfected with 5 μg pEFmlink-MKK6DD (Alonso *et al*, 2000) or pEFmlink together with pcDNA3.1-hygro. Cells were selected with 100 μg/ml of hygromycin. Pools were selected for MKK6DD expression by Western blotting. HEK293 cells or MEFs were transiently transfected with 10 μg Rab5, Rab5Q79L or Myc-Rab5S34N in pCMV (Stenmark *et al*, 1994), HA-RN-tre (Lanzetti *et al*, 2000) or EEA1 (de Renzis *et al*, 2002) in pcDNA3.1 or Myc-p38α in pEFmlink (Ambrosino *et al*, 2003). MEFs were transiently transfected with either 1 or 10 μg of pEFmlink-MKK6DD, as indicated. Cells were collected 48 h after changing the transfection medium (see above) and used either for immunofluorescence and radioligand binding assays or to prepare cell lysates.

The neuroblastoma cell line SH-SY5Y was grown in Dulbecco's minimal essential medium (Gibco-BRL) supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. All-*trans* retinoic acid (Sigma) was added every 2 days at a concentration of 10 μM during 6 days for SH-SH-5Y differentiation. For transfection experiments, cells were plated and 24 h later were transfected with 10 μg pEFmlink-MKK6DD or the pEFmlink vector using the Amaxa nucleofector system according to the manufacturer's instructions (Amaxa Biosystem). Cell lysates were prepared 48 h after the transfection.

Cell stimulation, preparation of cell lysates and Western blotting analysis

Cells were serum-starved for 16 h before stimulation with 10 μM Damgo (Sigma) or 10 μM morphine (Sigma) in serum-free medium at 37°C for 5 min. For UV stimulation, subconfluent cells were

treated with UV using a Stratalinker apparatus and then incubated for 30 min (Alonso *et al*, 2000). Preparation of cell lysates and Western blotting were performed as described previously (Ambrosino *et al*, 2003). The primary antibodies were Phospho-p38 MAPK and Phospho-Thr (Cell Signalling Technologies), p38α (Santa-Cruz), Phospho-Ser (Abcam), α tubulin (Sigma), MOR (DiaSorin), MKK6 (Alonso *et al*, 2000), Rab5 (Stenmark *et al*, 1994), EEA1 (raised in rabbits against full-length EEA1 expressed in insect cells), Rabenosyn-5 (raised in rabbits against full-length Rabenosyn-5 expressed in insect cells and affinity purified) and Syntaxin-13 (McBride *et al*, 1999). Binding of primary antibodies was detected using Alexa-680-coupled secondary antibodies and the Odyssey Imaging system (Ly-Cor). For the immunoprecipitation of endogenous EEA1 and Rabenosyn-5, HEK293-GFP-MOR and SH-SY5Y cells, expressing or not MKK6DD, were incubated for 30 min with 10 μM of either Damgo, morphine or SB203580.

Immunofluorescence

MEFs were grown on coverslips, transfected with wt EEA1 and the T1392A or T1392D mutants, fixed with 4% paraformaldehyde in PBS for 45 min at 4°C and permeabilized with 4% Triton X-100. Saturation was performed using 5% BSA in PBS for 1 h at 4°C. EEA1 was visualized using rabbit anti-EEA1 antibody, followed by incubation with secondary antibodies coupled to Alexa-488 (Molecular Probes). Images were taken with a Leica TCS SP2 confocal microscope.

Subcelullar fractionation

HeLa cells, MEFs and differentiated SH-SY5Y cells were transfected or stimulated as indicated, washed four times with ice-cold PBS, scraped and resuspended in swelling buffer (10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 0.25 M sucrose) containing phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μg/ml), pepstatin (10 μg/ml), sodium vanadate (1 mM) and aprotinin (10 μg/ml). Cells were lysed by repeated passages through a 27-gauge needle and the extracts were sequentially centrifuged first at 600 *g* for 12 min to remove nuclei and intact cells and then at 200 000 *g* for 1 h to separate the cytosol from the membranes.

Damgo binding assays

Cells were incubated for 30 min at 37°C with Damgo or morphine (1 μM, unless indicated otherwise), and washed three times with medium for 5 min at 4°C. ^{3}H-labelled Damgo binding (10 nM, 67 Ci/mmol) was performed as described (Macé *et al*, 1999). Nonspecific binding was determined in the presence of 1 μM of unlabelled Damgo. Experiments were performed in triplicate.

Site-directed mutagenesis

The EEA1 and Rabenosyn-5 mutants were generated with the QuickChange site-directed mutagenesis kit (Stratagene) using as templates pGEX-EEA1 (1257–1411), full-length EEA1 in pcDNA3.1 (de Renzis *et al*, 2002) and pGEX-Rabenosyn-5 (Nielsen *et al*, 2000). All mutations were confirmed by DNA sequencing.

Recombinant protein purification

GST-human p38α, GST-ATF2 (19–96) and MalE-MKK6DD (Alonso *et al*, 2000), GST-EEA1 (1257–1411; wt and T1392A mutant) (Christoforidis *et al*, 1999a), His-GDI and GST-Rabenosyn-5 (wt and S215A mutant) (Nielsen *et al*, 2000) were purified using either glutathione (GST), Talon (His) or amylose (MalE) beads from *Escherichia coli* BL21 (DE3) induced with isopropyl-1-thio-β-D-galactopyranoside.

p38 MAPK assays

Lysates prepared from Damgo- or UV-stimulated cells were incubated with rabbit anti-p38α antiserum (Alonso *et al*, 2000) coupled to protein A-Sepharose or with protein A-Sepharose alone overnight at 4°C. The kinase assays were performed in the presence of [γ-^{32}P]ATP (2 μCi) as described previously (Alonso *et al*, 2000). Phosphorylated ATF2 was visualized by autoradiography.

Purified GST-p38α (1 μg, unless indicated otherwise) was activated with MalE-MKK6DD (200 ng) in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT and 200 μM ATP for 1 h at 30°C. Kinase assays were carried out using 200 ng of the active p38 MAPK in the same buffer as indicated above but containing 2 μM microcystin, 100 μM ATP and 2 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The reaction was stopped by adding loading buffer and boiling for 5 min. Proteins were resolved by SDS–PAGE and detected by autoradiography.

In vitro recruitment assay of EEA1 on early endosomes

Recruitment of EEA1 (1257–1411) wt and T1392A mutant to early endosomes was performed as described previously (Christoforidis *et al*, 1999b) using *in vitro*-translated EEA1 (TNT kit, Promega) and early endosomes purified from HeLa cells. Proteins bound to early endosomes were separated by SDS–PAGE and visualized by Western blotting and autoradiography.

Biotinylation and purification of cell surface proteins

HEK293 cells expressing GFP-MOR alone or together with MKK6DD, MEFs and SH-SY5Y cells were grown to 80% confluency and incubated with 10 μM Damgo or morphine, 50 μM H_2O_2 or 10 μM SB203580, as indicated. After four washes in PBS, cells were treated for 30 min at 4°C with 0.4 mg/ml of membrane-impermeable sulfo-NHS-SS-Biotin (Pierce) prepared in PBS. Cells were then washed three times in PBS and lysed in a buffer containing 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl and 10 mM Tris–HCl pH 7.4. The supernatants obtained after centrifugation at 10 000 *g* for 2 min were incubated with immobilized monomeric avidin according to the manufacturer's instructions (Pierce) to separate biotin-coupled cell surface proteins. After five washes in PBS, avidin-bound biotinylated proteins were resolved by SDS–PAGE and visualized by Western blotting.

Knockdown of p38α and p38β by siRNA

Human p38α and p38β siRNAs and the control luciferase siRNA were obtained from Ambion. HeLa cells were transfected with 100 nM of siRNA using the siPORT transfection buffer (Ambion). After 96 h, cells were scraped and membranes were prepared as described above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We are grateful to Franck Talmont (CNRS/UMR5089, Toulouse) for generously providing the GFP-MOR construct and to Markus Schmitt for help with preliminary EEA1 phosphorylation experiments. We thank Ana Cuadrado, Gilles Dietrich and Anne Ephrussi for critically reading the manuscript and Concetta Ambrosino, Nuria Ajenjo and members of the Nebreda lab for many helpful suggestions. GM was supported by an EC Marie-Curie postdoctoral fellowship and by the EMBL. MM was a recipient of fellowships from HFSP, Austrian Science Fund (Erwin Schrödinger fellowship) and Max Planck Society. This work was supported by the EMBL and CNIO (ARN) and by grants from HFSP (RG-2060/1999-M to MZ) and the European Union (HPRN-CT-2000-00081 to MZ).

References

Adams S, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR (2000) Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell* **6:** 109–116

Alonso G, Ambrosino C, Jones M, Nebreda AR (2000) Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. *J Biol Chem* **275:** 40641–40648

Ambrosino C, Mace G, Galban S, Fritsch C, Vintersten K, Black E, Gorospe M, Nebreda AR (2003) Negative feedback regulation of MKK6 mRNA stability by p38alpha mitogen-activated protein kinase. *Mol Cell Biol* **23:** 370–381

Callaghan J, Simonsen A, Gaullier JM, Toh BH, Stenmark H (1999) The endosome fusion regulator early-endosomal autoantigen 1 (EEA1) is a dimer. *Biochem J* **338:** 539–543

Cavalli V, Vilbois F, Corti M, Marcote M, Tamura K, Karin M, Arkinstall J, Gruenberg J (2001) The stress induced MAP kinase p38 regulates endocytic trafficking via GDI:Rab5 complex. *Mol Cell* **7:** 421–432

Christoforidis S, McBride HM, Burgoyne RD, Zerial M (1999a) The Rab5 effector EEA1 is a core component of endosome docking. *Nature* **397:** 621–625

Christoforidis S, Miaczynska M, Ashman K, Wilm M, Zhao L, Yip SC, Waterfield MD, Backer JM, Zerial M (1999b) Phosphatidylinositol-3-OH kinases are Rab5 effectors. *Nat Cell Biol* **1:** 249–252

de Renzis S, Sonnichsen B, Zerial M (2002) Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. *Nat Cell Biol* **4:** 124–133

Dumas JJ, Merithew E, Sudharshan E, Rajamani D, Hayes S, Lawe D, Corvera S, Lambright DG (2001) Multivalent endosome targeting by homodimeric EEA1. *Mol Cell* **8:** 947–958

Ferguson S (2001) Evolving concepts in G protein coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol rev* **53:** 1–24

Finn AK, Whistler JL (2001) Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* **32:** 829–839

Fratti RA, Chua J, Deretic V (2003) Induction of p38 mitogen-activated protein kinase reduces early endosome autoantigen 1 (EEA1) recruitment to phagosomal membranes. *J Biol Chem* **278:** 46961–46967

Hayakawa A, Hayes SJ, Lawe DC, Sudharshan E, Tuft R, Fogarty K, Lambright D, Corvera S (2004) Structural basis for endosomal targeting by FYVE domains. *J Biol Chem* **279:** 5958–5966, Epub 2003 Oct 5931

He L, Fong J, von Zastrow M, Whistler JL (2002) Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* **108:** 271–282

Horiuchi H, Lippe R, McBride HM, Rubino M, Woodman P, Stenmark H, Rybin V, Wilm M, Ashman K, Mann M, Zerial M (1997) A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. *Cell* **90:** 1149–1159

Huang CC, You JL, Wu MY, Hsu KS (2004) Rap1-induced p38 mitogen-activated protein kinase activation facilitates AMPA receptor trafficking via the GDI:Rab5 complex: potential role in (*S*)-3,5-dihydroxyphenylglycine-induced long-term depression. *J Biol Chem* **279:** 12286–12292

Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, von Zastrow M (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism *in vitro* and in the mammalian brain. *Mol Pharmacol* **53:** 377–384

Kieffer BL (1999) Opioids: first lessons from knockout mice. *Trends Pharmacol Sci* **20:** 19–26

Kieffer BL, Evans CJ (2002) Opioid tolerance—in search of the holy grail. *Cell* **108:** 587–590

Kutateladze TG, Capelluto DG, Ferguson CG, Cheever ML, Kutateladze AG, Prestwich GD, Overduin M (2004) Multivalent mechanism of membrane insertion by the FYVE domain. *J Biol Chem* **279:** 3050–3057

Lanzetti L, Rybin V, Malabarba MG, Christoforidis S, Scita G, Zerial M, Di Fiore PP (2000) The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5. *Nature* **408:** 374–377

Law PY, Wong YH, Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* **40:** 389–430

Lawe DC, Chawla A, Merithew E, Dumas J, Carrington W, Fogarty K, Lifshitz L, Tuft R, Lambright D, Corvera S (2002) Sequential roles for phosphatidylinositol 3-phosphate and Rab5 in tethering and fusion of early endosomes via their interaction with EEA1. *J Biol Chem* **277:** 8611–8617

Lawe DC, Sitouah N, Hayes S, Chawla A, Virbasius JV, Tuft R, Fogarty K, Lifshitz L, Lambright D, Corvera S (2003) Essential role of Ca^{2+}/calmodulin in Early Endosome Antigen-1 localization. *Mol Biol Cell* **14:** 2935–2945

Li J, Chen C, Huang P, Liu-Chen LY (2001) Inverse agonist up-regulates the constitutively active D3.49(164)Q mutant of the rat mu-opioid receptor by stabilizing the structure and blocking constitutive internalization and down-regulation. *Mol Pharmacol* **60:** 1064–1075

Ma W, Zheng WH, Powell K, Jhamandas K, Quirion R (2001) Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an *in vitro* and *in vivo* study. *Eur J Neurosci* **14:** 1091–1104

Macé G, Blanpied C, Emorine LJ, Druet P, Dietrich G (1999) Morphine-like activity of natural human IgG autoantibodies is because of binding to the first and third extracellular loops of the mu-opioid receptor. *J Biol Chem* **274:** 20079–20082

Matthes HWD, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dollé P, Tzavara E, Hanoune J, Roques BP, Kieffer BL (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. *Nature* **383:** 819–823

McBride HM, Rybin V, Murphy C, Giner A, Teasdale R, Zerial M (1999) Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. *Cell* **98:** 377–386

Mills IG, Jones AT, Clague MJ (1998) Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes. *Curr Biol* **8:** 881–884

Mu FT, Callaghan JM, Steele-Mortimer O, Stenmark H, Parton RG, Campbell PL, McCluskey J, Yeo JP, Tock EP, Toh BH (1995) EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine 'fingers' and contains a calmodulin-binding IQ motif. *J Biol Chem* **270:** 13503–13511

Nebreda A, Porras A (2000) p38 MAP kinases: beyond the stress response. *Trends Biochem Sci* **25:** 257–260

Nielsen E, Christoforidis S, Uttenweiler-Joseph S, Miaczynska M, Dewitte F, Wilm M, Hoflack B, Zerial M (2000) Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. *J Cell Biol* **151:** 601–612

Nitsche JF, Pintar JE (2003) Opioid receptor-induced GTPgamma35S binding during mouse development. *Dev Biol* **253:** 99–108

Patki V, Virbasius J, Lane WS, Toh BH, Shpetner HS, Corvera S (1997) Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA* **94:** 7326–7330

Pelkmans L, Fava E, Grabner H, Hannus M, Habermann B, Krausz E, Zerial M (2005) Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature* **436:** 78–86

Pfeffer SR, Dirac-Svejstrup AB, Soldati T (1995) Rab GDP dissociation inhibitor: putting rab GTPases in the right place. *J Biol Chem* **270:** 17057–17059

Porras A, Zuluaga S, Black E, Valladares A, Alvarez AM, Ambrosino C, Benito M, Nebreda AR (2004) p38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. *Mol Biol Cell* **15:** 922–933

Rybin V, Ullrich O, Rubino M, Alexandrov K, Simon I, Seabra MC, Goody R, Zerial M (1996) GTPase activity of Rab5 acts as a timer for endocytic membrane fusion. *Nature* **383:** 266–269

Sasaki T, Kikuchi A, Araki S, Hata Y, Isomura M, Kuroda S, Takai Y (1990) Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to smg p25A, a ras p21-like GTP-binding protein. *J Biol Chem* **265:** 2333–2337

Seachrist JL, Ferguson SS (2003) Regulation of G protein-coupled receptor endocytosis and trafficking by Rab GTPases. *Life Sci* **74:** 225–235

Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, Toh BH, Murphy C, Zerial M, Stenmark H (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* **394:** 494–498

Singhal PC, Bhaskaran M, Patel J, Patel K, Kasinath BS, Duraisamy S, Franki N, Reddy K, Kapasi AA (2002) Role of p38 mitogen-activated protein kinase phosphorylation and Fas–Fas ligand interaction in morphine-induced macrophage apoptosis. *J Immunol* **168:** 4025–4033

Sivars U, Aivazian D, Pfeffer SR (2003) Yip3 catalyses the dissociation of endosomal Rab–GDI complexes. *Nature* **425:** 856–859

Stenmark H, Aasland R, Driscoll PC (2002) The phosphatidylinositol 3-phosphate-binding FYVE finger. *FEBS Lett* **513:** 77–84

Stenmark H, Aasland R, Toh BH, D'Arrigo A (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J Biol Chem* **271:** 24048–24054

Stenmark H, Parton RG, Steele-Mortimer O, Lutcke A, Gruenberg J, Zerial M (1994) Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO J* **13:** 1287–1296

Sternini C, Spann M, Anton B, Keith DE, Jr, Bunnett NW, von Zastrow M, Evans C, Brecha NC (1996) Agonist-selective endocytosis of mu opioid receptor by neurons *in vivo*. *Proc Natl Acad Sci USA* **93:** 9241–9246

Wang JB, Johnson PS, Persico AM, Hawkins AL, Griffin CA, Uhl GR (1994) Human mu opiate receptor—cDNA and genomic clones, pharmacologic characterization and chromosomal assignment. *FEBS Lett* **338:** 217–222

Whistler JL, von Zastrow M (1998) Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc Natl Acad Sci USA* **95:** 9914–9919

Zerial M, McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* **2:** 107–117