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RESEARCH ARTICLE

Mitotic Golgi translocation of ERK1c is mediated by a PI4KIIIβ–14-3-3γ shuttling complex

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ABSTRACT

Golgi fragmentation is a highly regulated process that allows division of the Golgi complex between the two daughter cells. The mitotic reorganization of the Golgi is accompanied by a temporary block in Golgi functioning, as protein transport in and out of the Golgi stops. Our group has previously demonstrated the involvement of the alternatively spliced variants ERK1c and MEK1b (ERK1 is also known as MAPK3, and MEK1 as MAP2K1) in mitotic Golgi fragmentation. We had also found that ERK1c translocates to the Golgi at the G2 to M phase transition, but the molecular mechanism underlying this recruitment remains unknown. In this study, we narrowed the translocation timing to prophase and prometaphase, and elucidated its molecular mechanism. We found that CDK1 phosphorylates Ser343 of ERK1c, thereby allowing the binding of phosphorylated ERK1c to a complex that consists of PI4KIIIβ (also known as PI4KB) and the 14-3-3γ dimer (encoded by *YWHAB*). The stability of the complex is regulated by protein kinase D (PKD)-mediated phosphorylation of PI4KIIIβ. The complex assembly induces the Golgi shuttling of ERK1c, where it is activated by MEK1b, and induces Golgi fragmentation. Our work shows that protein shuttling to the Golgi is not completely abolished at the G2 to M phase transition, thus integrating several independent Golgi-regulating processes into one coherent pathway.

KEY WORDS: Golgi fragmentation, Golgi shuttling, ERK1c, CDK1, PKD

INTRODUCTION

The Golgi complex of eukaryotic cells is organized into stacks of cisternae, which are anchored in the perinuclear region. Once the cell enters mitosis, its one Golgi complex needs to properly divide between the two daughter cells (Wei and Seemann, 2009; Ouellet and Barral, 2012). The mechanism of this division includes fragmentation of the perinuclear stacks of Golgi cisternae. This occurs in two steps: the first is a dispersal of the perinuclear Golgi into a few smaller stacks, and the second is a breakdown of these fragments into small tubules and vesicles, which are spread throughout the cytosol (Wei and Seemann, 2009, 2010; Corda et al., 2012). With the progression of mitosis, the fragmented vesicles are randomly distributed between the emerging new cells. This is followed by fusion of the fragments into a full Golgi complex in each of the formed cells. Several studies have shown that inhibition of Golgi fragmentation results in attenuation of mitotic entry (Sutterlin et al., 2002; Preisinger et al., 2005; Shaul and Seger, 2006; Colanzi et al., 2007; Feinstein and Linstedt, 2007; Villeneuve et al., 2013). Therefore, it has been suggested that the proper segregation of the Golgi complex during mitosis serves as a 'mitotic checkpoint', essential for proper cell division.

Golgi fragmentation, being such an important mitotic step, is tightly regulated by a series of phosphorylations and other post-translational modifications. Several kinases, such as protein kinase D (PKD), Raf, MEK1 and MEK2 (MEK1/2, also known as MAP2K1 and MAP2K2), ERK1 and ERK2 (ERK1/2, also known as MAPK3 and MAPK1) and Myt1 (Wortzel and Seger, 2011; Kienzle et al., 2013; Villeneuve et al., 2013), as well as the polo-like kinase 3 (Lin et al., 2000; Sutterlin et al., 2001; Xie et al., 2004), are involved in the regulation of the first step of the fragmentation. The regulatory substrates and molecular mechanisms involved in the action of these kinases are not as yet understood, but might include phosphorylation of the Golgi structural protein GRASP55 (also known as GORASP2) by components of the ERK cascade (Jesch et al., 2001). The second step in the fragmentation is then regulated by cyclin-dependent kinase 1 (CDK1; Nurse, 2002), which is indirectly activated in the Golgi by Myt1, possibly downstream of the ERK cascade (Villeneuve et al., 2013). Upon activation, CDK1 induces the final fragmentation either by phosphorylating Golgi proteins, such as GM130 (also known as GOLGA2) (Lowe et al., 1998), Nir2 (also known as PITPNM1) (Litvak et al., 2004) and GRASP65 (also known as GORASP1) (Lin et al., 2000), or inhibiting the transport of phospholipids from the endoplasmic reticulum (ER) to the Golgi, by phosphorylation of transport machinery components (Bailly et al., 1991). However, several studies have suggested that there are additional modes of crosstalk between these kinases (Borysov and Guadagno, 2008; Chiu et al., 2012) and, therefore, more studies are required to fully delineate this important Golgi process.

The ERK cascade (Raf–MEK–ERK) is a signal transduction pathway that induces distinct, and even opposing, physiological processes, such as proliferation and differentiation, as well as apoptosis and cell migration (Wortzel and Seger, 2011). The use of specific MEK inhibitors and activated kinases has revealed that this cascade is involved in Golgi fragmentation (Acharya et al., 1998; Colanzi et al., 2000, 2003b; Kienzle et al., 2013; Villeneuve et al., 2013). Interestingly, although Raf-activated MEK has been found at the Golgi during mitosis (Colanzi et al., 2003b), ERK1/2 has not been detected at this compartment during the process. In addition, although ERK1/2 proteins seem to function on the outer surface of the Golgi membrane under some conditions (Torii et al., 2004), they do not seem to accumulate at the Golgi at any stage (Shaul and Seger, 2006). However, specific antibody against ERK that is monophosphorylated at Tyr204 (in human ERK1) did stain the Golgi at the G2 to M phase transition, but the identity of the stained molecules was initially obscure (Cha and Shapiro, 2001). The nature of these stained ERK molecules, as well as the identity of the MEK

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involved in the process were later resolved by the identification of an alternative ERK cascade, composed of the spliced variants of MEK1b, as well as the alternatively spliced ERK1 variant ERK1c (Aebersold et al., 2004; Shaul and Seger, 2006; Shaul et al., 2009).

MEK1b is a primate alternatively spliced isoform of MEK1 (Seger et al., 1992; Zheng and Guan, 1993), which is formed by elimination of exon 5 of the gene, so that it lacks 26 amino acids within its kinase domain. It was initially considered as an inactive kinase, because it failed to phosphorylate ERK1/2 (Zheng and Guan, 1993), but was later shown to phosphorylate ERK1c during mitosis (Shaul et al., 2009). ERK1c is an alternatively spliced isoform of ERK1, which is formed by inclusion of the stop-codon-containing intron 7 of the gene. The formed protein therefore contains 18 unique amino acids in its C-terminus, instead of the 46 C-terminal amino acids of ERK1. Our group has previously shown that the expression of these proteins is increased in mitosis, and that ERK1c accumulates in the Golgi during prophase. Using overexpression, as well as knockdown of ERK1c, we have shown that these two proteins are indeed essential for the Golgi fragmentation (Shaul and Seger, 2006; Shaul et al., 2009). Furthermore, we have shown that MEK1b phosphorylates ERK1c preferentially on its regulatory Tyr residue (Shaul and Seger, 2006). These results strongly suggest that the cascade involved in the regulation of Golgi fragmentation is composed of MEK1b and ERK1c. However, the mechanism that allows the accumulation of these proteins in the Golgi at the correct time to regulate mitosis is not yet clear.

In this paper, we identify ERK1c as a new substrate of CDK1 during mitosis and demonstrate that ERK1c is phosphorylated by CDK1 on Ser343 at the unique C-terminal region. This phosphorylation allows the interaction of the kinase with a complex of PI4KIIIβ (also known as PI4KB) and the dimer of 14-3-3γ (encoded by *YWHAB*), which escort it to the Golgi; there it meets MEK1b, which activates it. We also show that the phosphorylation of Ser294 of PI4KIIIβ by PKD is a central step in regulating the formation of the complex. These results add PI4KIIIβ and 14-3-3γ as new components in the regulation of Golgi fragmentation and emphasize that, unlike previously thought, CDK1, PKD and ERK1c act within one pathway to regulate mitotic Golgi fragmentation.

RESULTS

ERK1c is translocated to the Golgi in mitosis

Previous work from our group has demonstrated the unique ability of ERK1c to accumulate in the Golgi and, consequently, regulate Golgi fragmentation during mitosis (Shaul and Seger, 2006). However, in those reports we primarily utilized overexpressed, and not the endogenous, protein. In order to further study the molecular mechanisms that govern the activity of ERK1c in the Golgi it was important to follow the endogenous ERK1c and its interacting proteins. For this purpose, we first resorted to the specific anti-ERK1c antibody that was previously developed (Aebersold et al., 2004). The suitability of the antibody for cell staining was confirmed using competition with the antigenic peptide as well as lack of staining of mouse cells, as ERK1c is only expressed in primates (Fig. S1A,B). Using this, as well as antibody staining for the Golgi marker GM130, we found that in G1 and S phase, the expression of ERK1c is low, and diffuse in the cytoplasm, whereas in G2 the expression is higher, but is still cytoplasmic. However, at prophase, and even more so in prometaphase, ERK1c changed its distribution and colocalized with GM130 in the Golgi. This continued to be the case in metaphase, during which time the Golgi undergoes fragmentation (Fig. 1A). Staining against phosphorylated histone H3 (pHis) confirmed the stages of mitosis, and similar results were obtained in HB2 cells (Fig. S1C).

To further verify the mitotic Golgi translocation of ERK1c, we used a sucrose density gradient to fractionate membrane vesicles from nocodazole-treated and non-treated HeLa cells. Nocodazole causes the cells to arrest in prometaphase, which is the time in which the Golgi undergoes fragmentation (Colanzi et al., 2003b). In this experiment, GM130 was used as a Golgi marker, MEK1 as a cytoplasmic marker and calnexin as an ER marker, showing a separation of the membranes. As expected, in the control non-treated cells, ERK1c was localized in the cytoplasmic fraction, with no detectable colocalization with GM130 (Fig. 1B), whereas upon nocodazole treatment ERK1c was detected mainly in the Golgi fraction. Taken together, these results confirm that endogenous ERK1c does shift its localization from the cytoplasm to the Golgi. Moreover, unlike the more general times suggested (G2 and M phase; Shaul and Seger, 2006), we now show that the elevation in ERK1c expression occurs in G2, whereas the translocation starts at prophase and peaks at prometaphase.

Use of ImageStream to detect and quantify Golgi localization during the cell cycle

We then developed a more quantitative method to follow the Golgi translocation of ERK1c. For this purpose we used multispectral imaging flow-cytometry (ImageStream) analysis, which is able to follow protein distribution in a large number of cells and quantify it. To establish this system, we first synchronized HeLa cells using a double-thymidine block. Most of the cells reached mitosis 9.5 h after the block release, as indicated by the Draq5 staining of the DNA. This timing was delayed by inhibiting CDK1 or MEK1/2 by treatment with the flavopiridol and U0126 inhibitors, respectively (Fig. S1D). As the 4N peak is a mixed population of G2 and mitotic cells, we further defined the mitotic cells by gating the nuclear area and the intensity of the Draq5 staining. This gating resulted in three subpopulations, which represent cells in G2, cells in prophase and prometaphase, and cells in mitosis (metaphase to telophase; Fig. S1E). This was validated by pHis staining that, as expected, stained all prophase and mitotic cells (Fig. S1F,G). In order to quantify the colocalization by the ImageStream, we used the 'Bright Detail Similarity' (BDS) index, which is considered significant above 1.5 (George et al., 2006; Lowman et al., 2010; Phadwal et al., 2012). To verify that this holds true in our system, we stained for the nuclear protein CCDC86 that showed poor localization to GM130 (mean BDS, 0.24±0.002, n=3), whereas the mean BDS (n=3) for the Golgi protein GRASP55 with GM130 was 1.23±0.009 (Fig. S2A, B). Therefore, the use of a cutoff of 1.5 should indeed be a good parameter for colocalization in our system.

Use of ImageStream to quantify ERK1c translocation and study its regulation

We then undertook to use ImageStream to quantify the Golgi translocation of ERK1c. Thus, HeLa cells were synchronized at the peak of mitosis and stained with anti-ERK1c and -GM130 antibodies, as well as Draq5, and analyzed by ImageStream. As expected, in G2 the endogenous ERK1c was found to be localized all over the cytoplasm, but at prophase and more so in later stages of mitosis, it shifted to the Golgi (Fig. S2C). Quantification of the cells using the parameters above indeed showed that the ERK1c and GM130 BDS correlation is mostly below 1.5 in G2 cells (more than 70% of the cells), and this substantially changed in most mitotic cells (Fig. S2D). These results confirm that ImageStream can be

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

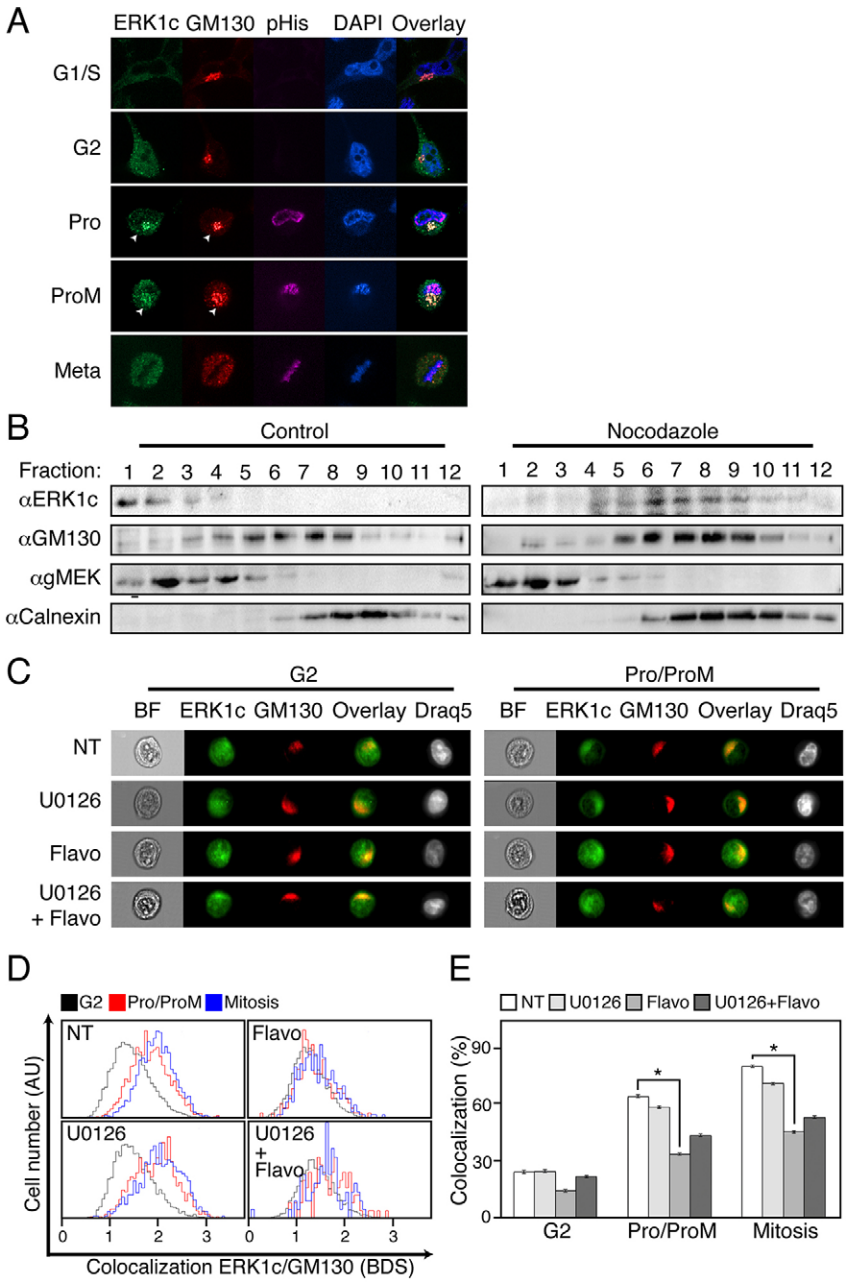

Fig. 1. The translocation of endogenous ERK1c to the Golgi depends on the activity of CDK1. (A) HeLa cells were synchronized and immediately fixed, or released for 9 h and then fixed. The cells were stained for ERK1c, GM130, histone 3 phosphorylated at Ser28 (pHis) and with DAPI. The arrowheads indicate the Golgi. (B) The postnuclear supernatants of the nocodazole-treated or non-treated (control) HeLa cells were subjected to sucrose density gradient centrifugation. The gradient was split into 12 equal-volume fractions from top to bottom. Each fraction was analyzed by SDS-PAGE, followed by western blotting using the indicated antibodies. gMEK, general MEK. (C) HeLa cells were synchronized by release from thymidine block for 9 h, and then were either treated with 10 µM flavopiridol (Flavo), 5 µM U0126, or both for 1 h, or left untreated (NT) as control. The cells were fixed and stained for ERK1c and GM130 and with Draq5. Cells were analyzed by multispectral imaging flow cytometry (ImageStream). BF, brightfield image. (D) The colocalization of ERK1c and GM130 was quantified using the BDS index, and was measured at G2, prophase and prometaphase (Pro/ProM), and during the rest of mitosis (metaphase to telophase) for each treatment. AU, arbitrary scale. (E) Quantification of the percentage of cells with a BDS above 1.5 of every population in each treatment (mean±s.e.m., n=3). *P<0.01 for the difference between the NT and the Flavo in Pro/ProM and mitosis.

used as a reliable quantitative tool to detect Golgi translocation in our system.

Next, we attempted to use this system to study the regulation of the subcellular localization of ERK1c. Given that MEK1b (Shaul et al., 2009) and CDK1 (Lowe et al., 1998; Lin et al., 2000; Takizawa and Morgan, 2000) have been previously reported to regulate Golgi fragmentation, we used their selective inhibitors to follow the role of these kinases in the regulation of ERK1c localization. Thus, synchronized HeLa cells were treated with U0126, flavopiridol, or both. As expected (Villeneuve et al., 2013), inhibition of MEK attenuated cell cycle progression, whereas inhibition of CDK1, or both kinases led to a complete cell cycle delay (Fig. S1D). Although both inhibitors had the same effect on cell cycle progression, their effect on the localization of ERK1c was different. We found that during G2, the distribution of ERK1c was diffuse regardless of the inhibitions (Fig. 1C–E). In prophase and prometaphase, ERK1c translocated to the Golgi in the non-treated and in the U0126-treated cells. By contrast, CDK1 inhibition (alone or with U0126) attenuated the Golgi translocation of ERK1c. Similar results were generated using the inhibitors PD184352 and roscovitine to inhibit MEK1/2 and CDK1, respectively (Fig. S2E,F). Taken together, our results clearly show that endogenous ERK1c translocates to the Golgi at the beginning of mitosis, and this translocation is regulated by CDK1.

The C-terminal region of ERK1c regulates its localization

We then went on to study the molecular mechanisms by which CDK1 influences ERK1c localization. Given that ERK1c differs from ERK1, which does not localize to the Golgi (Aebersold et al., 2004), only in its C-terminal region, we hypothesized that the unique C-terminal sequence of ERK1c is responsible for its Golgi translocation. To test this, we generated several mutations in the C-terminus of ERK1c: ERK1c lacking the 18 amino acids in the C-terminus (ΔCter-ERK1) and ERK1c in which the 18 amino acids

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

were replaced by 18 Ala residues (ERK1c-Ala) (Fig. 2A). ERK1c, ΔCter-ERK1, ERK1c-Ala and ERK1, all conjugated to GFP, were transfected into HeLa cells, which were then starved or treated with nocodazole, fixed, and stained for ERK1c and GM130. In mitosis, ERK1c was mainly detected in Golgi vesicles, whereas all other constructs, as well as ERK1c in non-treated cells, were found to be diffusely localized all over the cells (Fig. 2B). These results suggest that the composition of the C-terminal amino acids is required for the Golgi translocation of ERK1c.

CDK1 phosphorylates the C-terminus of ERK1c

Owing to the importance of CDK1 activity, as well as the C-terminus of ERK1c, for the subcellular localization of the latter, we hypothesized that the translocation of ERK1c into the Golgi is regulated by a direct CDK1 phosphorylation. Indeed, the C-terminal sequence of ERK1c contains the consensus phosphorylation site of CDK1 (Litvak et al., 2004) (Ser343 and Pro344; Fig. 2A). In order to examine whether CDK1 can indeed phosphorylate Ser343, we performed an *in vitro* phosphorylation assay. Purified GST-tagged ERK1c, the ERK1c C-terminus and ERK1 were subjected to phosphorylation by an active CDK1–Cyclin-B1 complex, or BSA control. Indeed, the C-terminus of ERK1c was phosphorylated to ∼5% stoichiometry and the full-length proteins were phosphorylated to ∼15% stoichiometry. The higher stoichiometry of the full-length protein is probably due to other Ser-Pro sequences in the kinase domain of ERK (positions 74 and 263 in human ERK1 and ERK1c; Fig. 3A). To verify the specific phosphorylation of CDK1 on Ser343, we used non-phosphorylatable (Ser343Ala, S343A) and phosphomimetic (Ser343Asp, S343D) mutants conjugated to GFP, and transfected them into HeLa cells. The proteins were then precipitated, and subjected to an *in vitro* phosphorylation assay with active CDK1–Cyclin-B1 complex. As expected, the phosphorylation of both mutants was reduced as compared to the phosphorylation of the wild-type (WT) protein (Fig. 3B). These results confirm that ERK1c is an *in vitro* substrate of CDK1, which phosphorylates mainly Ser343 at the unique C-terminal region of ERK1c, although other phosphorylation sites exist.

To verify and follow the phosphorylation of Ser343, we generated an antibody against this phosphorylated residue in ERK1c, and showed that it is specific to the phosphorylated protein (Fig. S3A,B). Then, we used this antibody in western blot analysis of extracts from nocodazole- or double-thymidine-block-treated cells, and indeed found an increased recognition of the 41-kDa ERK1c band (Fig. 3C). When CDK1 activity was inhibited by flavopiridol, the antibody-detected phosphorylation of ERK1c in mitosis was substantially reduced (Fig. 3D). Furthermore, using ImageStream analysis, we found that the phosphorylation of ERK1c Ser343 (pSer343-ERK1c) was low at interphase, increased at prophase and prometaphase, peaked at metaphase, and then decreased in anaphase and telophase (Fig. 3E,F; Fig. S3C,D). The Golgi localization of pSer343-ERK1c roughly correlated with the localization of the total protein shown above in the beginning of mitosis (Fig. S3E) and to the rate of phosphorylation. However, the localization was not reduced in late mitosis, indicating that the translocation is dependent on the phosphorylation, whereas the detachment is not. Interestingly, at both prophase and prometaphase, we detected two sub-populations of cells: those showing low pSer343 staining with a diffused distribution and those with high pSer343 staining with Golgi localization (Fig. S3F). Taken together, these results clearly indicate that the mitotic Ser343-ERK1c phosphorylation occurs on endogenous proteins starting at prophase, when the phosphorylation directs Golgi translocation.

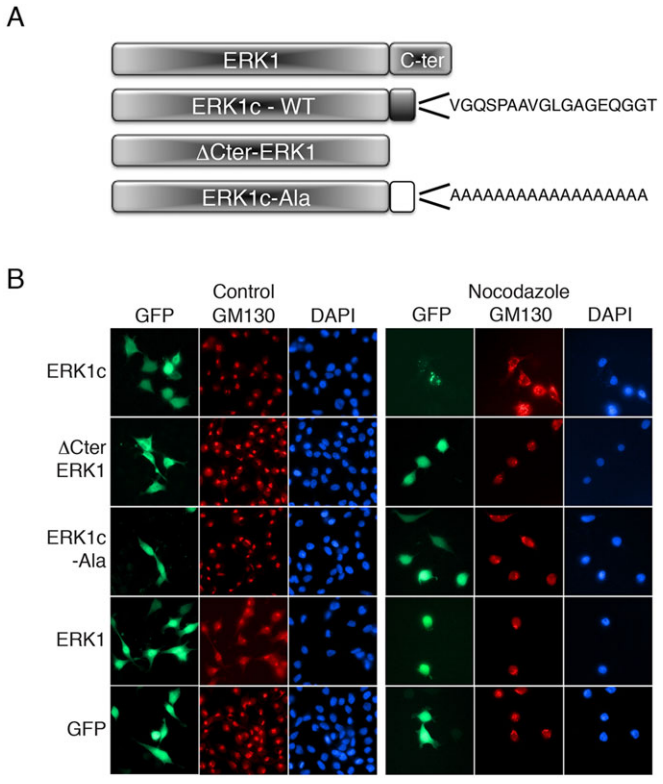

Fig. 2. The unique C-terminus of ERK1c regulates its translocation to the Golgi during mitosis. (A) Schematic representation of ERK1, ERK1c and the mutant constructs. (B) HeLa cells were transfected for 24 h with the indicated GFP-tagged constructs. Then the cells were either starved, or treated with 100 ng/ml nocodazole for another 16 h. This was followed by fixing of the cells, and staining for GFP, GM130 and with DAPI.

Phosphorylation of Ser343 is important for the activity of ERK1c in cells

We next tested whether inhibition of CDK1 activity affects the activity of ERK1c. For this purpose, HeLa cells overexpressing GFP–ERK1 or GFP–ERK1c were synchronized and treated with U0126, flavopiridol, or both, for 1 h. The cells were then harvested and the activity of both kinases towards the general substrate, myelin basic protein (MBP), was determined. We found that the activity of both ERK1 and ERK1c was increased in G2 and mitosis, although to different extents (Fig. 3G). Inhibition of MEK resulted in inhibition of both proteins, whereas inhibition of CDK1 inhibited only the activity of ERK1c. Moreover, we also found that ERK1c (but not ERK1) interacted with CDK1, and that this interaction was increased towards mitosis (Fig. S4A–E). Thus, these results suggest that CDK1 interacts with, and phosphorylates, ERK1c Ser343, and that this phosphorylation affects the localization and the activity of ERK1c during mitosis.

The phosphorylation state of Ser343 regulates the localization and activity of ERK1c

Given that CDK1 activity is necessary for ERK1c translocation to the Golgi, we surmised that the ERK1c Ser343 phosphorylation would affect its distribution. In order to test this hypothesis, HeLa cells were grown on coverslips, and transfected with GFP-tagged ERK1c and its mutants Ser343Ala and Ser343Asp. The cells were

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

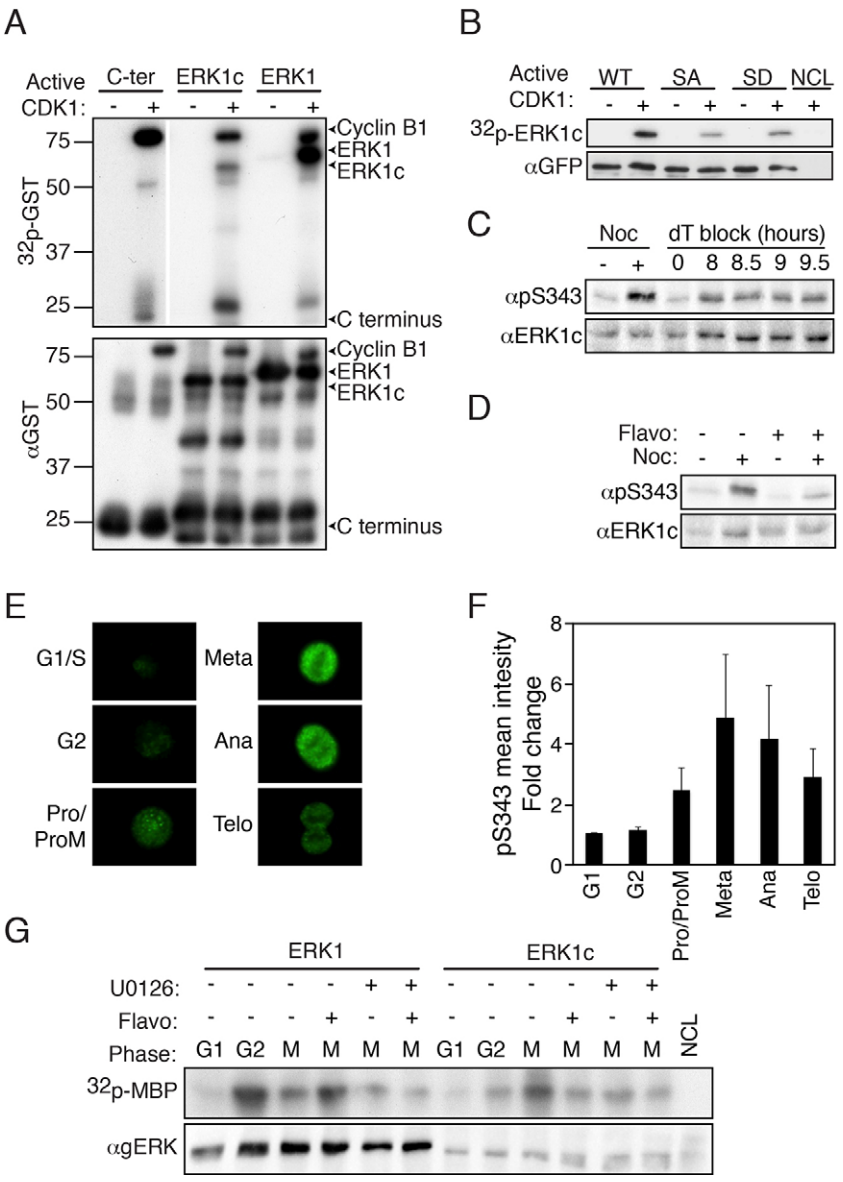

Fig. 3. CDK1 specifically phosphorylates Ser343 in the C-terminus of ERK1c. (A) GST-tagged ERK1, ERK1c and the C-terminus of ERK1c (C-ter) (5 µg) were subjected to an *in vitro* phosphorylation assay with or without the active CDK1–cyclin-B1 complex. (B) HeLa cells were transfected for 48 h with GFP-tagged ERK1c WT, or ERK1c in which Ser343 was mutated to either Ala or Asp (SA and SD, respectively). The GFP proteins were immunoprecipitated, and subjected to *in vitro* phosphorylation assay with or without the active CDK1–cyclin-B1 complex. NCL, no cell lysate. (C) HeLa cells were either starved, stimulated with 100 ng/ml nocodazole (Noc) for 16 h or synchronized with a double-thymidine block, and harvested at the indicated times after release. Cells were blotted with antibodies against pSer343-ERK1c and ERK1c. (D) HeLa cells were either starved or stimulated with 100 ng/ml nocodazole, in the presence of 10 µM flavopiridol. Lysates were blotted with antibodies against pSer343-ERK1c and ERK1c. (E) Synchronized HeLa cells were fixed at the peak of mitosis, and then stained with anti-ERK1c and GM130 antibodies, and Draq5. Cells were analyzed by multispectral imaging flow cytometry (ImageStream). Cells from G1 or S (G1/S), G2, prophase and prometaphase (Pro/ProM), metaphase (Meta), anaphase (Ana) and telophase (Telo) were selected by their DNA structure according to their Draq5 staining. (F) The pSer343 ERK1c intensity was measured for G1, G2, and mitotic (prophase to telophase) populations. Results are mean±s.e.m., *n*=3. (G) HeLa cells were transfected with GFP–ERK1 or GFP–ERK1c. After 24 h, the cells were synchronized by double-thymidine block for an additional 48 h. Cells were released from the block for the indicated times, and treated with 10 µM flavopiridol, 5 µM U0126, or both for 1 h before the harvest. The GFP proteins were immunoprecipitated, and subjected to an *in vitro* phosphorylation assay against MBP. gERK, general ERK.

then treated with nocodazole, fixed, and stained for GM130 and pHis, and with DAPI. The distribution of WT ERK1c in the non-treated interphase cells was found to be either diffused all over the cell, or weakly associated with the Golgi (Fig. 4A, control). The non-phosphorylatable ERK1c, Ser343Ala, was mainly diffused all over the cell, and the distribution of the phosphomimetic mutant Ser343Asp was similar to that of WT ERK1c, although in some cells it was more localized in the Golgi. Upon nocodazole treatment, WT ERK1c, as well as the Ser343Asp mutant were mainly colocalized with GM130, manifesting their complete Golgi translocation (Fig. 4A, nocodazole). However, the Ser343Ala mutant failed to translocate and still showed a diffuse localization even in the various stages of mitosis. Moreover, the Golgi in these overexpressing cells was not fragmented. These results indicate that the phosphorylation of Ser343 by CDK1 is necessary for the Golgi accumulation, as well as the consequent fragmentation. As MEK inhibition had no effect on the translocation of ERK1c to the Golgi (Fig. 1), we then examined whether MEK1b phosphorylates ERK1c at the Golgi. Indeed, overexpressed MEK1b was found at the Golgi even in interphase cells, while MEK1 was diffuse in the cytoplasm (Fig. S4F,G). Hence, we conclude that ERK1c is first phosphorylated by CDK1 to induce its Golgi localization, and consequently is phosphorylated and activated by MEK1b.

In order to further validate this assumption, we examined the effect of Ser343 mutation on the activation of ERK1c by MEK1b. Overexpression of Ser343 mutants prevented synchronization of the cells (data not shown), and therefore we induced full activation of ERK1c by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) stimulation (Aebersold et al., 2004). In our previous work, we have shown the specificity of MEK1b as a unique activator of ERK1c, whereas MEK1/2 did not significantly phosphorylate ERK1c under any conditions (Shaul et al., 2009). Therefore, Golgi MEK1b mediates the phosphorylation and activation of ERK1c, even upon TPA stimulation. Overexpression of WT ERK1c as well as its Ser343Ala and Ser343Asp mutants in HeLa cells followed by serum starvation and stimulation with TPA, resulted in induction of both double and mono-phosphorylations of WT ERK1c. The Ser343Ala mutant was not phosphorylated at all, whereas the Ser343Asp mutant was hyper-phosphorylated on its TEY motif (Fig. 4B,C). We next measured the activity of WT ERK1c and its

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

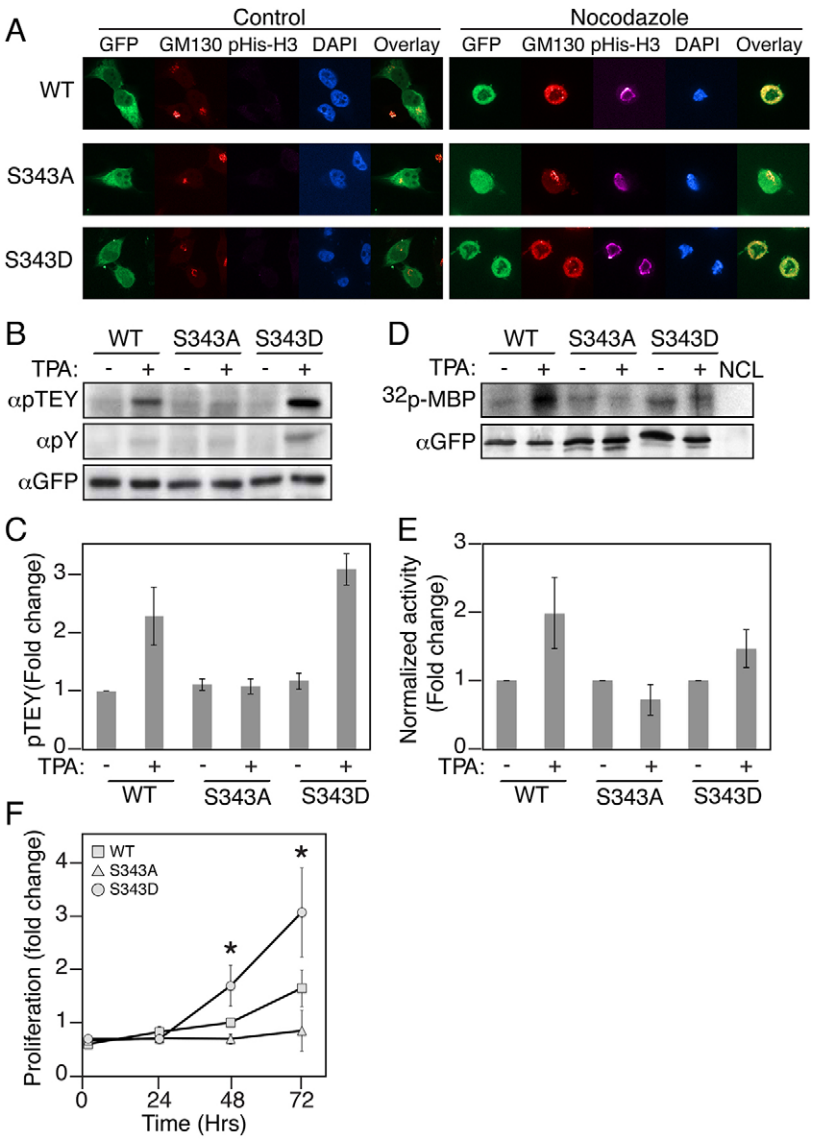

Fig. 4. The phosphorylation of Ser343 affects the activity of ERK1c and the proliferation. (A) HeLa cells were transfected for 24 h with the indicated GFP-tagged ERK1c constructs, followed by either starvation, or treatment with 100 ng/ml nocodazole for additional 16 h. The cells were then fixed and stained for GM130, histone 3 phosphorylated at Ser10 (pHis-H3 S10) and DAPI. (B) HeLa cells were transfected for 24 h with GFP-tagged ERK1c WT, Ser343Ala, or Ser343Asp and then starved for additional 16 h. The cells were then stimulated with 250 nM TPA for 15 min, and western blot analysis of the cell extracts was performed with the indicated antibodies. (C) Quantification of the pTEY results in B. The bar graphs represent the mean±s.e.m. of five distinct experiments. (D) HeLa cells were transfected as described above. GFP proteins were immunoprecipitated, and subjected to an *in vitro* phosphorylation assay with MBP as the substrate. NCL, no cell lysate. (E) Quantification of the relative activity of WT ERK1c, in D. The bar graphs represent mean±s.e.m. from five distinct experiments. (F) HeLa cells were seeded in 12-well plates and transfected with GFP-tagged ERK1c WT, Ser343Ala, and Ser343Asp mutants. The cells were grown for 72 h with 1% FCS, then fixed and stained with Methylene Blue. The relative absorbance is a measure of proliferation. This experiment was performed three times in quadruplets and mean±s.e.m. are shown. **P*<0.001.

mutants towards MBP. For this purpose, GFP-tagged ERK1c WT, Ser343Ala and Ser343Asp were immunoprecipitated and subjected to an *in vitro* phosphorylation assay towards MBP. As expected, the activity of the WT ERK1c was increased upon stimulation and Ser343Ala ERK1c was not active at all, whereas the Ser343Asp ERK1c was active in both resting and stimulated cells (Fig. 4D,E). These results suggest that the phosphorylation state of Ser343 affects both the activation and the activity of ERK1c.

Owing to the fact that ERK1c regulates Golgi fragmentation, and thereby, mitotic progression (Shaul and Seger, 2006), we speculated that overexpression of the non-phosphorylated and phosphomimetic proteins would affect the proliferation rate of the overexpressed cells. Indeed, in our previous work, we have shown that overexpression or knockdown of ERK1c results in acceleration and attenuation of cell cycle progression, respectively (Shaul and Seger, 2006). Similarly, we found here that the proliferation of cells expressing Ser343Ala ERK1c was reduced as compared to those expressing the WT protein, whereas cells expressing the Ser343Asp ERK1c demonstrated a higher rate of proliferation (Fig. 4F). It should be noted that overexpression of proteins might result in dysregulation of their activity, and therefore the results might have been compromised by this effect. However, the characterization of Ser343 mutants by the various methods used (Fig. 4), does indicate that the Ser343 phosphorylation affects ERK1c localization and activity and, therefore, cell cycle progression and proliferation.

PI4KIIIβ and 14-3-3γ regulate the mitotic Golgi translocation of ERK1c

We then attempted to examine the molecular mechanism that mediates the ERK1c translocation. In view of the fact that ERK1c does not have any Golgi localization motif or domain for interaction with Golgi lipids or proteins, we hypothesized that the phosphorylation of Ser343 is a part of the Golgi-targeting mechanism. Phosphorylation has been implicated in the shuttling of a large number of proteins in various systems (Schmierer and Hill, 2005; Youn and Shin, 2006; Chuderland et al., 2008). Such a system has even been described for the Golgi of cycling cells (not mitotic) where, during fission of vesicles from the trans-Golgi network (TGN), the shuttling of the protein BARS (also known as Ctbp1) was dependent on its phosphorylation by PAK1–PAK3. This phosphorylation induces formation of a shuttling complex, which consists of a PI4KIIIβ and 14-3-3γ dimer that accompanies BARS to the Golgi (Valente et al., 2012). We then went on to

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

examine whether a similar system also mediates the translocation of ERK1c. First, we tested whether ERK1c interacts with the PI4KIIIβ–14-3-3γ complex. By using co-immunoprecipitation, we found an interaction between ERK1c and PI4KIIIβ–14-3-3γ in cells overexpressing GFP–ERK1c. This interaction was increased towards mitosis and reached a peak by 9 h, just before the peak of mitosis (Fig. 5A). Similar results were obtained by blocking cells at mitotic entry using a nocodazole treatment (Fig. 5B). Furthermore, this interaction was specific for ERK1c, as GFP–ERK1 failed to interact with PI4KIIIβ–14-3-3γ in the synchronized cells (Fig. 5C).

To confirm these results, we performed a proximity ligation assay (PLA), which is a useful tool to determine interaction or close proximity of endogenous proteins (see Materials and Methods, and controls in Fig. S4H). Similar to the co-immunoprecipitation results, no substantial interactions were detected between the endogenous ERK1c and PI4KIIIβ–14-3-3γ in unsynchronized cells (Fig. 5D,E, control). However, nocodazole treatment resulted in a marked increase in both interactions (Fig. 5D,E, nocodazole). As would be expected from the fragmented pattern of the Golgi at this stage, the interactions were seen all over the cytoplasm. Finally, we examined whether the ERK1c Ser343 phosphorylation is required for the interaction. For this purpose, we performed the PLA analysis after overexpression of either the GFP–ERK1c-WT or the GFP–ERK1c-Ser343Ala mutant. As demonstrated above for the endogenous protein, the WT ERK1c interaction with PI4KIIIβ–14-3-3γ was increased in mitosis, whereas the Ser343Ala mutant failed to interact (Fig. 5F,G). These results indicate that Ser343 phosphorylation in mitosis induces its binding with PI4KIIIβ and 14-3-3γ.

To further substantiate the role of the interacting complex in the Golgi translocation of ERK1c, we knocked down PI4KIIIβ and 14-3-3γ using small interfering siRNA (siRNA) in HeLa cells (Fig. 6A) and examined the ability of ERK1c to translocate to the Golgi. As expected, nocodazole treatment induced Golgi translocation of ERK1c and Golgi fragmentation in the control siRNA-treated cells. However, knocking down either PI4KIIIβ or 14-3-3γ, resulted in a reduced translocation of ERK1c to the Golgi, whereas knocking down both PI4KIIIβ and 14-3-3γ together completely abolished the Golgi translocation of ERK1c, and prevented the fragmentation of the Golgi (Fig. 6B,C). These

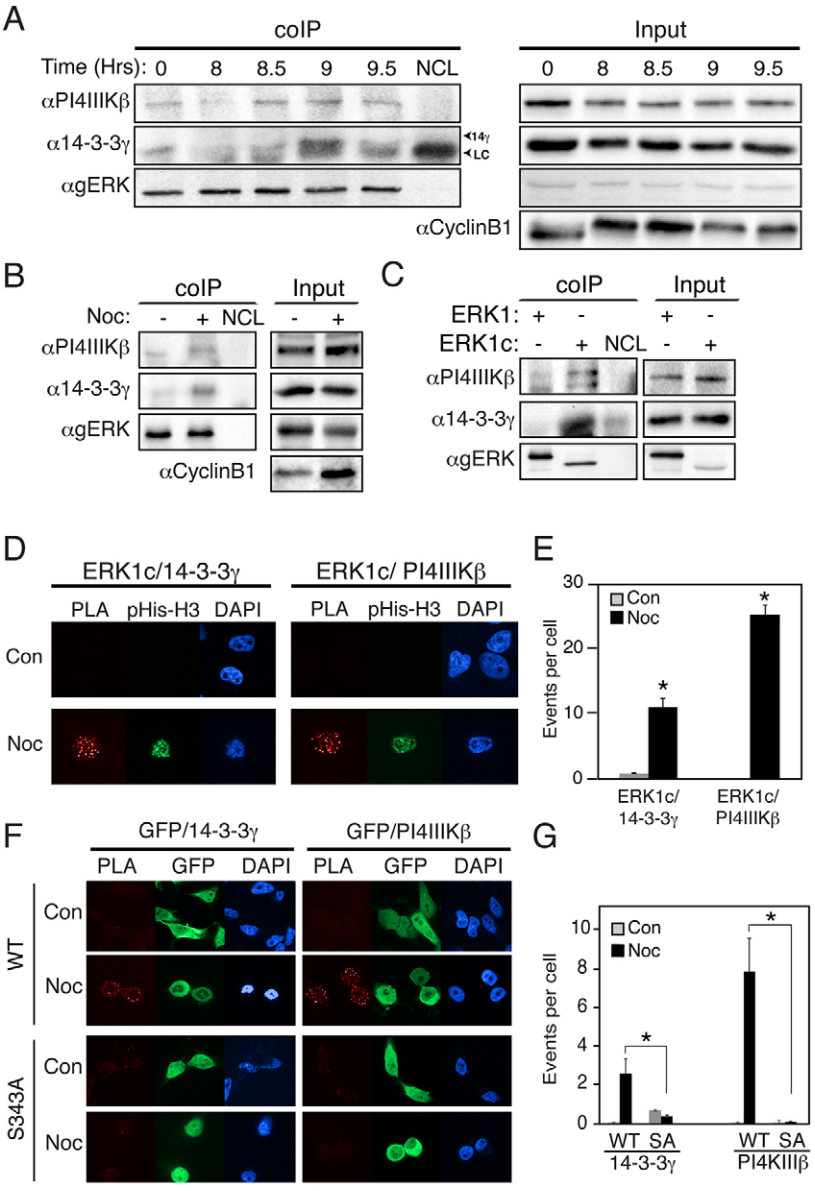

Fig. 5. ERK1c interacts with PI4KIIIβ and 14-3-3γ upon mitotic stimulation. (A) HeLa cells were transfected with GFP–ERK1c for 24 h, and then synchronized by double-thymidine block for an additional 48 h. Cells were released from the block for the indicated times, and the GFP proteins were co-immunoprecipitated (coIP), and subjected to western blot analysis for their interaction with PI4KIIIβ and 14-3-3γ. LC, light chain of the antibody used; NCL, no cell lysate; gERK, general ERK. (B) HeLa cells were transfected with GFP–ERK1c for 24 h, and then synchronized by nocodazole (Noc) treatment for 16 h. GFP proteins were co-immunoprecipitated, and blotted as above. (C) HeLa cells were treated as in A, and transfected with GFP-tagged ERK1 or ERK1c. The GFP proteins were co-immunoprecipitated, and subjected to western blot analysis for their interaction with PI4KIIIβ and 14-3-3γ. (D) A proximity ligation assay (PLA) demonstrated a mitotic-induced interaction of ERK1c with PI4KIIIβ–14-3-3γ. HeLa cells were grown on slides to 70% confluence, serum starved (control), or treated with 100 ng/ml nocodazole for 16 h. The cells were then fixed, subjected to a PLA with the indicated antibodies, and stained with antibody against histone 3 phosphorylated at Ser28 (pHis-H3). The cells were visualized by using confocal spinning disc microscopy. The experiment was repeated three times, with more than 100 cells were counted in each treatment. (E) Quantification of the interactions described in D was performed using ImageJ. The data shown represents mean±s.e.m. *P<0.0005. (F) HeLa cells were transfected for 24 h with GFP-tagged ERK1c WT or Ser343Ala (SA), and then treated with 100 ng/ml nocodazole for an additional 16 h, or left untreated (control). The cells were then fixed, subjected to a PLA with the indicated antibodies, and visualized by using a confocal spinning disc microscopy. The experiment was repeated three times, with more than 100 cells were counted in each treatment. (G) Quantification of the interactions described in F was performed using ImageJ. The data shown represent mean±s.e.m. *P<0.0005.

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

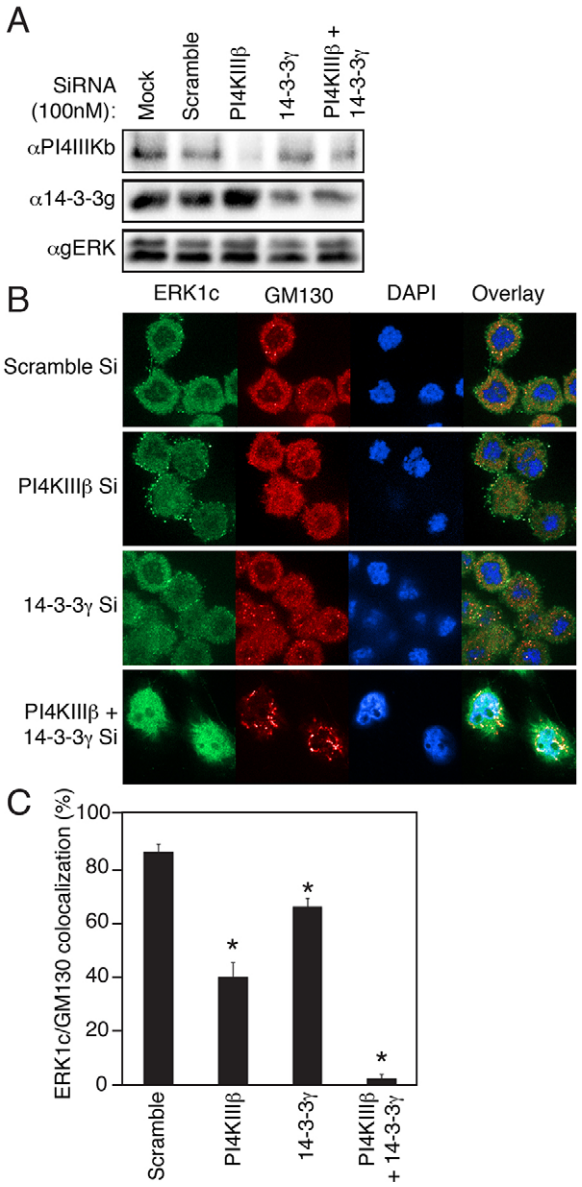

Fig. 6. Knockdown of PI4KIIIβ and 14-3-3γ affects the Golgi translocation of ERK1c. (A) HeLa cells were transfected with 100 nM of scrambled siRNA, or siRNA against PI4KIIIβ and 14-3-3γ or both. After 48 h the cells were harvested and subjected to western blot analysis for PI4KIIIβ, 14-3-3γ as well as with antibody against general ERK (gERK). (B) Cells grown on coverslips were transfected with siRNA (Si) as above. After 24 h, cells were either starved or treated with nocodazole (100 ng/ml) for an additional 16 h. Cells were then fixed and stained for ERK1c, GM130 and with DAPI. Slides were visualized by using a confocal spinning disc microscopy. (C) Quantification of ERK1c–GM130 localization. The data shown represent mean±s.e.m. (n=3). *P<0.05.

results validate that 14-3-3γ and PI4KIIIβ serve as a means to induce ERK1c translocation to the Golgi and therefore should be considered as new regulators of Golgi fragmentation.

PI4KIIIβ phosphorylation by PKD1 regulates its binding to ERK1c

It has been previously shown that the interaction between PI4KIIIβ and 14-3-3γ depends on the phosphorylation of Ser294 of PI4KIIIβ by PKD (Hausser et al., 2006). Furthermore, PKD is phosphorylated and activated upon nocodazole treatment (Fuchs et al., 2009), and its signaling has been reported to be important for mitotic Golgi fragmentation upstream of the ERK cascade in an indirect manner (Kienzle et al., 2013). We therefore undertook to study whether the phosphorylation of PI4KIIIβ by PKD is necessary for the mitotic Golgi translocation of ERK1c. We found that the interaction between ERK1c and WT PI4KIIIβ is indeed increased upon nocodazole stimulation, whereas the PI4KIIIβ-Ser294Ala mutant that cannot be regulated by PKD, demonstrated only basal interaction in cycling cells, which was reduced upon nocodazole stimulation (Fig. 7A,B). Interestingly, kinase dead PI4KIIIβ (Asp656Ala) interacted with ERK1c regardless of the stimulation, suggesting that the kinase activity of PI4KIIIβ is not required for the process (Fig. 7A,B). The importance of PKD phosphorylation to the assembly of the shuttling complex was verified by inhibition of PKD activity with 10 µM kb NB 142-70 (Ni et al., 2013), which indeed prevented the assembly of ERK1c with PI4KIIIβ but not with 14-3-3γ, as assessed by a co-immunoprecipitation assay (Fig. 7C,D) and by PLA (Fig. 7E,F). These results indicate that PI4KIIIβ serves as a shuttling platform for ERK1c that requires PKD phosphorylation but not lipid kinase activity. The assembly of ERK1c with the shuttling complex is sequential, first ERK1c interacts with 14-3-3γ, and only then do they interact with phosphorylated PI4KIIIβ. Thus, the three Golgi fragmentation regulating signaling pathways, the ERK1c cascade, CDK1, and PKD signaling, which were originally assumed to act independently, integrate into one coherent pathway.

DISCUSSION

In this study, we elucidated the molecular mechanism that targets ERK1c to the Golgi during mitosis. We have shown that the phosphorylation of Ser343 by CDK1 in the unique C-terminal region of ERK1c regulates the Golgi translocation of ERK1c. The phosphorylation of the ERK1c TEY motif is dependent on the Ser343-phosphorylation-induced Golgi translocation but is not required for the translocation itself. Our results best fit a model in which the expression of ERK1c in cycling cells is low and is localized diffusely all over the cell. In late G2, the amount of ERK1c in the cells increases, followed by its Ser343-phosphorylation by CDK1 in prophase and prometaphase. pSer343-ERK1c then binds to 14-3-3γ, which further interacts with phosphorylated PI4KIIIβ, thus forming a shuttling complex that escorts ERK1c to the Golgi. The assembly of the shuttling complex is regulated by PKD, which phosphorylates PI4KIIIβ on Ser294. In the Golgi, ERK1c meets active MEK1b, which phosphorylates it on the activatory TEY motif and, consequently, induces ERK1c activation to facilitate Golgi fragmentation (Fig. 8).

It has been previously suggested that the ERK cascade regulates Golgi fragmentation by direct phosphorylation of GRASP55 and GRASP65 (Jesch et al., 2001). In our previous studies, we found that ERK1c, rather than ERK1/2, is the ERK isoform that is found in the Golgi during mitosis (Shaul and Seger, 2006). Thus, it was initially hypothesized that ERK1c might act by phosphorylating the GRASP proteins. However, our recent preliminary results do not support this assumption, as we did not detect substantial phosphorylation of GRASP proteins by ERK1c in *in vitro* phosphorylation assays (data not shown). Moreover, here we show that ERK1c translocates to the Golgi only at prophase, and therefore, it is unlikely to execute the GRASP phosphorylation, which has already started at G2. Instead, it is possible that GRASP proteins are phosphorylated by the other MAPK family member, JNK2, which has recently been shown to mediate GRASP65 phosphorylation at G2 (Cervigni et al., 2015). The substrates of

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

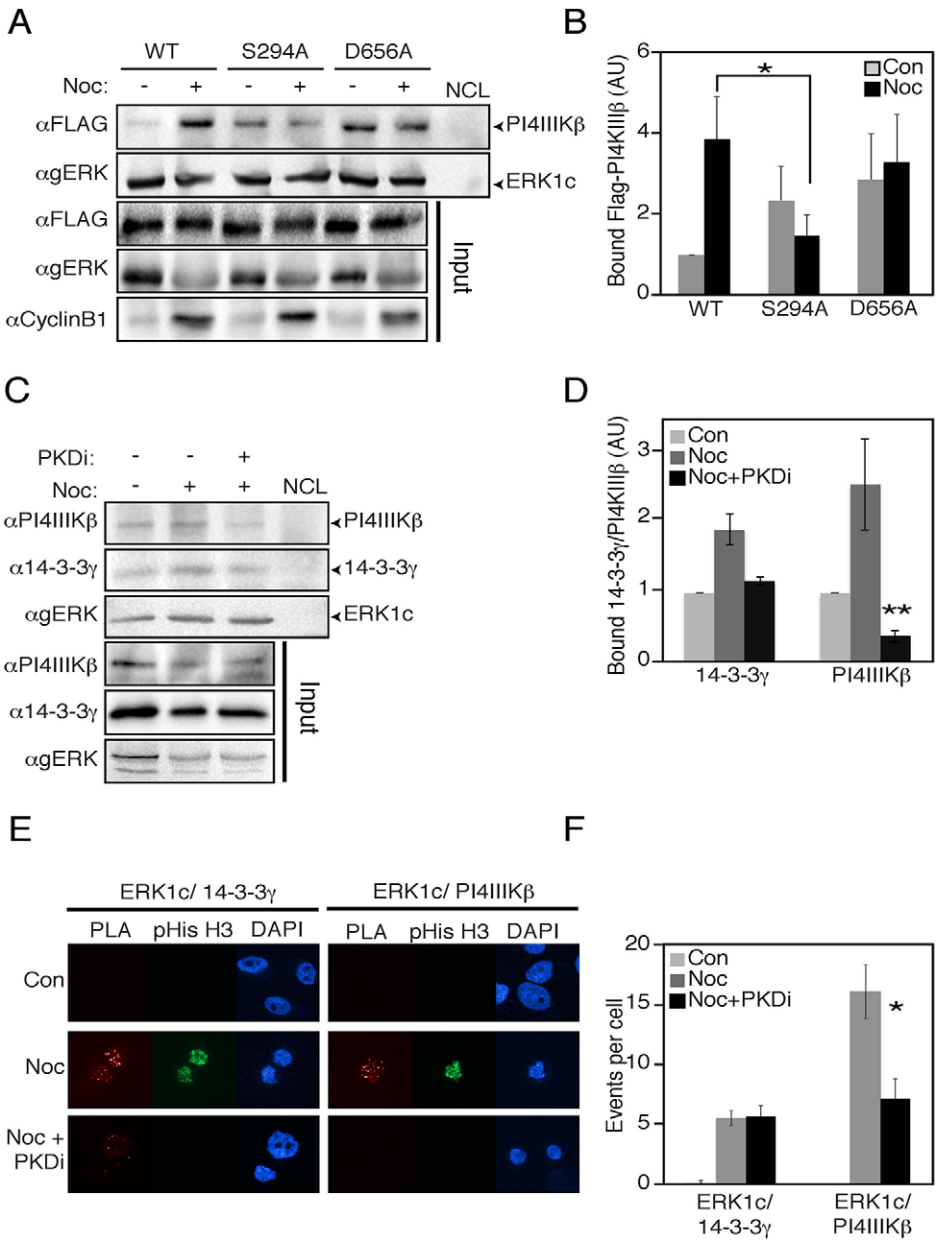

Fig. 7. PKD phosphorylation of PI4KIIIβ is essential for the shuttling complex formation. (A) HeLa cells were transfected with GFP–ERK1c and Flag-tagged PI4KIIIβ WT, Ser294Ala and Asp656Ala for 24 h, and then synchronized by nocodazole (Noc) treatment for 16 h. GFP proteins were co-immunoprecipitated, and blotted with the indicated antibodies. gERK, general ERK; NCL, no cell lysate. (B) The amount of bound PI4KIIIβ for the experiment described in A was quantified using Image Lab (Bio-Rad). Results shown represent mean±s.e.m. (n=3). *P<0.01. (C) HeLa cells were transfected with GFP–ERK1c for 24 h, and then synchronized by nocodazole treatment for 16 h. Cells were treated with 10 µM kb Nb 142-70 (PKDi) for 1 h. GFP proteins were co-immunoprecipitated, and blotted with the indicated antibodies. (D) The amount of bound PI4KIIIβ for the experiment described in A was quantified using Image Lab. Results shown represent mean±s.e.m. (n=4). **P<0.05. (E) A PLA demonstrated a mitotic-induced interaction of ERK1c with PI4KIIIβ–14-3-3γ. HeLa cells were grown on slides to 70% confluence, serum starved, and either treated with 100 ng/ml nocodazole for 16 h with or without 10 µM kb Nb 142-70 for 1 h, or left untreated (control). The cells were then fixed, subjected to a PLA with the indicated antibodies, and stained with antibody against histone 3 phosphorylated at Ser28 (pHis-H3). The cells were visualized by using a confocal spinning disc microscope. The experiment was repeated three times, with more than 100 cells were counted in each treatment. (F) Quantification of the interactions for the experiment described in E was performed using ImageJ. The data shown represent means± s.e.m. *P<0.01.

ERK1c at prophase and prometaphase are yet to be identified, but our initial results suggest that ERK1c might be involved in releasing the Golgi from fragmentation-inhibiting components at the onset of mitosis.

Mitotic Golgi fragmentation is a spatio-temporal regulated process, required for proper division of the Golgi between the two daughter cells, and is considered to be a mitotic checkpoint (Shaul and Seger, 2006; Colanzi et al., 2007; Feinstein and Linstedt, 2007) that is regulated in part by Raf and MEK (Kano et al., 2000; Colanzi et al., 2003a; Feinstein and Linstedt, 2007; Villeneuve et al., 2013). Interestingly, it has been noticed that this effect is generated independently of ERK1/2, the main ERK isoforms downstream of MEK1/2 (Acharya et al., 1998; Colanzi et al., 2000, 2003a). However, it has been shown that mono-phosphorylated ERK does exist in the mitotic Golgi, suggesting that the effect of MEK1/2 might be mediated by other ERK-like proteins (Cha and Shapiro, 2001). Indeed, our group has identified ERK1c, a unique primate splicing variant of ERK1 (Aebersold et al., 2004), that is highly expressed and activated during the G2 and M stages of the cell cycle (Shaul and Seger, 2006). We also noticed that the elevated expression and activation of ERK1c is accompanied by a massive translocation of ERK1c to the Golgi. At the Golgi, ERK1c is activated by MEK1b (Shaul et al., 2009) and, consequently, induces and/or regulates Golgi fragmentation. The increased expression in ERK1c, along with the timing of translocation to the Golgi in prophase, suggests that the translocation of ERK1c to the Golgi is a regulated process.

There are several known mechanisms of protein targeting and shuttling to the Golgi. One of the main mechanisms is protein ubiquitylation. This process has been shown to regulate the redistribution of a protein into the endocytic system, and especially to the Golgi. Ste2p, Ste6p, Fur4p and Gap1p, among other proteins, undergo ubiquitylation that induces translocation to the Golgi in yeasts (Galan et al., 1996; Hicke and Riezman, 1996; Kolling and Losko, 1997; Helliwell et al., 2001; Risinger and Kaiser, 2008). Although we have previously shown that ERK1c is mono-ubiquitylated in over-condensed cells, and suggested that this

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

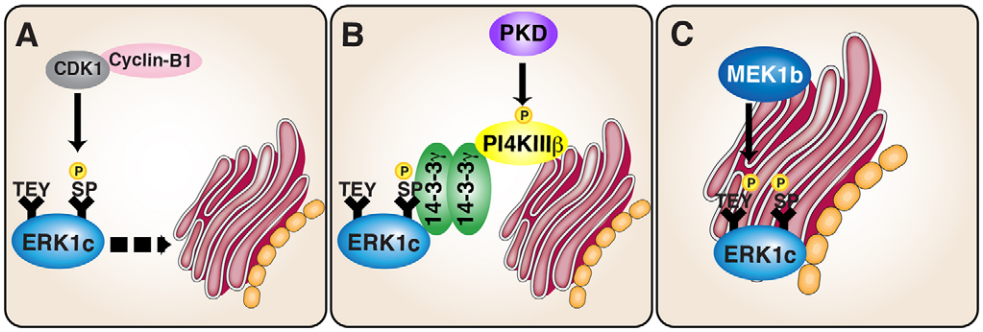

Fig. 8. A model of ERK1c-induced translocation to the Golgi. (A) During late G2, CDK1 becomes active, and phosphorylates ERK1c on Ser343 in its unique C-terminus. (B) Phosphorylated ERK1c interacts with a complex of PI4KIIIβ and 14-3-3γ, which mediates the Golgi translocation. (C) In the Golgi, ERK1c is phosphorylated by MEK1b, becomes active and induces mitotic Golgi fragmentation.

ubiquitylation might target ERK1c to the Golgi (Aebersold et al., 2004), we did not detect ubiquitylation of ERK1c during mitosis (data not shown). This indicates that the translocation in mitosis is distinct from that in other stages of the cell cycle. Another mechanism of Golgi targeting is through a KDEL motif in the protein. The KDEL receptor is found in the cis-Golgi complex, where it attracts KDEL-containing proteins that escape from the ER. Subsequently, the receptor redirects these proteins back to the ER as part of the anterograde transport (Pfeffer, 2007; Capitani and Sallese, 2009). Several other proteins, like the glycosyltransferases, translocate to the Golgi owing to their interaction with Golgi-targeting domains of other proteins or by interacting with various golgins, such as GM130, Giantin, and GRASP55 and GRASP65 (Petrosyan et al., 2012). Additionally, some of the Golgi proteins seem to have specific targeting sequence and domains, distinct from the classical KDEL (Kjer-Nielsen et al., 1999; Lee and Pohajdak, 2000; Brown et al., 2001; Shi et al., 2004), with the exact sequences and their common mechanism of action still, as yet, obscure. More recently, it has been shown that phosphorylation can also serve as a Golgi-targeting mechanism (Valente et al., 2012). In that study, it was demonstrated that phosphorylation of BARS on Ser147 by PAK1–PAK3 induces BARS interaction with the 14-3-3γ dimer and PI4KIIIβ which, together, serve as a Golgi shuttling complex. However, this mechanism was suggested to act specifically in the vesiculization of the Golgi, which is not very active during mitosis (Takatsu et al., 2013). Here, we demonstrate that the PI4KIIIβ–14-3-3γ complex does affect mitotic Golgi translocation of ERK1c. The interaction of ERK1c with this complex depends on both the phosphorylation of Ser343 by the mitotic CDK1 and the phosphorylation of PI4KIIIβ by PKD that coordinate the timely translocation to the Golgi.

CDK1 is a potent kinase with ~80 known *in vivo* substrates, and an additional 300 potential targets (Enserink and Kolodner, 2010). Phosphorylation by CDK1 might affect activity, stability, interaction or localization of a given substrate. The change in the localization of a protein (not necessarily a Golgi protein) can occur either indirectly through the action of phosphorylation-binding proteins, as has been demonstrated for Pds1 and Ndc10 (Enserink and Kolodner, 2010), or directly, due to the phosphorylation by CDK1. An example of the latter effect is HuR, which has been found to be phosphorylated by CDK1 in order to induce its nuclear localization and retention by 14-3-3θ (Kim et al., 2008). Other proteins, such as Ect2 (Matthews et al., 2012) and greatwall kinase (Gwl, also known as MASTL) (Wang et al., 2013), are phosphorylated by CDK1 at prophase, inducing their nuclear export and activation. Finally, CDK1 phosphorylates the protein survivin (also known as BIRC5), a component of the chromosomal passenger complex (CPC), to induce the CPC translocation to the centromeres at prometaphase, allowing chromosome bi-orientation (Tsukahara et al., 2010). Our findings in this paper add ERK1c to the group of substrates that are spatially regulated by CDK1.

In summary, we have elucidated the molecular mechanism that targets ERK1c to the Golgi during mitosis. We show that the unique C-terminal region facilitates the Golgi translocation during mitosis. This timely translocation is achieved by CDK1-induced phosphorylation of Ser343. The phosphorylated ERK1c interacts with 14-3-3γ, which further interacts with PKD-phosphorylated PI4KIIIβ, to form a Golgi-shuttling complex essential for ERK1c activation and Golgi fragmentation. These data combine the actions of three main regulatory components of Golgi fragmentation, which were thought to act independently, into one pathway. It is very likely that other regulatory components, such as Plk3, Myt1 and GRASP55 feed into the same pathway by regulating the same or other interacting components. Thus, a crosstalk between the ERK cascade, CDK1 and PKD is essential for proper mitotic Golgi fragmentation.

MATERIALS AND METHODS

Cell culture, transfection and synchronization

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). HB2 cells were cultured in the same medium, with the addition of hydrocortisone (0.5 mg/ml) and insulin (10 µg/ml). All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HeLa cells were transfected with plasmids using polyethylenimine and with siRNAs using Dharmafect. Cells were synchronized at the G1/S boundary by double-thymidine block (Merrill, 1998). In short, HeLa cells were treated with 2.5 mM thymidine in DMSO, washed twice with PBS, grown for 8 h in regular medium, treated again with 2.5 mM thymidine for 16 h and then washed with PBS. This marks time 0, after which the cells were grown under regular conditions. HeLa cells were also synchronized at the M phase, using 100 ng/ml nocodazole for 16 h.

Antibodies and reagents

Thymidine, nocodazole, myelin basic protein (MBP), propidium iodide, polyethylenimine, PD184352, ATP, and antibodies against phosphorylated (p)ERK, tyrosine-phosphorylated (pY)-ERK, general ERK, general MEK and flag-M2 were purchased from Sigma-Aldrich. Antibodies against GST, PI4KIIIβ, 14-3-3γ, CDK1 and CyclinB1, as well as Protein A/G PLUS–agarose beads and kb Nb 142-70 and roscovitine were obtained from Santa Cruz Biotechnology. [γ32P]-ATP was from Perkin Elmer; Draq5 from BioStatus, (Leicestershire, UK); Alexa Fluor® 488 and 568 as well as DAPI was from Invitrogen. UO126 was from Calbiochem; flavopiridol and anti-calnexin antibody was from Enzo Life Science (Farmingdale, NY); active CDK1–CyclinB1 from Upstate (Lake Placid, NY); anti-GFP antibody mouse monoclonal antibody from Roche; rabbit polyclonal anti-GFP antibody was from Abcam; anti-GM130 antibody from MBL; anti-pHis-H3 S10 antibody was from CST (Boston, MA), anti-pHis-H3 S28 Alexa-Fluor-488-conjugated antibody from BD Biosciences; anti-CCDC86 antibody was from Bethyl Laboratories; the developing substrate NBT/BCIP was

Journal of Cell Science (2015) 128, 4083-4095 doi:10.1242/jcs.170910

from Promega. Secondary antibodies conjugated to Cy2, rhodamine, alkaline phosphatase, Alexa Fluor® 647 and horseradish peroxidase were from Jackson ImmunoResearch Laboratories; Dharmafect was from Dharmacon (Thermo Fisher Scientific, MA, USA). The anti-ERK1c antibody was prepared by the antibody unit of the Weizmann Institute of Science, as described previously (Aebersold et al., 2004), and the rabbit polyclonal anti-pSer343 antibody against the peptide [EVGQ(pS)PAAVGLGAGC] was prepared by GenScript (NJ, USA).

DNA constructs and mutations

GFP–ERK1 and GFP–ERK1c were cloned in pEGFP-C1 (Clontech, Mountain View, CA) as previously described (Shaul and Seger, 2006). The GFP-tagged ΔCter–ERK1 was produced by PCR amplification of the ERK1 1–1017 into pEGFP-C1 vector between XhoI and EcoRI sites. Point mutations were performed by site-directed mutagenesis. GST–ERK1 and GST–ERK1c were cloned in pGEX-2T vector (GE Healthcare, Buckinghamshire, UK) as previously described (Shaul and Seger, 2006), The GST-tagged C-terminus of ERK1c was produced by PCR amplification of the ERK1c C-terminus into pGEX-4T-1 vector between EcoRI and XhoI sites (GE Healthcare, Buckinghamshire, UK). The GST protein was purified according to the manufacturer's instructions and eluted from the glutathione beads using 10 mM of reduced glutathione, plasmids for expression of Flag-tagged PI4KIIIβ WT, S294A and D656A were produced as previously described (Hausser et al., 2005).

Immunoprecipitation

Cells were grown to 70% confluence, and then serum-starved (0.1% FBS for 16 h). After stimulation or other treatments, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and once with Buffer A (50 mM β-glycerophosphate pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1mM dithiothreitol, and 0.1 mM sodium vanadate). The cells were then scraped into Buffer H (50 mM β-glycerophosphate pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1mM dithiothreitol, 0.1 mM sodium vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 µg/ml pepstatin A, and 1 mM Benzamidin) (0.5 ml/plate), sonicated (50 W, 2×7 s) and centrifuged (15,000 ***g***, 15 min). Supernatants were then incubated for 2 h (4°C, with rotation) with protein-A/G–agarose beads (Santa Cruz Biotechnology) pre-linked with specific antibodies (1 h, 23°C). The bound protein-A/G beads were washed three times with ice-cold co-immunoprecipitation-washing buffer. The immunoprecipitated beads were then resuspended with sample buffer and boiled; the resolved proteins were analyzed by western blotting with the indicated antibodies.

In vitro kinase assay

Immunoprecipitated ERKs attached to protein-A/G beads were used as kinases by mixing them with MBP (5 µg/reaction). Similarly 0.3 µg active CDK1–CyclinB1 complex was incubated with GST-tagged ERK1, ERK1c and the ERK1c C-terminus (0.5 µg per reaction), or with immunoprecipitated ERKs attached to protein-A/G beads. Buffer RM [RM at threefold concentration is 30 mM $MgCl_2$, 4.5 mM DTT, 75 mM β-glycerophosphate pH 7.3, 0.15 mM sodium vanadate, 3.75 mM EGTA, 30 µM calmidazolium, and 2.5 mg/ml bovine serum albumin (BSA)] containing 100 µM γ[^{32}P]ATP (4000 cpm/pmol), was added to the reaction at a final volume of 30 µl and incubated for 20 min at 30°C with shaking. The reaction was terminated by adding 10 µl of 4× sample buffer, and the phosphorylated proteins were resolved on SDS-PAGE and subjected to autoradiography and western blot analysis with the proper antibodies.

Immunofluorescence microscopy

Cells were fixed in 3% paraformaldehyde in PBS (20 min, 23°C), and incubated with 2% bovine serum albumin (BSA) in PBS (15 min, 23°C), followed by permeabilization with Triton X-100 (0.1% in PBS, 5 min, 23°C). The fixed cells were then incubated with the primary antibodies (1 h, 23°C), washed three times with PBS and incubated with rhodamine-conjugated secondary antibody (1 h, 23°C) and DAPI. Slides were visualized by using a fluorescent microscope (Olympus BX51, ×40 magnification, PA, USA), or by spinning disc confocal microscopy (×60 magnification Zeiss, Jena, Germany). Background correction and contrast adjustment of raw data images were performed, using Photoshop (Adobe, CA).

Multispectral imaging flow-cytometry analysis

Synchronized cells were fixed with methanol, and stained, as described above, using anti-ERK1c antibody, anti-GM130 antibody, for the Golgi, and Draq5, for the DNA. Cells were imaged using multispectral imaging flow cytometry (ImageStreamX flow-cytometer; Amnis Corp., Seattle, WA). For multispectral imaging flow cytometry, ~1×10^4–2×10^4 cells were collected from each sample and data were analyzed using image analysis software (IDEAS 4.0; Amnis Corp.). Images were compensated for fluorescent dye overlap by using single-stained controls. Cells were gated for single cells, using the area and aspect ratio features, and for focused cells, using the Gradient RMS feature, as previously described (George et al., 2006). Cells were gated for G2 and M based on Draq5 intensity. The G2 and M population was further gated for prophase, prometaphase and mitotic (metaphase-telophase) populations using two parameters: the bright detail intensity of Draq5 staining (intensity of localized bright areas, subtracted for the local background), and the area of the 50% highest intensity pixels of the Draq5 staining. The different mitotic stages were further defined by the nuclear morphology, as previously described (Merrill, 1998). For detailed gating of the mitotic phases, please see the Fig. S3 legend. The colocalization of ERK1c and the GM130 was quantified using the bright detail similarity feature [the log transformed Pearson's correlation coefficient of the localized bright spots in the two input images (George et al., 2006)]. Images of cells with similarity values above 1.5 were considered to display a localized ERK1c in the Golgi.

Proximity ligation assay

Protein–protein interactions were detected by using a Duolink PLA kit [Olink Bioscience, Uppsala, Sweden; Lowder et al., 2011), according to the manufacturer's protocol. Briefly, cells were grown, fixed, and permeabilized, as described in the immunofluorescence microscopy section. The samples were then incubated with primary antibodies against two examined proteins (1 h, 23°C), washed (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.05% Tween 20), and then incubated with specific probes (1 h, 37°C), followed by DAPI staining to visualize nuclei and then another wash (0.2 M Tris-HCl pH 7.5, 0.15 M NaCl). The signal was visualized as distinct fluorescent spots by using spinning disc confocal microscopy. The number of PLA events was counted automatically in ImageJ, by the 'analyze particles' feature. Each field was counted twice: first, for the number of nuclei in the field, and second, for the number of PLA events. Then the average number of events was calculated per treatment. More than 100 cells were counted per treatment. Background correction, contrast adjustment, and the quantification of the fluorescence signal were performed using Photoshop and ImageJ software.

Golgi extraction on non-continuous sucrose gradients

Cells from three 15-cm^2 confluent plates were harvested in buffer H. Cells were centrifuged for 4 min at 200 ***g*** and 4°C, and resuspended in 750 µl buffer H. Homogenization of the cells was performed by 50 strokes with manual pestles, and 20 strokes of a 1 ml syringe, and then centrifuged for 6 min at 1700 ***g*** and 4°C. A non-continuous sucrose gradient was prepared by loading 800 µl of 0.5, 0.8, 1, 1.2 and 1.5 M sucrose solutions in buffer H. The supernatant (800 µl) was loaded on top of the gradient and the sample was centrifuged in an Ultra-centrifuge for 2 h at 4°C and 100,000 ***g*** (35,000 rpm, SW-T40i rotor). Finally, 12 fractions of 400 µl were collected carefully from the top.

Proliferation assay

Cell proliferation was determined using a Methylene Blue assay. For this purpose, HeLa cells were transfected with the indicated plasmids, and incubated for 48 or 72 h in 1% FCS-containing medium. Cells were fixed in 4% paraformaldehyde at room temperature, then washed once in 0.1 M sodium borate buffer, pH 8.5, and, thereafter, incubated with 1% Methylene Blue in 0.1 M sodium borate buffer, pH 8.5, for 10 min. Excess of stain was washed out with double distilled water, and the stain was extracted with 0.1 M HCl (0.4 ml/well) for 2 h at room temperature with shaking.

Thereafter, aliquots of each sample were transferred into a 96-well plate, and absorbance at 495 nm was determined using an ELISA reader.

FACS analysis

Cells were trypsinized, washed with PBS, and fixed in ice-cold methanol overnight. The samples were resuspended in 0.5 ml of staining solution (0.1% Triton X-100, 33 mM sodium-citrate, 100 µg/ml RNase and 50 µg/ml propidium iodide). The cells were analyzed in a LSR II (Becton Dickinson, NJ, USA) machine, and the percentage of cells at different stages was calculated using the FlowJo software.

Statistical analysis

Data are expressed as mean±s.e.m. Statistical evaluation was carried out using functional analysis and Student's *t*-test (two-tailed) to test for differences between the control and experimental results. Values of $P<0.05$ were considered statistically significant. The ImageStream statistical analysis was performed using a two-way ANOVA, with an *a priori* contrast between the groups.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

I.W. was responsible for designing and performing experiments, as well as writing the paper. T.H. performed experiments. Z.P. performed the ImageStream analysis. A.H. supplied reagents. R.S. designed experiments, wrote the paper and supervised the work.

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Supplementary information

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References

Acharya, U., Mallabiabarrena, A., Acharya, J. K. and Malhotra, V. (1998). Signaling via mitogen-activated protein kinase kinase (MEK1) is required for Golgi fragmentation during mitosis. *Cell* **92**, 183-192.

Aebersold, D. M., Shaul, Y. D., Yung, Y., Yarom, N., Yao, Z., Hanoch, T. and Seger, R. (2004). Extracellular signal-regulated kinase 1c (ERK1c), a novel 42-kilodalton ERK, demonstrates unique modes of regulation, localization, and function. *Mol. Cell. Biol.* **24**, 10000-10015.

Bailly, E., McCaffrey, M., Touchot, N., Zahraoui, A., Goud, B. and Bornens, M. (1991). Phosphorylation of two small GTP-binding proteins of the Rab family by p34cdc2. *Nature* **350**, 715-718.

Borysov, S. I. and Guadagno, T. M. (2008). A novel role for Cdk1/cyclin B in regulating B-raf activation at mitosis. *Mol. Biol. Cell* **19**, 2907-2915.

Brown, D. L., Heimann, K., Lock, J., Kjer-Nielsen, L., van Vliet, C., Stow, J. L. and Gleeson, P. A. (2001). The GRIP domain is a specific targeting sequence for a population of trans-Golgi network derived tubulo-vesicular carriers. *Traffic* **2**, 336-344.

Capitani, M. and Sallese, M. (2009). The KDEL receptor: new functions for an old protein. *FEBS Lett.* **583**, 3863-3871.

Cervigni, R. I., Bonavita, R., Barretta, M. L., Spano, D., Ayala, I., Nakamura, N., Corda, D. and Colanzi, A. (2015). JNK2 controls fragmentation of the Golgi complex and the G2/M transition through phosphorylation of GRASP65. *J. Cell Sci.* **128**, 2249-2260.

Cha, H. and Shapiro, P. (2001). Tyrosine-phosphorylated extracellular signal–regulated kinase associates with the Golgi complex during G2/M phase of the cell cycle: evidence for regulation of Golgi structure. *J. Cell Biol.* **153**, 1355-1368.

Chiu, C.-Y., Kuo, K.-K., Kuo, T.-L., Lee, K.-T. and Cheng, K.-H. (2012). The activation of MEK/ERK signaling pathway by bone morphogenetic protein 4 to increase hepatocellular carcinoma cell proliferation and migration. *Mol. Cancer Res.* **10**, 415-427.

Chuderland, D., Konson, A. and Seger, R. (2008). Identification and characterization of a general nuclear translocation signal in signaling proteins. *Mol. Cell* **31**, 850-861.

Colanzi, A., Deerinck, T. J., Ellisman, M. H. and Malhotra, V. (2000). A specific activation of the mitogen-activated protein kinase kinase 1 (MEK1) is required for Golgi fragmentation during mitosis. *J. Cell Biol.* **149**, 331-340.

Colanzi, A., Suetterlin, C. and Malhotra, V. (2003a). Cell-cycle-specific Golgi fragmentation: how and why? *Curr. Opin. Cell Biol.* **15**, 462-467.

Colanzi, A., Sutterlin, C. and Malhotra, V. (2003b). RAF1-activated MEK1 is found on the Golgi apparatus in late prophase and is required for Golgi complex fragmentation in mitosis. *J. Cell Biol.* **161**, 27-32.

Colanzi, A., Hidalgo Carcedo, C., Persico, A., Cericola, C., Turacchio, G., Bonazzi, M., Luini, A. and Corda, D. (2007). The Golgi mitotic checkpoint is controlled by BARS-dependent fission of the Golgi ribbon into separate stacks in G2. *EMBO J.* **26**, 2465-2476.

Corda, D., Barretta, M. L., Cervigni, R. I. and Colanzi, A. (2012). Golgi complex fragmentation in G2/M transition: an organelle-based cell-cycle checkpoint. *IUBMB Life* **64**, 661-670.

Enserink, J. M. and Kolodner, R. D. (2010). An overview of Cdk1-controlled targets and processes. *Cell Div.* **5**, 11.

Feinstein, T. N. and Linstedt, A. D. (2007). Mitogen-activated protein kinase kinase 1-dependent Golgi unlinking occurs in G2 phase and promotes the G2/M cell cycle transition. *Mol. Biol. Cell* **18**, 594-604.

Fuchs, Y. F., Eisler, S. A., Link, G., Schlicker, O., Bunt, G., Pfizenmaier, K. and Hausser, A. (2009). A Golgi PKD activity reporter reveals a crucial role of PKD in nocodazole-induced Golgi dispersal. *Traffic* **10**, 858-867.

Galan, J. M., Moreau, V., Andre, B., Volland, C. and Haguenauer-Tsapis, R. (1996). Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. *J. Biol. Chem.* **271**, 10946-10952.

George, T. C., Fanning, S. L., Fitzgeral-Bocarsly, P., Medeiros, R. B., Highfill, S., Shimizu, Y., Hall, B. E., Frost, K., Basiji, D., Ortyn, W. E. et al. (2006). Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. *J. Immunol. Methods* **311**, 117-129.

Hausser, A., Storz, P., Märtens, S., Link, G., Toker, A. and Pfizenmaier, K. (2005). Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. *Nat. Cell Biol.* **7**, 880-886.

Hausser, A., Link, G., Hoene, M., Russo, C., Selchow, O. and Pfizenmaier, K. (2006). Phospho-specific binding of 14-3-3 proteins to phosphatidylinositol 4-kinase III beta protects from dephosphorylation and stabilizes lipid kinase activity. *J. Cell Sci.* **119**, 3613-3621.

Helliwell, S. B., Losko, S. and Kaiser, C. A. (2001). Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. *J. Cell Biol.* **153**, 649-662.

Hicke, L. and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* **84**, 277-287.

Jesch, S. A., Lewis, T. S., Ahn, N. G. and Linstedt, A. D. (2001). Mitotic phosphorylation of Golgi reassembly stacking protein 55 by mitogen-activated protein kinase ERK2. *Mol. Biol. Cell* **12**, 1811-1817.

Kano, F., Takenaka, K., Yamamoto, A., Nagayama, K., Nishida, E. and Murata, M. (2000). MEK and Cdc2 kinase are sequentially required for Golgi disassembly in MDCK cells by the mitotic Xenopus extracts. *J. Cell Biol.* **149**, 357-368.

Kienzle, C., Eisler, S. A., Villeneuve, J., Brummer, T., Olayioye, M. A. and Hausser, A. (2013). PKD controls mitotic Golgi complex fragmentation through a Raf-MEK1 pathway. *Mol. Biol. Cell* **24**, 222-233.

Kim, H. H., Abdelmohsen, K., Lal, A., Pullmann, R., Jr., Yang, X., Galban, S., Srikantan, S., Martindale, J. L., Blethrow, J., Shokat, K. M. et al. (2008). Nuclear HuR accumulation through phosphorylation by Cdk1. *Genes Dev.* **22**, 1804-1815.

Kjer-Nielsen, L., van Vliet, C., Erlich, R., Toh, B. H. and Gleeson, P. A. (1999). The Golgi-targeting sequence of the peripheral membrane protein p230. *J. Cell Sci.* **112**, 1645-1654.

Kolling, R. and Losko, S. (1997). The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein. *EMBO J.* **16**, 2251-2261.

Lee, S. Y. and Pohajdak, B. (2000). N-terminal targeting of guanine nucleotide exchange factors (GEF) for ADP ribosylation factors (ARF) to the Golgi. *J. Cell Sci.* **113**, 1883-1889.

Lin, C.-Y., Madsen, M. L., Yarm, F. R., Jang, Y.-J., Liu, X. and Erikson, R. L. (2000). Peripheral Golgi protein GRASP65 is a target of mitotic polo-like kinase (Plk) and Cdc2. *Proc. Natl. Acad. Sci. USA* **97**, 12589-12594.

Litvak, V., Argov, R., Dahan, N., Ramachandran, S., Amarilio, R., Shainskaya, A. and Lev, S. (2004). Mitotic phosphorylation of the peripheral Golgi protein Nir2 by Cdk1 provides a docking mechanism for Plk1 and affects cytokinesis completion. *Mol. Cell* **14**, 319-330.

Lowder, M. A., Appelbaum, J. S., Hobert, E. M. and Schepartz, A. (2011). Visualizing protein partnerships in living cells and organisms. *Curr. Opin. Chem. Biol.* **15**, 781-788.

Lowe, M., Rabouille, C., Nakamura, N., Watson, R., Jackman, M., Jämsä, E., Rahman, D., Pappin, D. J. C. and Warren, G. (1998). Cdc2 kinase directly phosphorylates the cis-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. *Cell* **94**, 783-793.

Lowman, X. H., McDonnell, M. A., Kosloske, A., Odumade, O. A., Jenness, C., Karim, C. B., Jemmerson, R. and Kelekar, A. (2010). The proapoptotic function of Noxa in human leukemia cells is regulated by the kinase Cdk5 and by glucose. *Mol. Cell* **40**, 823-833.

Matthews, H. K., Delabre, U., Rohn, J. L., Guck, J., Kunda, P. and Baum, B. (2012). Changes in Ect2 localization couple actomyosin-dependent cell shape changes to mitotic progression. *Dev. Cell* **23**, 371-383.

Merrill, G. F. (1998). Cell synchronization. *Methods Cell Biol.* **57**, 229-249.

Ni, Y., Sinnett-Smith, J., Young, S. H. and Rozengurt, E. (2013). PKD1 mediates negative feedback of PI3K/Akt activation in response to G protein-coupled receptors. *PLoS ONE* **8**, e73149.

Nurse, P. M. (2002). Nobel lecture: cyclin dependent kinases and cell cycle control. *Biosci. Rep.* **22**, 487-499.

Ouellet, J. and Barral, Y. (2012). Organelle segregation during mitosis: lessons from asymmetrically dividing cells. *J. Cell Biol.* **196**, 305-313.

Petrosyan, A., Ali, M. F. and Cheng, P.-W. (2012). Glycosyltransferase-specific Golgi-targeting mechanisms. *J. Biol. Chem.* **287**, 37621-37627.

Pfeffer, S. R. (2007). Unsolved mysteries in membrane traffic. *Annu. Rev. Biochem.* **76**, 629-645.

Phadwal, K., Alegre-Abarrategui, J., Watson, A. S., Pike, L., Anbalagan, S., Hammond, E. M., Wade-Martins, R., McMichael, A., Klenerman, P. and Simon, A. K. (2012). A novel method for autophagy detection in primary cells: impaired levels of macroautophagy in immunosenescent T cells. *Autophagy* **8**, 677-689.

Preisinger, C., Körner, R., Wind, M., Lehmann, W. D., Kopajtich, R. and Barr, F. A. (2005). Plk1 docking to GRASP65 phosphorylated by Cdk1 suggests a mechanism for Golgi checkpoint signalling. *EMBO J.* **24**, 753-765.

Risinger, A. L. and Kaiser, C. A. (2008). Different ubiquitin signals act at the Golgi and plasma membrane to direct GAP1 trafficking. *Mol. Biol. Cell* **19**, 2962-2972.

Schmierer, B. and Hill, C. S. (2005). Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads. *Mol. Cell. Biol.* **25**, 9845-9858.

Seger, R., Seger, D., Lozeman, F. J., Ahn, N. G., Graves, L. M., Campbell, J. S., Ericsson, L., Harrylock, M., Jensen, A. M. and Krebs, E. G. (1992). Human T-cell mitogen-activated protein kinase kinases are related to yeast signal transduction kinases. *J. Biol. Chem.* **267**, 25628-25631.

Shaul, Y. D. and Seger, R. (2006). ERK1c regulates Golgi fragmentation during mitosis. *J. Cell Biol.* **172**, 885-897.

Shaul, Y. D., Gibor, G., Plotnikov, A. and Seger, R. (2009). Specific phosphorylation and activation of ERK1c by MEK1b: a unique route in the ERK cascade. *Genes Dev.* **23**, 1779-1790.

Shi, X., Lappin, D. F. and Elliott, R. M. (2004). Mapping the Golgi targeting and retention signal of Bunyamwera virus glycoproteins. *J. Virol.* **78**, 10793-10802.

Sutterlin, C., Lin, C.-Y., Feng, Y., Ferris, D. K., Erikson, R. L. and Malhotra, V. (2001). Polo-like kinase is required for the fragmentation of pericentriolar Golgi stacks during mitosis. *Proc. Natl. Acad. Sci. USA* **98**, 9128-9132.

Sutterlin, C., Hsu, P., Mallabiabarrena, A. and Malhotra, V. (2002). Fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells. *Cell* **109**, 359-369.

Takatsu, H., Katoh, Y., Ueda, T., Waguri, S., Murayama, T., Takahashi, S., Shin, H.-W. and Nakayama, K. (2013). Mitosis-coupled, microtubule-dependent clustering of endosomal vesicles around centrosomes. *Cell Struct. Funct.* **38**, 31-41.

Takizawa, C. G. and Morgan, D. O. (2000). Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. *Curr. Opin. Cell Biol.* **12**, 658-665.

Torii, S., Kusakabe, M., Yamamoto, T., Maekawa, M. and Nishida, E. (2004). Sef is a spatial regulator for Ras/MAP kinase signaling. *Dev. Cell* **7**, 33-44.

Tsukahara, T., Tanno, Y. and Watanabe, Y. (2010). Phosphorylation of the CPC by Cdk1 promotes chromosome bi-orientation. *Nature* **467**, 719-723.

Valente, C., Turacchio, G., Mariggiò, S., Pagliuso, A., Gaibisso, R., Di Tullio, G., Santoro, M., Formiggini, F., Spanò, S., Piccini, D. et al. (2012). A 14-3-3gamma dimer-based scaffold bridges CtBP1-S/BARS to PI(4)KIIIbeta to regulate post-Golgi carrier formation. *Nat. Cell Biol.* **14**, 343-354.

Villeneuve, J., Scarpa, M., Ortega-Bellido, M. and Malhotra, V. (2013). MEK1 inactivates Myt1 to regulate Golgi membrane fragmentation and mitotic entry in mammalian cells. *EMBO J.* **32**, 72-85.

Wang, P., Galan, J. A., Normandin, K., Bonneil, E., Hickson, G. R., Roux, P. P., Thibault, P. and Archambault, V. (2013). Cell cycle regulation of Greatwall kinase nuclear localization facilitates mitotic progression. *J. Cell Biol.* **202**, 277-293.

Wei, J.-H. and Seemann, J. (2009). Mitotic division of the mammalian Golgi apparatus. *Semin. Cell Dev. Biol.* **20**, 810-816.

Wei, J.-H. and Seemann, J. (2010). Unraveling the Golgi ribbon. *Traffic* **11**, 1391-1400.

Wortzel, I. and Seger, R. (2011). The ERK cascade: distinct functions within various subcellular organelles. *Genes Cancer* **2**, 195-209.

Xie, S., Wang, Q., Ruan, Q., Liu, T., Jhanwar-Uniyal, M., Guan, K. and Dai, W. (2004). MEK1-induced Golgi dynamics during cell cycle progression is partly mediated by Polo-like kinase-3. *Oncogene* **23**, 3822-3829.

Youn, J. H. and Shin, J.-S. (2006). Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion. *J. Immunol.* **177**, 7889-7897.

Zheng, C. F. and Guan, K. L. (1993). Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. *J. Biol. Chem.* **268**, 23933-23939.