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CORRECTION

LRRK1-phosphorylated CLIP-170 regulates EGFR trafficking by recruiting p150^{Glued} to microtubule plus ends

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We apologise to the authors and readers for any confusion that this error might have caused.

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RESEARCH ARTICLE

LRRK1-phosphorylated CLIP-170 regulates EGFR trafficking by recruiting p150^{Glued} to microtubule plus ends

Shin Kedashiro[1], Strahil Iv. Pastuhov[1], Tomoki Nishioka[2], Takashi Watanabe[2], Kozo Kaibuchi[2], Kunihiro Matsumoto[1,*] and Hiroshi Hanafusa[1]

ABSTRACT

The binding of ligand to epidermal growth factor receptor (EGFR) causes the receptor to become activated and stimulates the endocytosis of EGFR. Early endosomes containing activated EGFR migrate along microtubules as they mature into late endosomes. We have recently shown that LRRK1, which is related to the familial Parkinsonism gene product Park8 (also known as LRRK2), regulates this EGFR transport in a manner dependent on LRRK1 kinase activity. However, the downstream targets of LRRK1 that might modulate this transport function have not been identified. Here, we identify CLIP-170 (also known as CLIP1), a microtubule plus-end protein, as a substrate of LRRK1. LRRK1 phosphorylates CLIP-170 at Thr1384, located in its C-terminal zinc knuckle motif, and this promotes the association of CLIP-170 with dynein–dynactin complexes. We find that LRRK1-mediated phosphorylation of CLIP-170 causes the accumulation of p150^{Glued} (also known as DCTN1) a subunit of dynactin, at microtubule plus ends, thereby facilitating the migration of EGFR-containing endosomes. Thus, our findings provide new mechanistic insights into the dynein-driven transport of EGFR.

KEY WORDS: CLIP-170, Dynein–dynactin, EGFR trafficking, LRRK1, Microtubule

INTRODUCTION

The interaction of the epidermal growth factor receptor (EGFR) with the epidermal growth factor (EGF) ligand causes a variety of cell signaling events to be activated, including those required for cell proliferation, survival and differentiation (Citri and Yarden, 2006; Lemmon and Schlessinger, 2010). Additionally, the binding of ligand to EGFR stimulates the endocytosis of the complex. Internalized receptors are first associated with early endosomes, which then mature into late endosomes, and EGFR is ultimately targeted to the lysosomes for degradation (Katzmann et al., 2002; Woodman and Futter, 2008; Madshus and Stang, 2009; Sorkin and von Zastrow, 2009; Platta and Stenmark, 2011; Tomas et al., 2014). This endosomal transport determines the intensity and duration of EGFR signaling (Miaczynska et al., 2004; Scita and Di Fiore, 2010). An important determinant of endocytic trafficking is the coupling of endocytic organelles with microtubule-dependent motors (Caviston and Holzbaur, 2006; Soldati and Schliwa, 2006; Akhmanova and Hammer, 2010; Hunt and Stephens, 2011). The minus-end-directed motor dynein is responsible for the early-to-late endosome transport of EGFR along microtubules (Driskell et al., 2007). However, the initiation step at which EGFR-containing early endosomes are coupled to the dynein motor complex has not been well characterized.

Cytoplasmic linker protein 170 (CLIP-170, also known as CLIP1) was originally proposed to link endosomes to microtubules (Pierre et al., 1992) and later was identified as the first plus-end tracking protein (+TIP) (Perez et al., 1999). CLIP-170 directly binds to p150^{Glued} (also known as DCTN1), which is another +TIP member, and recruits it to the growing microtubule plus ends (Valetti et al., 1999; Lansbergen et al., 2004; Watson and Stephens, 2006). p150^{Glued} is a large subunit of dynactin, which is a protein complex required for dynein-mediated microtubule-based transport (Schroer, 2004; Kardon and Vale, 2009). Because +TIPs have been proposed to promote the initial interaction of membranous organelles with microtubules (Pierre et al., 1992; Vaughan et al., 2002), CLIP-170 and p150^{Glued}, localized at the plus ends, have been implicated in the initiation of dynein-mediated retrograde transport along microtubules (Vaughan et al., 1999). Consistent with this, CLIP-170 has been shown recently to be involved in the capture of melanosomes for minus-end-directed transport along microtubules in *Xenopus* melanophores (Lomakin et al., 2009). In addition, plus-end-associated p150^{Glued} regulates the initiation of retrograde axonal transport in neurons (LIoyd et al., 2012; Moughamian and Holzbaur, 2012). These findings demonstrate that p150^{Glued}, recruited by CLIP-170 to microtubule plus ends, plays an important role in the initiation step of dynein-driven transport. CLIP-170 contains an N-terminal and a C-terminal functional domain, which flank a central coiled-coil region. The former of these consists of two conserved cytoskeleton-associated protein glycine-rich (CAP-Gly) domains that allow CLIP-170 to bind to microtubules. However, the C-terminal cargo-binding domain can inhibit the interaction between CLIP-170 and microtubules by binding to the N-terminal domain through two C-terminal motifs that are predicted to be Zn^{2+}-binding zinc knuckles (Lansbergen et al., 2004; Galjart, 2005; Weisbrich et al., 2007). p150^{Glued} is comprised of a single CAP-Gly domain that mediates its interaction with the second CLIP-170 C-terminal zinc knuckle domain (Lansbergen et al., 2004; Hayashi et al., 2007; Weisbrich et al., 2007). However, it has been unclear how CLIP-170 regulates p150^{Glued} localization at growing microtubule plus ends during the process of dynein-mediated transport.

We have recently demonstrated that LRRK1 regulates the trafficking of EGFR from the early to late endosomes by controlling the motility of EGFR-containing early endosomes

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(Hanafusa et al., 2011; Ishikawa et al., 2012). LRRK1 is related to the familial Parkinsonism gene product Park8 (also known as LRRK2) and belongs to the ROCO family of proteins. Contained within LRRK1 are a Ras of complex proteins (ROC) GTPase domain and a MAPKKK-like kinase domain (Bosgraaf and Van Haastert, 2003). Although we have shown that LRRK1 kinase activity is required for the motility of EGFR-containing endosomes, the downstream targets of LRRK1 that mediate these functions have not been identified. In this study, we identify CLIP-170 as a substrate of LRRK1. We find that LRRK1 phosphorylates CLIP-170 at Thr1384, located in its second zinc knuckle domain. Significantly, we show that the LRRK1-associated phosphorylation of CLIP-170 Thr1384 facilitates the interaction of CLIP-170 with dynein–dynactin complexes and promotes the accumulation of p150^{Glued} at microtubule plus ends. This, in turn, stimulates the dynein-driven transport of EGFR-containing endosomes. Our findings provide the first evidence that the phosphorylation of CLIP-170 plays an important role in the transport of EGFR by dynein–dynactin complexes.

RESULTS

LRRK1 phosphorylates CLIP-170 at Thr1384 located in the second zinc knuckle motif

Our previous results suggested that LRRK1 regulates the motility of EGFR-containing endosomes in a manner dependent on LRRK1 kinase activity (Hanafusa et al., 2011; Ishikawa et al., 2012). What is the target of LRRK1 phosphorylation that mediates this process? In a previous screen for LRRK1-binding proteins, we identified CLIP-170, a protein that is involved in dynein-mediated processes (Galjart, 2005; Wu et al., 2006; Akhmanova and Steinmetz, 2008; Lomakin et al., 2009; Hanafusa et al., 2011; Moughamian et al., 2013). We therefore examined the possibility that LRRK1 might regulate the motility of EGFR-containing endosomes by phosphorylating CLIP-170. First, we confirmed the interaction between LRRK1 and CLIP-170. Co-immunoprecipitation experiments revealed that endogenous LRRK1 was associated with endogenous CLIP-170 (Fig. 1A). Next, we examined whether CLIP-170 is a substrate of LRRK1. Because full-length recombinant CLIP-170 proteins obtained from *Escherichia coli* were not soluble, CLIP-170 proteins were immunopurified from HEK293 cells expressing FLAG–CLIP-170 and subjected to *in vitro* kinase assays. We took GFP-tagged LRRK1 that had been expressed and immunopurified from Cos7 cells and incubated this with CLIP-170 *in vitro*. We observed phosphorylation of CLIP-170 (Fig. 1B, lanes 1, 2). Strong phosphorylation of CLIP-170 was further observed when the protein was co-incubated with a hyperactive LRRK1 mutant, LRRK1(Y944F) (Ishikawa et al., 2012), but no phosphorylation was observed with a kinase-inactive mutant, LRRK1(K1243M) (Fig. 1B, lanes 3, 4). These results suggest that LRRK1 is one of the CLIP-170 kinases.

To identify the site(s) within CLIP-170 that are phosphorylated by LRRK1, two purified recombinant GST–CLIP-170 fragments, N and C (Fig. 1C), were incubated with LRRK1(Y944F) or LRRK1(K1243M). Both fragments were phosphorylated by LRRK1(Y944F) but not by LRRK1(K1243M) (Fig. 1C). The C-terminus of CLIP-170 showed greater phosphorylation by LRRK1 than the N-terminus (Fig. 1C, lanes 2, 4). We next determined potential phosphorylation sites in CLIP-170-N and -C. We incubated GST–CLIP-170-N and -C with LRRK1(Y944F) under *in vitro* kinase conditions and subjected the products to analysis by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). Five phosphorylation sites were found in CLIP-170-N (Thr13, Thr27, Thr36, Thr191 and Thr459) and six in CLIP-170-C (Thr1099, Ser1188, Ser1190, Ser1225, Ser1318 and Thr1384). Among these, Thr1384, located in the second zinc knuckle domain (Fig. 1D), produced the highest score (Table 1; supplementary material Fig. S1). To confirm that CLIP-170 Thr1384 is the major site of LRRK1 phosphorylation, we used site-directed mutagenesis to produce GST–CLIP-170-C(T1384A), in which Thr1384 is replaced with alanine. *In vitro* kinase assays showed that phosphorylation of GST–CLIP-170-C(T1384A) by wild-type LRRK1 or LRRK1(Y944F) was greatly reduced compared with that of wild-type GST–CLIP-170-C (Fig. 1D, lanes 2, 4–6). These results suggest that Thr1384, located in the C-terminal second zinc knuckle domain of CLIP-170, is the major phosphorylation site for LRRK1.

LRRK1 phosphorylation of CLIP-170 Thr1384 affects its interaction with the dynein–dynactin complex

The second zinc knuckle motif of CLIP-170 is important for its interaction with p150^{Glued}, a subunit of dynactin that is crucial for dynein motor activity (Lansbergen et al., 2004; Hayashi et al., 2007; Weisbrich et al., 2007). Because the Thr1384 site is located in the second zinc knuckle domain, we asked whether CLIP-170 Thr1384 phosphorylation affects its binding to dynein–dynactin complexes. To address this question, we generated mutations of CLIP-170-C at the T1384 residue that were designed to mimic either the dephosphorylated residue [GST–CLIP-170-C(T1384A)] or the phosphorylated residue [GST–CLIP-170-C(T1384E)], and we performed GST pull-down assays from lysates of HEK293 cells. Under these conditions, wild-type GST–CLIP-170-C and GST–CLIP-170-C(T1384A) interacted with endogenous p150^{Glued} or dynein intermediate chain (DIC, also known as DYNC1I1) very weakly (Fig. 2A, lanes 2, 3). In contrast, GST–CLIP-170C(T1384E) associated strongly with p150^{Glued} and DIC (Fig. 2A, lane 4), emphasizing the importance of CLIP-170 Thr1384 phosphorylation in the interaction between CLIP-170 and dynein–dynactin complexes.

We further examined these interactions using full-length CLIP-170. HEK293 cells were transfected with GFP–CLIP-170, GFP–CLIP-170(T1384A) or GFP–CLIP-170(T1384E), and lysates were subjected to immunoprecipitation with anti-GFP antibodies. Consistent with previous reports (Lansbergen et al., 2004; Lee et al., 2010), endogenous p150^{Glued} and DIC coprecipitated with wild-type GFP–CLIP170 (Fig. 2B, lane 2). The interaction of DIC with CLIP-170 is dependent on p150^{Glued} because depletion of p150^{Glued} by small interfering (si)RNA abolished the interaction between DIC and p150^{Glued} (supplementary material Fig. S2). The association of GFP–CLIP-170(T1384A) with p150^{Glued} or DIC was slightly reduced compared with that of wild-type GFP–CLIP-170 (Fig. 2B, lane 3). In contrast, the T1384E mutation resulted in a higher association of CLIP-170 with p150^{Glued} and DIC (Fig. 2B, lane 4), confirming the results obtained with the GST pull-down assay.

Next, we examined the effect of LRRK1 phosphorylation on the association of CLIP-170 with the dynein–dynactin complex. Knockdown of LRRK1 by siRNA resulted in a slight reduction in the association between wild-type GFP–CLIP-170 and endogenous p150^{Glued} or DIC (Fig. 2C, lanes 2, 3), suggesting that LRRK1 is involved in these interactions. Furthermore, we found that the interaction of GFP–CLIP-170(T1384E) with p150^{Glued} and DIC was increased compared with that of wild-type GFP–CLIP-170,

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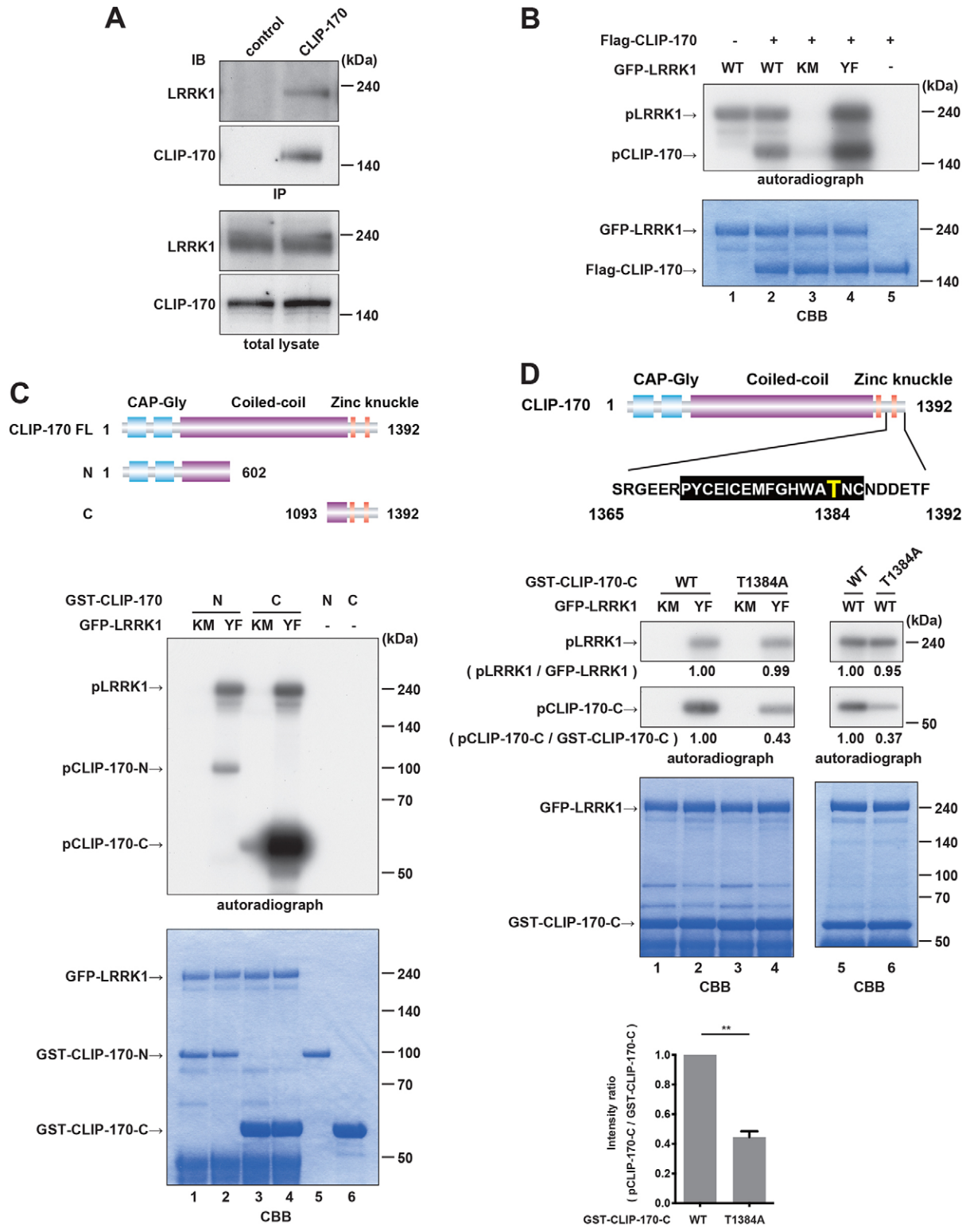

Fig. 1. See next page for legend.

and was not affected by LRRK1 depletion (Fig. 2C, lanes 4, 5). Taken together, these results suggest that LRRK1 promotes the interaction of CLIP-170 with dynein–dynactin complexes by phosphorylating CLIP-170 Thr1384 in the second zinc knuckle domain.

LRRK1 phosphorylation of CLIP-170 Thr1384 promotes the accumulation of p150^{Glued} at microtubule plus ends

CLIP-170 is known to be a plus-end tracking protein (+TIP) that associates specifically with the ends of growing microtubules and is required for processive microtubule growth (Perez et al., 1999;

Journal of Cell Science

Journal of Cell Science (2015) 128, 385–396 doi:10.1242/jcs.161547

Fig. 1. LRRK1 phosphorylates CLIP-170 at Thr1384. (A) Interaction of LRRK1 with CLIP-170. Cos7 cells were immunoprecipitated (IP) with control mouse anti-IgG or anti-CLIP-170 antibodies, followed by immunoblotting (IB) with anti-LRRK1 and anti-CLIP-170 antibodies. Molecular-mass markers are shown in kDa. (B) LRRK1-mediated phosphorylation of CLIP-170 in an *in vitro* kinase assay. Cos7 cells were transfected with wild-type (WT) GFP–LRRK1, a kinase-inactive GFP–LRRK1 K1243M (KM) or a hyperactive GFP–LRRK1 Y944F (YF), as indicated. LRRK1 proteins were immunoprecipitated with anti-GFP antibodies. We purified CLIP-170 proteins from HEK293 cells expressing FLAG–CLIP-170 using anti-FLAG antibodies followed by FLAG peptide elution. Immunoprecipitated LRRK1 proteins were incubated with CLIP-170 proteins in the presence of [γ-^{32}P]ATP for 20 min at 30˚C. Autophosphorylated LRRK1 (pLRRK1) and phosphorylated CLIP-170 (pCLIP-170) were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) to visualize total amounts of both LRRK1 and CLIP-170 proteins. (C) LRRK1-mediated phosphorylation of CLIP-170 fragments. Schematic diagram of CLIP-170 and deletion constructs of CLIP-170 are shown in the upper panel. GFP–LRRK1 KM and YF were prepared as above and incubated with recombinant GST–CLIP-170-N or GST–CLIP-170-C proteins purified from *E. coli*. *In vitro* kinase assays were carried out as in B. (D) LRRK1-mediated phosphorylation of CLIP-170 at Thr1384. A schematic diagram of CLIP-170 and the sequence around the C-terminal second zinc knuckle region (black box) are shown in the upper panel. Wild-type GFP–LRRK1 and KM and YF mutants were prepared as above and incubated with recombinant GST–CLIP-170-C or GST–CLIP-170-C T1384A proteins purified from *E. coli*. *In vitro* kinase assays were carried out as in B. Relative levels of phosphorylation by LRRK1 YF are shown in the lower panel. The levels of phosphorylated CLIP-170-C were quantified and normalized to those of total GST–CLIP-170-C. Data show the mean±s.e.m. (data are combined from five independent experiments); $^{**}P<0.01$ (Welch's *t*-test).

Komarova et al., 2002; Vaughan, 2004; Bieling et al., 2008). We therefore investigated whether phosphorylation of CLIP-170 Thr1384 affects its localization on microtubules and/or the microtubule growth rate. We depleted endogenous human (h)CLIP-170 in HeLa S3 cells using siRNA (Fig. 3A) and expressed various siRNA-resistant GFP-tagged mouse (m)CLIP-170 constructs. Expression levels of the different GFP–mCLIP-170 proteins were similar to those of endogenous CLIP-170 (Fig. 3B). Under these conditions, as reported previously (Perez et al., 1999), wild-type GFP–mCLIP-170 was localized in a 'comet tail' pattern at the microtubule plus ends and colocalized with EB1 (also known as MAPRE1), a +TIP marker (Morrison et al., 1998) (Fig. 3C). This localization pattern is similar to that seen with endogenous CLIP-170 (Fig. 3C). The staining patterns of both GFP–mCLIP-170(T1384A) and GFP–mCLIP-170(T1384E) on microtubule plus ends were indistinguishable from that of wild-type CLIP-170 (Fig. 3D). To examine microtubule growth, we examined the behavior of GFP–mCLIP-170 by time-lapse microscopy (supplementary material Movie 1). This live-cell imaging analysis of GFP–mCLIP-170 demonstrated that neither the T1384A nor the T1384E mutation had any effect on the speed of the comets decorated by GFP–mCLIP-170 (supplementary material Fig. S3). These results suggest that phosphorylation of CLIP-170 at Thr1384 affects neither its plus-end localization nor microtubule growth.

Previous studies have shown that CLIP-170 is required for the recruitment of p150^{Glued} to growing microtubule plus ends (Valetti et al., 1999; Lansbergen et al., 2004; Watson and Stephens, 2006). We therefore examined the localization of endogenous p150^{Glued} in HeLa S3 cells that had been depleted of endogenous hCLIP-170 and that expressed various GFP–mCLIP-170 constructs. When wild-type GFP–mCLIP-170 was expressed, p150^{Glued} colocalized with GFP–mCLIP-170 at the microtubule plus ends (Fig. 4A). This localization pattern is consistent with previous observations (Vaughan et al., 1999; Lansbergen et al., 2004). Interestingly, we found that expression of GFP–mCLIP-170(T1384A) decreased the peak intensity of p150^{Glued} staining at CLIP-170-decorated microtubule tips, compared with that of cells expressing wild-type GFP–mCLIP-170 (Fig. 4A,B). In contrast, recruitment of p150^{Glued} at microtubule plus ends was significantly increased in cells expressing GFP–mCLIP-170(T1384E) (Fig. 4A,B). These results suggest that phosphorylation of CLIP-170 increases the recruitment of p150^{Glued} to the microtubule plus ends.

We further examined whether LRRK1 is important for the targeting of p150^{Glued} to microtubule tips. We observed no difference in the distribution of wild-type GFP–mCLIP170 between control and LRRK1-depleted cells (Fig. 4C; supplementary material Fig. S4A). Furthermore, LRRK1 depletion did not affect the microtubule growth rate (supplementary material Fig. S3). However, accumulation of p150^{Glued} at microtubule tips was decreased in LRRK1-siRNA-treated cells compared with control siRNA-treated cells (Fig. 4C,D). Thus, LRRK1 is necessary for the efficient recruitment of p150^{Glued} to microtubule plus ends.

Table 1. Identification of LRRK1 phosphorylation sites of CLIP-170-N and CLIP-170-C by LC-MS/MS

Sequence[a]	pRS site probabilities[b]	XCorr[c]	MH$^+$ [Da][d]	Identified site(s)
Phosphorylation sites of GST–CLIP-170-N				
TPp**T**AVVAPVEK	T(1): 0.0; T(3): 100.0	2.63	1191.60515	T27
TPTAVVAPVEKp**T**ISSEK	T(1): 0.0; T(3): 0.0; T(12): 99.7; S(14): 0.2; S(15): 0.2	2.04	1836.93242	T36
p**T**ASESISNLSEAGSIK	T(1): 99.9; S(3): 0.1; S(5): 0.0; S(7): 0.0; S(10): 0.0; S(14): 0.0	1.96	1673.76250	T191
APp**T**KILKPGSTALK	T(3): 100.0; S(10): 0.0; T(11): 0.0	1.83	1504.84989	T13
VEEESITKGDLEVAp**T**VSEK	S(5): 0.0; T(7): 0.0; T(15): 100.0; S(17): 0.0	1.75	2143.00577	T459
Phosphorylation sites of GST–CLIP-170-C				
GEERPYCEICEMFGHWAp**T**NCNDDETF[e]	Y(6): 0.0; T(18): 100.0; T(25): 0.0	8.18	3363.22007	T1384
VEMMSEAALNGNGDDLNNYDp**S**DDQEK	S(5): 0.0; Y(19): 0.2; S(21): 99.8	2.85	2986.12809	S1318
SIp**S**ITSALLTEKDAELEK	S(1): 0.4; S(3): 98.8; T(5): 0.4; S(6): 0.4; T(10): 0.0	2.17	2028.01535	S1190
p**S**ISITSALLTEK	S(1): 100.0; S(3): 0.0; T(5): 0.0; S(6): 0.0; T(10): 0.0	2.14	1342.68913	S1188
SLHp**S**VVQTLESDKVK	S(1): 0.6; S(4): 99.4; T(8): 0.0; S(11): 0.0	2.11	1749.87650	S1225
SKELLp**T**VENQK	S(1): 0.0; T(6): 100.0	1.97	1368.67803	T1099

[a]The peptide in the protein sequence. Phosphoseryl and phosphothreonyl residues are denoted as pS and pT, respectively, and are shown in bold. [b]An estimation of the probability (0–100%) for the respective site being truly phosphorylated. [c]Cross-correlation score (Xcorr) of the peptide is based on the match of the obtained product ion spectrum to the theoretical product ion spectrum for the corresponding peptide contained in the database. [d]The average mass for nano-electrospray tandem mass analysis. [e]The C-terminal amino acid residue.

Journal of Cell Science (2015) 128, 385–396 doi:10.1242/jcs.161547

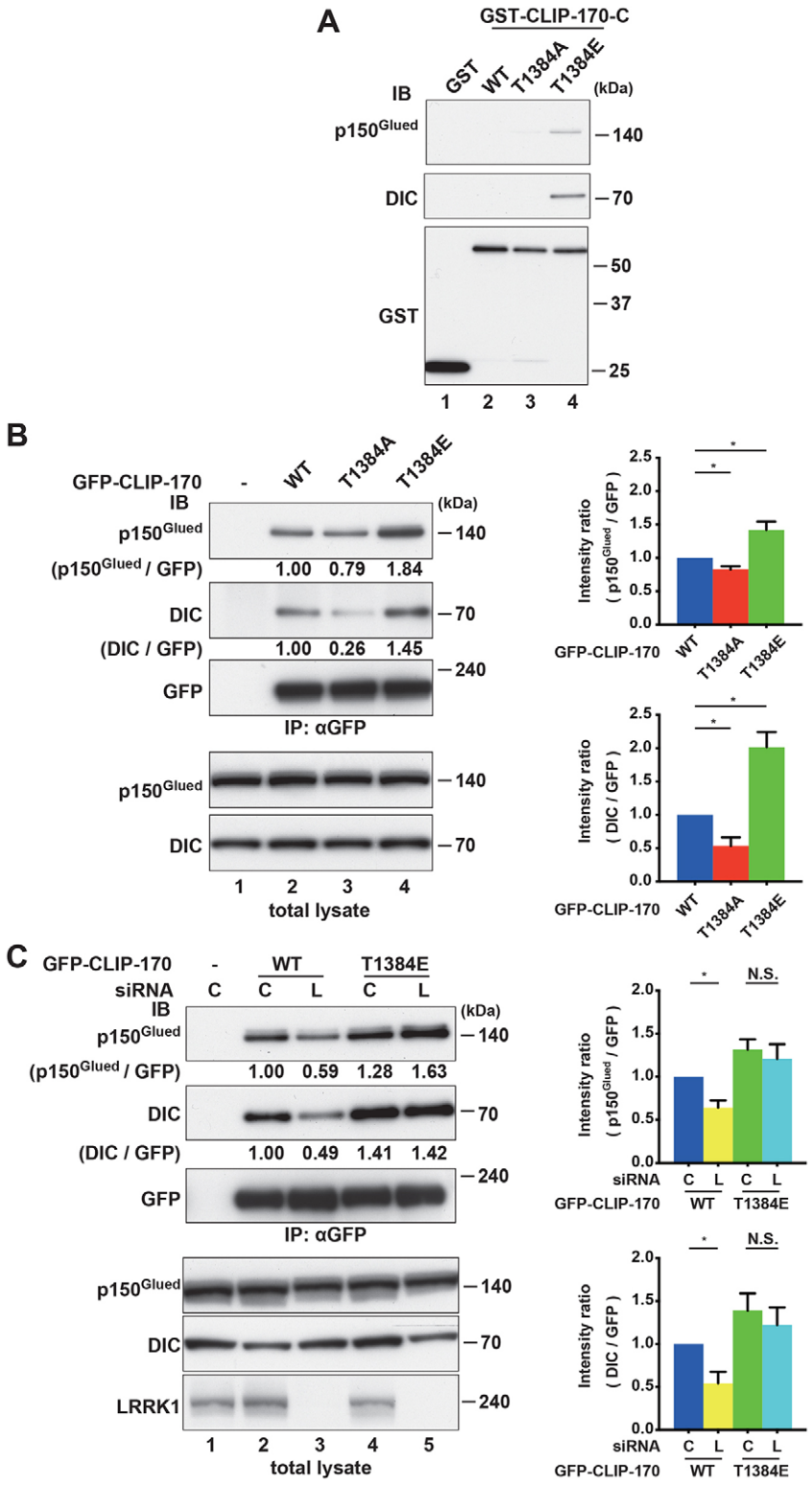

Fig. 2. CLIP-170 Thr1384 phosphorylation enhances its association with dynein–dynactin complexes. (A) Interaction of CLIP-170-C with dynactin and dynein. A GST pull-down assay was performed on lysates of HEK293 cells using GST alone, wild-type (WT) GST-CLIP-170-C or T1384A or T1384E GST-CLIP-170-C mutants. GST fusion proteins, dynactin and dynein were detected by immunoblotting (IB) with anti-GST, anti-p150^{Glued} and anti-dynein intermediate chain (DIC) antibodies, as indicated. Molecular-mass markers are shown in kDa. (B) Interaction of CLIP-170 with dynactin and dynein. HEK293 cells were transfected with wild-type GFP–CLIP-170 or GFP–CLIP-170 T1384A or T1384E mutants, as indicated. Complex formation was detected by immunoprecipitation (IP) with anti-GFP (α-GFP) antibodies, followed by immunoblotting with anti-p150^{Glued} and anti-DIC antibodies. Total cell lysates were immunoblotted with anti-p150^{Glued} and anti-DIC antibodies. Relative levels of association were quantified and are shown in the right panels. The levels of p150^{Glued} and DIC were quantified and normalized to those of GFP. The data show the mean±s.e.m. (combined from five independent experiments); *P<0.05 (one-way ANOVA). (C) Effect of LRRK1 depletion on the interaction of CLIP-170 with dynactin and dynein. HEK293 cells were treated with control ('C') or LRRK1 ('L') siRNA and then transfected with wild-type GFP–CLIP-170 or GFP–CLIP-170 T1384E, as indicated. Complex formation was detected as in B. Total cell lysates were immunoblotted with anti-p150^{Glued}, anti-DIC and anti-LRRK1 antibodies. Relative levels of association (right panels) were determined as in B. Data show the mean±s.e.m. (combined from seven independent experiments); *P<0.05; N.S., not significant (one-way ANOVA).

Importantly, we found that when the phosphorylation-mimicking mutant GFP–mCLIP170(T1384E) was expressed, the localization of p150^{Glued} at microtubule tips was unaffected by LRRK1 depletion (Fig. 4E,F). Taken together, these results suggest that LRRK1-mediated phosphorylation of CLIP-170 at Thr1384 promotes the recruitment of dynein–dynactin complexes to the plus ends of microtubules.

Transport of EGFR-containing endosomes depends on LRRK1-mediated phosphorylation of CLIP-170 Thr1384

Recent studies have shown that CLIP-170-dependent localization of p150^{Glued} at microtubule plus ends is required for the efficient initiation of dynein-mediated transport (Moughamian et al., 2013). One attractive potential role for LRRK1-mediated phosphorylation of CLIP-170 is the promotion of the transport

Journal of Cell Science (2015) 128, 385–396 doi:10.1242/jcs.161547

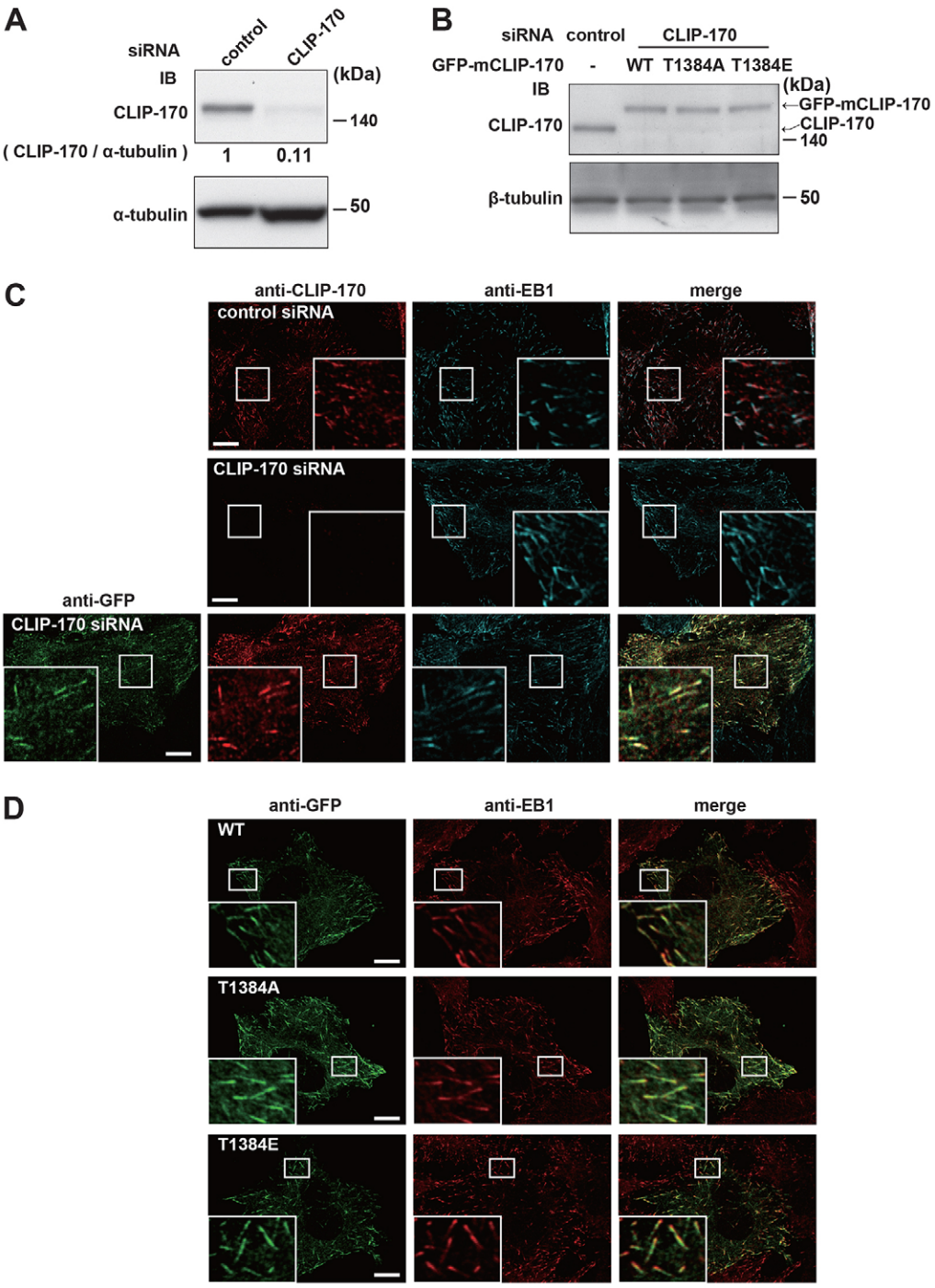

Fig. 3. Effect of Thr1384 phosphorylation on CLIP-170 plus-end localization. (A) Effect of CLIP-170 siRNA. HeLa S3 cells were treated with control or CLIP-170 siRNA, as indicated. Cell lysates were immunoblotted (IB) with anti-CLIP-170 and anti-α-tubulin antibodies. Relative amounts of CLIP-170 are shown. The CLIP-170 bands were quantified and normalized to those of α-tubulin. Molecular-mass markers are shown in kDa. (B) Expression levels of CLIP-170. HeLa S3 cells were treated with control or CLIP-170 siRNA and then transfected with GFP–mCLIP-170 constructs [wild type (WT) or T1384A or T1384E mutants], as indicated. Cell lysates were immunoblotted with anti-CLIP-170 and anti-β-tubulin antibodies. (C) CLIP-170 images. HeLa S3 cells were treated with control or CLIP-170 siRNA and then transfected with GFP–mCLIP-170, as indicated. The cells were stained with anti-GFP (green), anti-CLIP-170 (red) and anti-EB1 (cyan) antibodies. The boxed regions are magnified in insets. (D) The effect of Thr1384 phosphorylation on CLIP-170 plus-end localization. HeLa S3 cells were treated with CLIP-170 siRNA and then transfected with mouse GFP–mCLIP-170 constructs (wild type or T1384A or T1384E mutants), as indicated. The cells were stained with anti-GFP (green) and anti-EB1 (red) antibodies. The boxed regions are magnified in insets. Scale bars: 10 μm.

of EGFR-containing endosomes, which would result from increased localization of dynein–dynactin complexes to the plus ends of microtubules. Indeed, long-range rapid movement of EGFR-containing endosomes is reported to depend on dynein (Driskell et al., 2007). First, to confirm this, we analyzed the motility of EGFR-containing endosomes in cells lacking $p150^{Glued}$. Using time-lapse confocal fluorescence microscopy, the movement of EGF-containing endosomes was followed starting at 10 min after a brief pulse of fluorescent EGF (Alexa-Fluor-647–EGF), followed by washing. Under these conditions, we could detect Alexa-Fluor-647–EGF bound to EGFR that had been endocytosed and localized to endosomes (Hanafusa et al., 2011). In control cells, long-range rapid movement of Alexa-Fluor-647–EGF from the cell periphery to the cell center was frequently observed (Fig. 5A,H; supplementary material Movie 2). As expected, depletion of $p150^{Glued}$ by siRNA (supplementary material Fig. S4B) resulted in the disruption of the long-range movement of Alexa-Fluor-647–EGF (Fig. 5B,H; supplementary material Movie 3). In control cells, 15% of Alexa-Fluor-647–EGF-positive endosomes moved >4.0 μm during the imaging period (30 s), but this fraction decreased to only 2.4% in $p150^{Glued}$-depleted cells (Fig. 5I). Thus, loss of $p150^{Glued}$ seems to perturb the function of dynactin in the dynein-mediated retrograde transport of EGFR-containing endosomes.

Journal of Cell Science (2015) 128, 385–396 doi:10.1242/jcs.161547

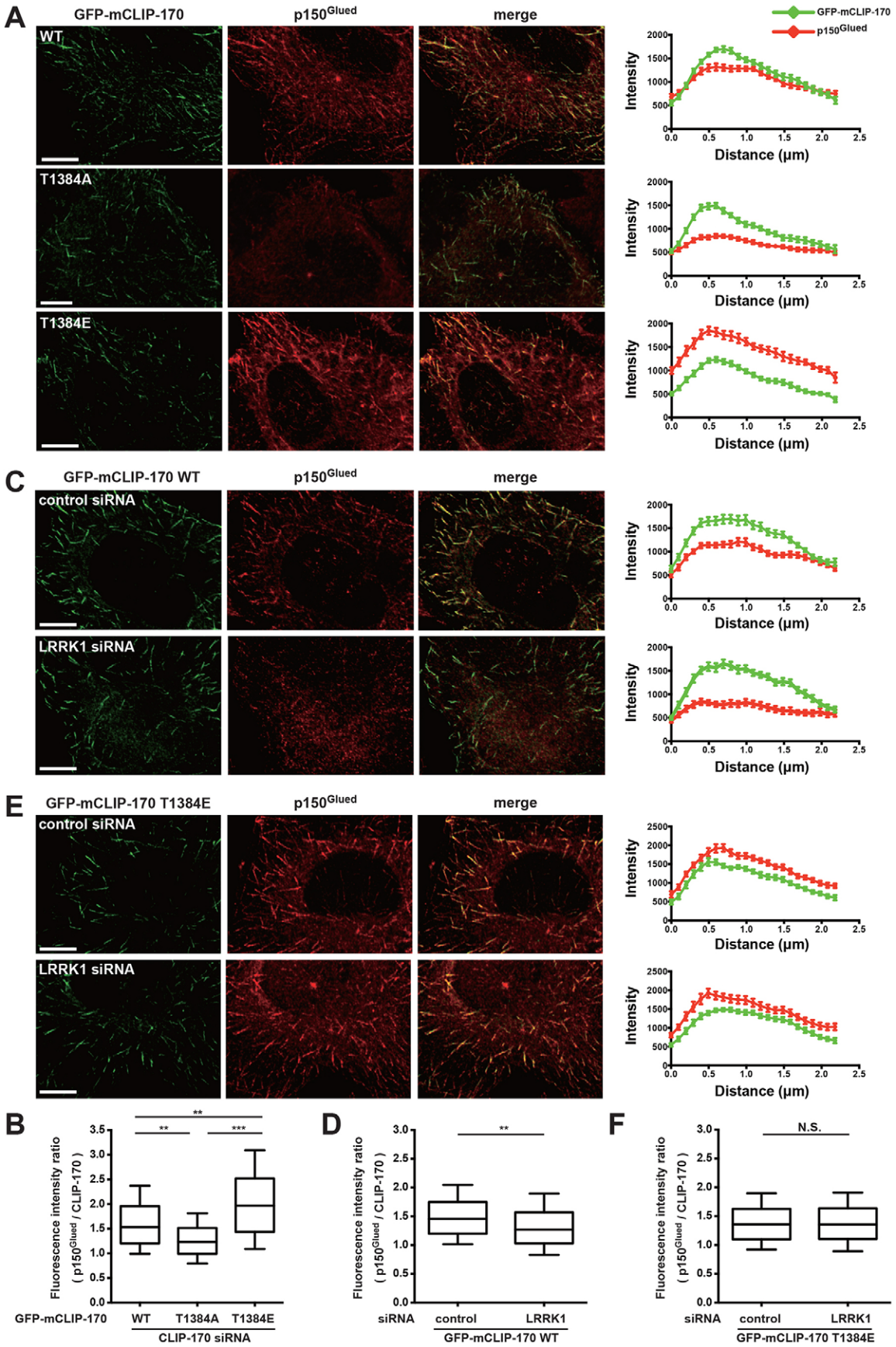

Fig. 4. See next page for legend.

Journal of Cell Science (2015) 128, 385–396 doi:10.1242/jcs.161547

Fig. 4. Effects of CLIP-170 Thr1384 phosphorylation on p150^{Glued} plus-end localization. (A,C,E) Localization of p150^{Glued} at microtubule plus ends. HeLa S3 cells were treated with CLIP-170 siRNA (A), control siRNA or LRRK1 siRNA (C,E) and then transfected with GFP–mCLIP-170 constructs [wild type (WT) or T1384A or T1384E mutants], as indicated. The cells were stained with anti-GFP (green) and anti-p150^{Glued} (red) antibodies. Distributions of CLIP-170 and p150^{Glued} at microtubule tips (n=20) were analyzed by line scans across the area. Scale bars: 10 μm. Average staining intensity was quantified and is shown in the panels on the right. Data show the mean±s.e.m. (B,D,F) Quantification of p150^{Glued} localization on microtubule tips. Relative amounts of p150^{Glued} bound to GFP–mCLIP-170-decorated microtubule tips were calculated as the ratio of the integrated intensities of p150^{Glued}:GFP in individual tips. n=2500–3000 tips in 10 cells (250–300 tips/cell). Analysis was performed using the same set of cells as shown in A,C,E. The boundaries of the box indicate the 25th and 75th percentiles, and whiskers indicate the 10th and 90th percentiles. The median is shown by a line. **P<0.01; ***P<0.001; N.S., not significant (Kruskal–Wallis test and Mann–Whitney U test).

Furthermore, we found that endogenous LRRK1 is associated with endogenous p150^{Glued} (Fig. 5C). These results suggest that LRRK1 participates in the dynein-dependent transport of EGFR-containing endosomes.

Next, we asked whether CLIP-170 itself is important for the motility of EGFR-containing endosomes. When hCLIP-170 was knocked down in HeLa S3 cells by siRNA, the motility of Alexa-Fluor-647–EGF was significantly decreased (Fig. 5D,H,I; supplementary material Movie 4). To investigate the functional relevance of CLIP-170 Thr1384 phosphorylation in the transport of EGFR-containing endosomes, we expressed an siRNA-resistant mCLIP-170, a non-phosphorylatable mutant T1384A or a phospho-mimicking mutant T1384E in hCLIP-170-depleted cells. We found that wild-type mCLIP-170 and mCLIP-170(T1384E) were able to rescue the long-range rapid movement of Alexa-Fluor-647–EGF, whereas mCLIP-170(T1384A) was not (Fig. 5E–I; supplementary material Movies 5–7). Furthermore, the extent of long-range movement of Alexa-Fluor-647–EGF in mCLIP-170(T1384A)-expressing cells was similar to that observed in p150^{Glued}-depleted cells (Fig. 5H,I). These results suggest that phosphorylation of CLIP-170 Thr1384 is required for dynein-mediated transport of EGFR-containing endosomes.

We have demonstrated previously that LRRK1 kinase activity is required for the motility of EGFR-containing endosomes (Hanafusa et al., 2011; Ishikawa et al., 2012). Consistent with this, expression of a kinase-inactive LRRK1(K1243M) inhibited the long-range rapid movement of Alexa-Fluor-647–EGF (Fig. 5J,K). If LRRK1 regulates the transport of EGFR-containing endosomes by phosphorylating CLIP-170 at Thr1384, we predicted that the phospho-mimicking mutant mCLIP-170(T1384E) should be able to compensate for the inhibition of LRRK1 kinase activity during this process. We found that expression of RFP–mCLIP-170(T1384E), but not of RFP–mCLIP-170(T1384A), suppressed the defect in the long-range rapid movement of Alexa-Fluor-647–EGF in cells expressing GFP–LRRK1(K1243M) (Fig. 5J,K). Taken together, these results suggest that LRRK1-mediated phosphorylation of CLIP-170 Thr1384 is required for its ability to facilitate the long-range movement of EGFR-containing endosomes.

DISCUSSION

We previously found that LRRK1 kinase activity is essential for EGFR intracellular trafficking (Hanafusa et al., 2011; Ishikawa et al., 2012). In the present study, we demonstrate that LRRK1 phosphorylates CLIP-170 at Thr1384 within its C-terminal zinc knuckle domain. LRRK1-mediated phosphorylation of CLIP-170 at Thr1384 increases its association with dynein–dynactin complexes, and thereby promotes the accumulation of these complexes at microtubule plus ends, facilitating the dynein-driven transport of EGFR-containing endosomes.

CLIP-170 contains two CAP-Gly domains in its N-terminus and two tandem repeated zinc knuckle motifs in its C-terminus (Pierre et al., 1992). p150^{Glued} also contains a CAP-Gly domain in its N-terminus (Akhmanova and Steinmetz, 2008). The CAP-Gly domains of CLIP-170 and p150^{Glued} have been implicated in highly dynamic and regulated biological processes such as dynein–dynactin motor movement (Perez et al., 1999; Vaughan et al., 2002; Akhmanova and Hoogenraad, 2005; Lansbergen and Akhmanova, 2006; Bieling et al., 2008). The p150^{Glued} CAP-Gly domain contributes to the targeting of dynein–dynactin complexes to the plus ends by interacting with the C-terminal second zinc knuckle domain of CLIP-170 (Vaughan et al., 1999; Lansbergen et al., 2004; Weisbrich et al., 2007). Free in solution, the first zinc knuckle domain of CLIP-170 binds to its own N-terminal CAP-Gly domain. Once CLIP-170 binds to microtubules, however, the exposed second zinc knuckle domain might serve to recruit dynein–dynactin complexes to the plus ends of microtubules by interacting with p150^{Glued}. In addition to CLIP-170, p150^{Glued} directly binds to EB1 and α-tubulin through its CAP-Gly domain (Ligon et al., 2003; Akhmanova and Steinmetz, 2008). These interactions are mutually exclusive and compete with one another with comparable moderate affinities (Hayashi et al., 2007; Weisbrich et al., 2007). These moderate, but specific interactions are important for the quick disruption and reformation of complexes in response to the movements of their target molecules. These multiple and partly competing interaction modes of the CAP-Gly domain appear to be ideal for the recruitment and clustering of different molecular functions at a common location, such as the growing microtubule end. We speculate that the modest increase in the affinity of CLIP-170 for p150^{Glued} caused by LRRK1-mediated phosphorylation is sufficient to shift these interactions towards complex formation and the initiation of dynein-mediated transport.

CLIP-170 can be phosphorylated on multiple residues (Rickard and Kreis, 1991; Choi et al., 2002; Yang et al., 2009). Previous studies have demonstrated that CLIP-170 is phosphorylated in the third serine-rich region adjacent to the second CAP-Gly microtubule-binding domain (Lee et al., 2010; Nakano et al., 2010). This phosphorylation induces conformational changes in CLIP-170 by increasing the intramolecular affinity of the N-terminus for the C-terminus, which promotes the dissociation of CLIP-170 from the microtubule plus ends (Lee et al., 2010). However, the LRRK1 phosphorylation sites are not located in the third serine-rich region. Here, we report that LRRK1 phosphorylation of CLIP-170 Thr1384 enhances its association with the dynein–dynactin complex without affecting CLIP-170 localization at the plus ends. Expression of a non-phosphorylatable mutant, CLIP-170(T1384A), led to a decrease in the accumulation of p150^{Glued} at microtubule plus ends, similar to that seen with an LRRK1 knockdown. A mutant that mimics constitutive phosphorylation of the Thr1384, CLIP-170(T1384E), significantly rescued the effect of LRRK1 depletion. This strongly indicates that LRRK1 participates in the recruitment of dynein–dynactin complexes at microtubule plus ends by phosphorylating CLIP-170. Experiments using time-lapse confocal fluorescence microscopy showed that CLIP-170(T1384A)-expressing cells are defective in EGFR transport

Journal of Cell Science (2015) 128, 385–396 doi:10.1242/jcs.161547

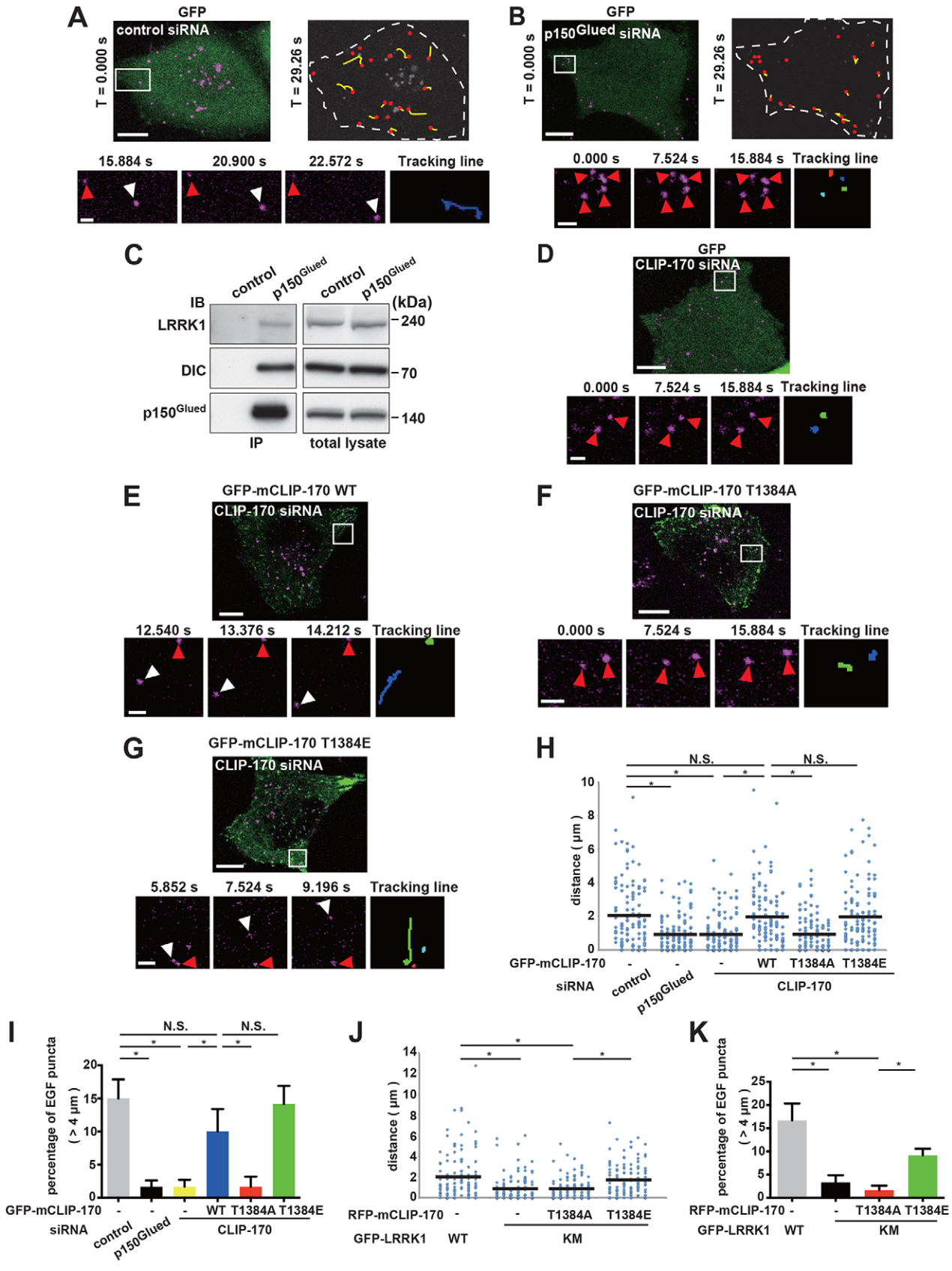

Fig. 5. See next page for legend.

Fig. 5. Effects of CLIP-170 Thr1384 phosphorylation on the transport of EGFR-containing endosomes. (A,B,D,E–G) CLIP-170 Thr1384 phosphorylation is required for the long-range movement of Alexa-Fluor-647–EGF. HeLa S3 cells were treated with control siRNA (A), p150^{Glued} siRNA (B) or CLIP-170 siRNA (D–G) and then transfected with GFP alone (A,B,D), wild-type (WT) GFP–mCLIP-170 (E), GFP–mCLIP-170 T1384A (F) or GFP–mCLIP-170 T1384E (G), as indicated. The cells were pulse-labeled with Alexa-Fluor-647–EGF. The movement of Alexa-Fluor-647–EGF-positive endosomes was observed by time-lapse confocal fluorescence microscopy, starting at 10 min after the initial exposure to Alexa-Fluor-647–EGF, with frames captured at 0.836-s intervals for 29.26 s. Upper panels show the images representing Alexa-Fluor-647–EGF (magenta) and GFP alone or GFP–mCLIP-170 (green) at 0 s. The lower left three panels represent stills from the boxed regions. Lower right panels show the tracking lines of Alexa-Fluor-647–EGF. White and red arrowheads indicate rapid moving and immobile Alexa-Fluor-647–EGF puncta, respectively. Images representing Alexa-Fluor-647–EGF (red circles) and tracks (yellow) at 29.26 s after imaging started are shown in the upper right panels (A,B). Scale bars: 10 μm (upper panels), 2 μm (lower panels). (C) Interaction of LRRK1 with p150^{Glued}. HEK293 cells were immunoprecipitated (IP) with control mouse anti-IgG or anti-p150^{Glued} antibodies, followed by immunoblotting (IB) with anti-LRRK1, anti-DIC and anti-p150^{Glued} antibodies. Molecular-mass markers are shown in kDa. (H,J) A mobility dot plot of randomly selected Alexa-Fluor-647–EGF-positive endosomes. HeLa S3 cells were treated with control siRNA, p150^{Glued} siRNA or CLIP-170 siRNA and then transfected with GFP alone or GFP–mCLIP-170 constructs [wild type (WT) or T1384A or T1384E mutants], as indicated (H). Alternatively, cells were transfected with GFP–LRRK1 (WT) or GFP–LRRK1 K1243M (KM) together with RFP alone or RFP–mCLIP-170 constructs (T1384A or T1384E mutants), as indicated (J). Mobility is defined as the distance of the trajectory of endosomes (n>120) during the 30-s observation periods, quantified using the Manual Tracking ImageJ plug-in. The horizontal bars indicate the mean of total endosomes (data are combined from six independent experiments); *P<0.05; N.S., not significant (Kruskal–Wallis test). (I,K) Quantification of long-range movement (>4.0 μm) of Alexa-Fluor-647–EGF-positive endosomes. Data show the mean±s.e.m. (six independent experiments with an average of 20 EGF-containing endosomes scored per experiment); *P<0.05; N.S., not significant (Kruskal–Wallis test).

and that expression of CLIP-170(T1384E) is able to suppress the inhibition of EGFR transport caused by expression of a kinase-inactive LRRK1(K1243M). These results indicate that LRRK1 phosphorylation of CLIP-170 Thr1384 is involved in the long-range movement of EGFR-containing endosomes.

Recently, Li et al. (Li et al., 2010) demonstrated that casein kinase 2 (CK2) phosphorylates CLIP-170 at Ser1318 near the first zinc knuckle domain during the G2/M phase. The first zinc knuckle region is important for the intramolecular association with its own N-terminal CAP-Gly domain, which causes conformational changes in the protein (Lansbergen et al., 2004; Galjart, 2005; Weisbrich et al., 2007). CK2 phosphorylation of CLIP-170 Ser1318 also enhances its interaction with p150^{Glued} during mitosis, which in turn facilitates the localization of CLIP-170 at the kinetochore, thereby stabilizing kinetochore–microtubule attachments (Li et al., 2010). Interestingly, we also identified Ser1318 as another LRRK1 phosphorylation site in our mass spectrometry analysis. The roles of LRRK1 phosphorylation at other sites await further investigation.

Microtubule plus ends continuously lengthen and shorten, a behavior known as dynamic instability (Mitchison and Kirschner, 1984; Kirschner and Mitchison, 1986). This dynamic behavior enables the microtubule plus end to search the cellular space until it achieves a productive interaction with an appropriate intracellular target, such as a vesicular cargo. Plus-end pools of CLIP-170 and p150^{Glued} have been implicated in this 'search-and-capture' mechanism in endomembranes prior to the initiation of intracellular transport (Pierre et al., 1992; Vaughan et al., 2002). However, removal of CLIP-170 or p150^{Glued} from the plus ends causes no defects in the efficiency of transferrin uptake or the motility of early endosomes or lysosomes in HeLa cells (Watson and Stephens, 2006; Kim et al., 2007; Dixit et al., 2008), raising questions about this hypothesis. More recently, it has been shown that CLIP-170 is important in the capture of dynamic microtubules during the initiation of minus-end-directed transport in *Xenopus* melanophores (Lomakin et al., 2009). Furthermore, p150^{Glued} is required for the enrichment of dynactin at plus ends and promotes the efficient initiation of retrograde transport by dynein in Cos7 cells and neurons (Vaughan et al., 2002; LIoyd et al., 2012; Moughamian and Holzbaur, 2012; Moughamian et al., 2013). These findings support a role for CLIP-170 and p150^{Glued} in membrane trafficking. Interestingly, we found that although LRRK1 is essential for the motility of EGFR-containing endosomes in HeLa cells, depletion of LRRK1 has no effect on the efficiency of transferrin uptake or the motility of early endosomes in the absence of EGF stimulation (Hanafusa et al., 2011; Ishikawa et al., 2012). Our present study further indicates that CLIP-170 and p150^{Glued} are required for LRRK1-mediated transport of EGFR-containing endosomes. Thus, CLIP-170 and p150^{Glued} localized at microtubule plus ends might be required only for high-flux trafficking events, such as the transport of melanosomes in *Xenopus* melanophores or EGFR-containing endosomes in HeLa cells. How can LRRK1-mediated phosphorylation of CLIP-170 facilitate the transport of a selective subset of EGFR-containing endosomes? Because LRRK1 interacts with EGFR, CLIP-170 and dynein–dynactin complexes, LRRK1 might recruit the dynein–dynactin complex to EGFR-containing endosomes prior to the loading of endosomes onto microtubules. LRRK1 phosphorylates CLIP-170, which would promote its association with LRRK1-bound dynein–dynactin complexes. LRRK1 phosphorylation of CLIP-170 would help to load EGFR-containing endosomes together with dynein–dynactin complexes onto growing microtubule plus ends. Only a subset of CLIP-170 molecules would become phosphorylated by LRRK1, and this phosphorylation would underlie the selective transport of EGFR-containing endosomes containing LRRK1 and dynein–dynactin complexes. Further investigation should provide new mechanistic insights into the initiation of the dynein-driven transport of EGFR.

MATERIALS AND METHODS

Plasmids and mutations

The vector encoding FLAG–CLIP-170 was kindly provided by Seiji Takashima (Osaka University, Japan). Mouse CLIP-170 cDNA was subcloned into the vectors pEGFP-C1 (Clontech) and pTagRFP-C (Evrogen). CLIP-170(T1384A) and CLIP-170(T1384E) were generated using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene, La Jolla, CA). Deletion constructs of CLIP-170 were generated by PCR-based mutagenesis and subcloned into the vector pGEX-6P-1 (GE Healthcare).

Antibodies and reagents

Rabbit antibodies against LRRK1 were generated by MBL (Medical & Biological Laboratories) by injecting rabbits with recombinant His-tagged LRRK1 corresponding to residues 1498–1873 of human LRRK1, coupled to keyhole limpet hemocyanin. The recombinant protein was expressed in *E. coli* using the pCold expression vector (Takara) and purified with His GraviTrap (GE Healthcare). Anti-CLIP-170 antibodies were as described previously (Fukata et al., 2002). Antibodies and their suppliers were as follows: anti-FLAG (M2, Sigma), anti-GFP (polyclonal antibody or RQ2, MBL), anti-DIC (74.1, Millipore), anti-p150^{Glued} (BD Transduction

Laboratories), anti-GST (polyclonal antibody, Santa Cruz Biotechnology), anti-CLIP-170 (H-300 or F-3, Santa Cruz Biotechnology), anti-EB1 (BD Transduction Laboratories), anti-α-tubulin (B-5-1-2, Sigma) and anti-β-tubulin (9F3, Cell Signaling Technology). Alexa-Fluor-647–EGF and mouse recombinant EGF were purchased from Molecular Probes Invitrogen (Carlsbad, CA) and TOYOBO (Osaka, Japan), respectively.

RNA interference

Control siRNA and stealth siRNA for human LRRK1 (target sequence starting at position 3165, 5′-GCAGGAACAGGAAAGTCACCATTTA-3′) were purchased from Invitrogen. siRNA for human hCLIP-170 (target sequence starting at position 55, 5′-GCACAGCTCTGAAGACACC-3′) and siRNA for p150^{Glued} (target sequence starting at position 2839, 5′-GATCGAGAGACAGTTATTA-3′) were purchased from Thermo Scientific. Annealed siRNAs were transfected into cells using RNAiMAX (Invitrogen). The transfected cells were analyzed at 72 h after transfection. For immunoprecipitation experiments, cells were transfected with control siRNA or LRRK1-specific siRNA using RNAiMAX, incubated overnight, and then transfected with wild-type GFP–CLIP-170 or GFP–CLIP-170(T1384E) in the indicated combinations, using FuGENE 6 reagent (Promega). For immunofluorescence experiments, cells were transfected with control siRNA, hCLIP-170 siRNA or p150^{Glued} siRNA using RNAiMAX and incubated for 72 h before fixation.

Immunoprecipitation and preparation of CLIP-170 proteins

For immunoprecipitation, cells were lysed in lysis buffer [20 mM MOPS pH 7.4, 10% glycerol, 0.15 M NaCl, 1% CHAPS, 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, phosphatase inhibitor cocktail 2 (Sigma) and protease inhibitor cocktail (Sigma)], followed by centrifugation at 15,000 ***g*** for 15 min. The supernatant was added to 10 µl (bed volume) of Dynabeads–Protein-G (Invitrogen) with the indicated antibodies and rotated for 10 min at room temperature. The beads were then washed three times with ice-cold phosphate-buffered saline (PBS) and subjected to immunoblotting or kinase assays. For preparation of full-length CLIP-170 proteins, immunoprecipitated FLAG–CLIP-170 was eluted with FLAG peptide (Sigma) in kinase buffer for 1 h at 4℃. For preparation of recombinant GST fusion proteins, GST–CLIP-170-N, GST–CLIP-170-C, GST–CLIP-170-C(T1384A) or GST–CLIP-170-C(T1384E) proteins were expressed in the *E. coli* strain BL21-CodonPlus (DE3)-RIPL and purified using glutathione–Sepharose (GE Healthcare) following the manufacturer's guidelines.

In vitro kinase assay

Immunocomplex kinase reactions of GFP–LRRK1, GFP–LRRK1(K1243M) or GFP–LRRK1(Y944F) with FLAG–CLIP-170 prepared from HEK293 cells were performed in a final volume of 20 µl containing 50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM DTT, 5 µCi of [γ-^{32}P]ATP and 100 µM ATP. Samples were incubated for 20 min at 30℃. Reactions were terminated by the addition of Laemmli sample buffer and boiling. GFP–LRRK1, GFP–LRRK1(K1243M), GFP–LRRK1(Y944F) or FLAG–CLIP-170 was resolved by SDS-PAGE. Autophosphorylation of LRRK1 and phosphorylation of CLIP-170 were detected by autoradiography.

GST fusion protein pull-down assay

For GST pull-down assays, 1 µg of soluble GST or GST fusion proteins bound to 20 µl of glutathione–Sepharose beads (75% slurry) were incubated with lysates from HEK293 cells for 1 h at 4℃. The beads were washed three times with cold PBS and harvested by brief centrifugation and finally suspended in Laemmli sample buffer and boiled. The assays were analyzed by immunoblotting with the indicated antibodies.

Fluorescence microscopy and image analysis

For immunofluorescence microscopy, cells grown on coverslips were treated as indicated and then fixed in 100% methanol for 10 min at −20℃. For endogenous CLIP-170 staining, cells were fixed in 100% methanol for 10 min at −20℃, followed by 4% paraformaldehyde for 20 min at room temperature. Cells were then permeabilized in 0.5% Triton X-100 and incubated with primary and secondary antibodies. The primary antibodies used were anti-GFP (polyclonal, 1:1000), anti-GFP (RQ2, 1:100), anti-p150^{Glued} (1:250), anti-CLIP-170-C (1:200) or anti-EB1 (1:250). The secondary antibodies used were Alexa-Fluor-488-, Alexa-Fluor-555- and Alexa-Fluor-647-conjugated goat anti-mouse-IgG, anti-rabbit-IgG or anti-rat-IgG antibodies, respectively (Molecular Probes). Confocal microscopy was performed using an Olympus FV1000 microscope. Linescan analysis and measurements of fluorescence intensity were performed using FV10-ASW2.1 software (Olympus). For quantification of p150^{Glued} localization on microtubule tips, the integrated fluorescence intensities of p150^{Glued} within GFP–mCLIP-170-stained tips were measured and calculated as the ratio of the integrated intensities of p150^{Glued}:GFP in individual tips (n=250–300 tips/cell). The region of GFP–mCLIP-170-stained tips was determined using CellProfiler 2.0 open software. For each series of experiments, microscope settings were optimized to achieve the brightest possible (but not saturated) images, and the settings were not changed during analysis. For live-cell fluorescence microscopy, cells were grown in 35-mm plastic-bottomed dishes. Time-lapse images were obtained with a laser-scanning confocal microscope. Imaging started at 10 min after the initial exposure to Alexa-Fluor-647–EGF, with frames captured at 0.836-s intervals for 29.26 s (35 frames). Tracking of Alexa-Fluor-647–EGF-positive endosomes was conducted using the 'Manual Tracking' ImageJ plug-in (National Institutes of Health).

Determination of phosphorylation sites by LC-MS/MS

For phosphorylation site analysis, immunoprecipitated proteins were subjected to a non-radioactive *in vitro* kinase assay and then eluted with guanidine solution (50 mM NH_4HCO_3, 7 M guanidine-HCl), followed by reduction, alkylation, demineralization and concentration. Next, proteins were digested with trypsin for 16 h at 37℃. From the peptide samples, phosphopeptides were enriched and captured using Titansphere® Phos-TiO Kit according to the manufacturer's instructions. Nano-electrospray tandem mass analysis was performed using a Q Exactive mass spectrometer (ThermoFisher Scientific Inc., Waltham, MA) system combined with a Paradigm MS4 HPLC system (Michrom BioResources Inc., Auburn, CA). Samples were injected onto the Advance nanoflow UHPLC/HTS-PAL system equipped with a MonoCap C18 Nano-flow column 0.1 mm×150 mm (GL Sciences, Japan). Reversed-phase chromatography was performed with a linear gradient (0 min, 5% B; 70 min, 40% B) of solvent A (H_2O with 0.1% formic acid) and solvent B (acetonitrile) at an estimated flow rate of 400 nl/min. Ionization was performed with an ADVANCE CaptiveSpray Source (Michrom BioResources Inc., Auburn, CA). A precursor ion scan was carried out using a 380–1900 mass to charge ratio (m/z) prior to MS/MS analysis. Raw data were analyzed using Proteome Discoverer™ software with the Sequest™ algorithm at 15 ppm precursor mass accuracy and 0.02 Da MS/MS tolerance. The peptide search was performed against UniProtKB *Homo sapiens* reference proteome dataset (release 2012_10) with a 1% false discovery rate cut-off. The most likely phosphorylation site localization was determined by using the PhosphoRS algorithm within the Proteome Discoverer software.

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Competing interests

The authors declare no competing interests.

Author contributions

S.K., K.M. and H.H. designed the study, analyzed the data and wrote the manuscript. T.N., T.W. and K.K. performed LC-MS/MS experiments. S.P. performed the statistical work using CellProfiler software. S.K. performed all of the other experiments.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.161547/-/DC1

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