AMPK phosphorylates GBF1 for mitotic Golgi disassembly

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Summary

In mammalian cells, the Golgi apparatus undergoes extensive fragmentation during mitosis; this is required not only for the partitioning of the complex but also for the process of mitosis. However, the molecular mechanism underlying the mitotic fragmentation of the Golgi is far from clear. Here, we show that AMP-activated protein kinase (AMPK) is phosphorylated and activated when cells enter mitosis. Activated AMPK phosphorylates GBF1, a guanine nucleotide exchange factor (GEF) for Arf-GTPases, disassociating GBF1 from the Golgi membrane and abolishing the action of GBF1 as an Arf1-GEF. We further demonstrate that the phosphorylation of AMPK and GBF1 is essential for Golgi disassembly and subsequent mitosis entry. These data suggest that AMPK–GBF1–Arf1 signaling is involved in the regulation of Golgi fragmentation during mitosis.

Key words: Golgi fragmentation, Mitosis, AMPK, GBF1, Arf1

Introduction

The Golgi apparatus is a continuous membranous system located at the pericentriolar region in mammalian cells (Marsh and Howell, 2002). Maintenance of the Golgi apparatus requires Golgi matrix proteins such as GM130 and GRASP65 (Nakamura et al., 1995; Barr et al., 1997), together with a constant membrane input from the endoplasmic reticulum (ER), a process involving two small GTPases, Sar1 and Arf1 (Ward et al., 2001; Altan-Bonnet et al., 2006). During mitosis, the Golgi undergoes extensive fragmentation through a multistage process that ensures not only the correct partitioning and inheritance of the Golgi, but also entry into mitosis (Sütterlin et al., 2002; Hidalgo Carcedo et al., 2004). Despite the fact that multiple protein kinases such as cyclin-dependent kinases, MAP kinase/ERK kinase 1, and polo-like kinase 1 are involved in the process through phosphorylation of downstream targets such as cis-Golgi matrix proteins and vesicle-tethering molecules (Acharya et al., 1998; Lowe et al., 1998; Lin et al., 2000; Preisinger et al., 2005), how these phosphorylation events eventually cause fragmentation of the Golgi remains elusive.

The small GTPase Arf1, which undergoes dynamic association and disassociation with the Golgi membrane, plays an important role in the maintenance of Golgi structure. In its GTP-bound membrane-associated form, Arf1 is active and recruits multiple cytosolic proteins to the Golgi membranes (Altan-Bonnet et al., 2004). Upon hydrolysis of its GTP to GDP, Arf1 becomes inactive and dissociates from membranes, which causes disorganization of the Golgi complex (Lippincott-Schwartz et al., 1990). The cycling of Arf1 between the Golgi membranes and the cytosol is strictly controlled by its specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). It has been suggested that mitotic Golgi disassembly and reassembly depends on the inactivation and reactivation of Arf1, which control the cycling of Golgi components into and out of the ER (Altan-Bonnet et al., 2003; Altan-Bonnet et al., 2006), although the requirement of Arf1 activity for mitotic Golgi fragmentation is inconclusive. In addition, it has been proposed that the mitotic increase of inactive Arf1 in the cytosol is due to a reduced rate of Arf1 membrane binding rather than a raised rate of dissociation from the membrane, suggesting a drop in Arf-GEF function rather than an enhancement in Arf-GAP activity during mitosis (Altan-Bonnet et al., 2003). Although the molecular mechanism underlying the mitotic decline in Arf-GEF activity is unclear, a recent study has shown that GBF1, a peripheral early Golgi-associated Arf1-GEF, can be phosphorylated by CDK1–cyclin-B in mitosis, resulting in its dissociation from the Golgi membranes followed by inactivation of Arf1 (Morohashi et al., 2010).

AMP-activated protein kinase (AMPK) is a serine-threonine kinase implicated in key cellular pathways including energy sensing and polarity establishment (Hardie et al., 1998; Mirouse et al., 2007; Zheng and Cantley, 2007). Under ATP-depleted conditions, AMPK is activated by an increase in AMP through binding of AMP to its regulatory γ subunit and the phosphorylation of its catalytic α subunit by AMPK kinases such as LKB1 (Hawley et al., 2003; Woods et al., 2003) and Ca^{2+}/calmodulin-dependent protein kinase kinase (Hawley et al., 2005; Woods et al., 2005). *In vivo* studies have discovered that AMPK may be in an ideal position to ensure cell division by mediating the mitosis-controlling functions of LKB1, because genetic interruption of AMPK activity leads to defective chromosomal segregation that eventually impedes the proper completion of mitotic cell division (Lee et al., 2007; Banko et al., 2011; Thaiparambil et al., 2012). The active form of AMPK can transiently associate with several

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mitotic structures including centrosomes, spindle poles and the midbody throughout the stages of mitosis (Vazquez-Martin et al., 2009b). Nevertheless, it has also been found that AMPK activation perturbs cell cycle progression by arresting cells at the G1 phase (Nagata et al., 2004; Igata et al., 2005), through suppression of mammalian target of rapamycin (mTOR) signaling (Gwinn et al., 2008). Thus, the temporal and spatial regulation of mitosis by AMPK remains elusive.

Interestingly, it was recently demonstrated that GBF1 is a novel AMPK substrate, and phosphorylation of GBF1 at Thr1337 by AMPK plays a critical role in ATP depletion-induced Golgi disassembly (Miyamoto et al., 2008). In this study, we have investigated the regulation of GBF1 by AMPK during mitosis, and determined its relevance to Golgi disassembly and the mitotic process. Our data support the conclusion that as a result of AMPK activation, phosphorylation of GBF1 at Thr1337 which disassociates GBF1 from the Golgi membranes and blocks its function as an Arf-GEF, is implicated in mitotic Golgi disassembly and the coordination of other events required for mitosis.

Results

AMPK is activated in mitotic cells

Cell division includes a series of energy-consuming events such as DNA replication, chromosomal segregation and cytokinesis. We initially examined the activity of AMPK in mitotic HEK293 cells by staining them with a specific anti-phospho-AMPKα^{Thr172}. We noted that the phospho-AMPK signals were only detectable in cells in mitosis and not in interphase (Fig. 1A), indicating AMPK activation in these cells. We further evaluated this activation by measuring the phosphorylation levels of AMPK and acetyl-CoA carboxylase, a typical substrate of AMPK (Sim and Hardie, 1988; Carling and Hardie, 1989), from western blots. Compared with cells in interphase showing low phospho-AMPK, mitotic cells arrested by nocodazole demonstrated a significant increase in both phospho-AMPK (Fig. 1B) and phospho-acetyl-CoA carboxylase (Fig. 1C). The mitotic enhancement of phospho-acetyl-CoA carboxylase was evidently attenuated by the AMPK inhibitor, Compound C, confirming the specificity of AMPK activation in the cells (Fig. 1C). These results suggest that AMPK is activated when cells enter mitosis.

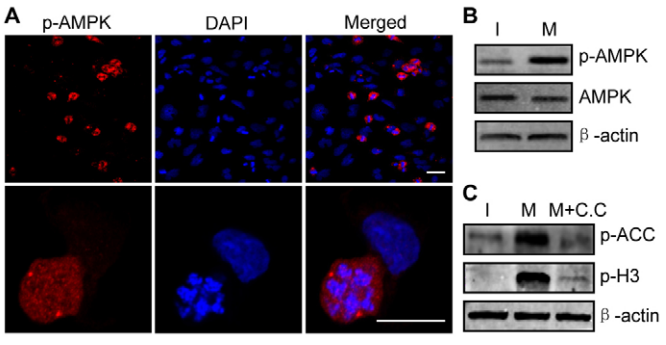

Fig. 1. AMPK is activated during mitosis. (**A**) HEK293 cells were left untreated or treated with nocodazole for 12 hours. Then the cells were stained with an anti-p-AMPK^{Thr172} antibody and imaged by confocal microscopy. Scale bars: 20 μm. (**B**,**C**) HEK293 cells were either untreated or treated with nocodazole with or without Compound C (C.C) for 16 hours. Then the cells were lysed and analyzed by western blotting using p-AMPK (B) or phospho-acetyl-CoA carboxylase (p-ACC) antibody (C). I, interphase; M, mitosis.

AMPK-dependent phosphorylation of GBF1 in mitosis

Deprivation of nutrients from cultured cells leads to phosphorylation of the Golgi-located GBF1 by activated AMPK, although an association of AMPK with the Golgi has not yet been seen (Miyamoto et al., 2008). To investigate whether activated AMPK in mitotic cells has the same action on GBF1, we assessed the phosphorylation of GBF1 and its dependence on AMPK during mitosis. Synchronized HEK293 cells were released from G1/S and GBF1 phosphorylation was determined with a phospho-AKT-substrate (PAS) antibody which recognizes the phosphorylation motif sequence of GBF1 (Miyamoto et al., 2008). A dramatic increase in phospho-GBF1 level occurred 8–10 hours after the cells were released from G1/S (Fig. 2A). The phosphorylation of GBF1 was well-coordinated with the phosphorylation of AMPK, and was followed by phosphorylation of histone-3 (p-H3), a typical marker for cells entering mitosis (Fig. 2A). Throughout the time-course, the total amount of GBF1 remained unchanged.

To gain further evidence about the phosphorylation of GBF1 and its dependence on AMPK during mitosis, we immunoprecipitated exogenously-expressed GBF1 and tested it with anti-PAS antibody in mitotic cells by nocodazole arrest. Our results showed that in nocodazole-treated mitotic cells, GBF1–GFP was significantly phosphorylated as demonstrated by increased PAS, and this phosphorylation was clearly prevented by expression of the inactive AMPK mutant (AMPK-DN–GFP) (Woods et al., 2000) or by addition of the AMPK inhibitor Compound C (Fig. 2B). We also performed an *in vitro* experiment to identify the mitotic phosphorylation of GBF1 by AMPK. GBF1–GFP was immunoprecipitated from cells that expressed it and cultured with interphase or mitotic cytosol. In some of the samples, the mitotic cytosol was either treated with Compound C or eluted by agarose-conjugated anti-AMPK antibody to delete AMPK. We found that the precipitated GBF1–GFP was phosphorylated by the mitotic cytosol but not the interphase cytosol. In addition, the phosphorylation of GBF1–GFP was abolished by Compound C addition to, or AMPK deletion from, the mitotic cytosol (Fig. 2C), indicating a specific effect of activated AMPK.

Finally, we confirmed the interaction between GBF1 and AMPK in mitotic cells by co-immunoprecipitation. In cells expressing GBF1–GFP, precipitation of GBF1 with a GFP antibody led to co-precipitation of endogenous AMPK only in nocodazole-treated mitotic cells (Fig. 2D). The formation of a complex of GBF1–GFP and AMPK was dependent on active AMPK and GBF1, because Compound C clearly inhibited the formation of the complex, and GFP itself did not precipitate AMPK (Fig. 2D).

Taken together, these results suggest that GBF1 is phosphorylated specifically by activated AMPK during mitosis.

Activation of AMPK and phosphorylation of GBF1 are essential to mitotic Golgi disassembly

Given that AMPK is activated and further phosphorylates Golgi-associated GBF1 during mitosis, we then evaluated the relevance of these events to Golgi disassembly and the process of mitosis. We first addressed whether inactivation of AMPK prevents the Golgi from fragmentation during mitosis. By releasing synchronized HEK293 cells from the G1/S phase, we observed

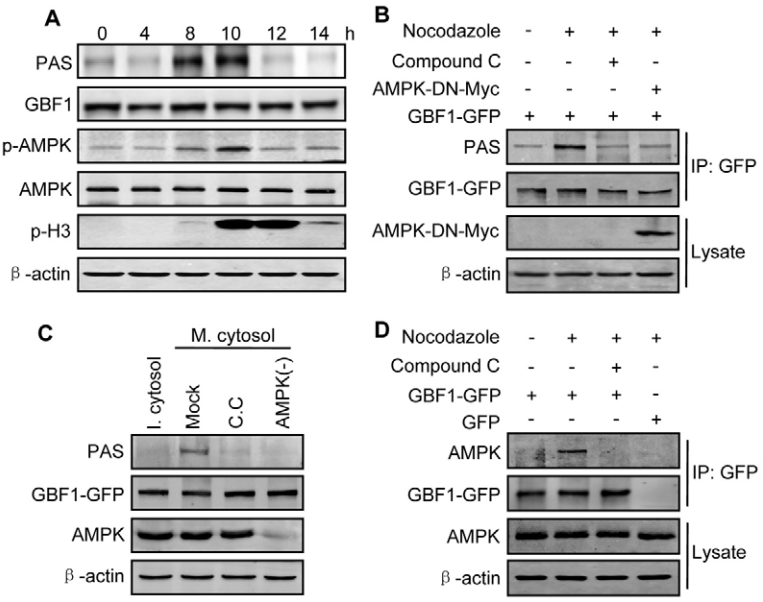

Fig. 2. Phosphorylation of GBF1 by AMPK in mitosis. (**A**) HEK293 cells were synchronized at the G1/S boundary by a double-thymidine block. At the indicated time points after release, the cells were harvested and analyzed by western blotting using the indicated antibodies. (**B**) HEK293 cells expressing GBF1–GFP with or without AMPK-DN–GFP were treated with nocodazole with or without Compound C for 16 hours. Then the cell lysates were precipitated with anti-GFP and the immunoprecipitates were subjected to western blotting using specific antibodies. (**C**) GBF1–GFP precipitated from GBF1-GFP-transfected HEK293 cells was incubated with HEK293 interphase cytosol, mitotic cytosol, or mitotic cytosol that had been treated with Compound C or depleted of AMPK. Then the precipitates were analyzed by western blotting. (**D**) Cells expressing GBF1–GFP were treated with nocodazole with or without Compound C for 16 hours. The cell lysates were immunoprecipitated with anti-GFP and analyzed by western blotting.

the morphology of the Golgi complex in cells treated with Compound C or expressing AMPK-DN. Compared to control cells in which the Golgi dispersed and eventually became the Golgi haze 10 hours after release, addition of Compound C or expression of inactive AMPK clearly inhibited the fragmentation of the Golgi and the complexes were maintained as perinuclear structures (Fig. 3A,B). We also performed AMPK RNAi and observed the effect on Golgi organization during mitosis. The efficiency of the designed siRNA was confirmed by western blotting (Fig. 3C). As expected, knockdown of AMPK dramatically abated the dispersal of the Golgi complex, as indicated by reduction in the formation of the Golgi haze (Fig. 3D,E).

The role of AMPK-mediated GBF1 phosphorylation in mitotic Golgi disassembly was then studied by introducing into HEK293 cells a GFP-tagged GBF1 mutant, GBF1T1337A–GFP, in which the Thr1337 was replaced by non-phosphorylatable alanine, because a previous study showed that GBF1 can be phosphorylated directly by AMPK at Thr1337 (Miyamoto et al., 2008). In HEK293 cells, GBF1T1337A–GFP localized to the perinuclear Golgi region. Ten hours after the cells were released from G1/S, as in AMPK-inactivated cells, GBF1-T1337A–GFP expression evidently suppressed the fragmentation of the Golgi as shown as reduced formation of the Golgi haze (Fig. 3F,G).

These results indicate that activation of AMPK and subsequent phosphorylation of GBF1 are essential for mitotic Golgi disassembly.

Activation of AMPK and phosphorylation of GBF1 are required for mitosis

To further address the significance of AMPK activation and GBF1 phosphorylation, we determined their effect on mitosis. First, we found that, 10 hours after releasing HEK293 cells from thymidine treatment, when many of the cells rounded-up and presented typical mitotic morphology, addition of Compound C or transfection with AMPK-DN or GBF1T1337A dramatically decreased the number of rounded cells, implying that entry to mitosis was inhibited (Fig. 4A). Results from flow cytometry showed that Compound C, AMPK-DN or GBF1T1337A

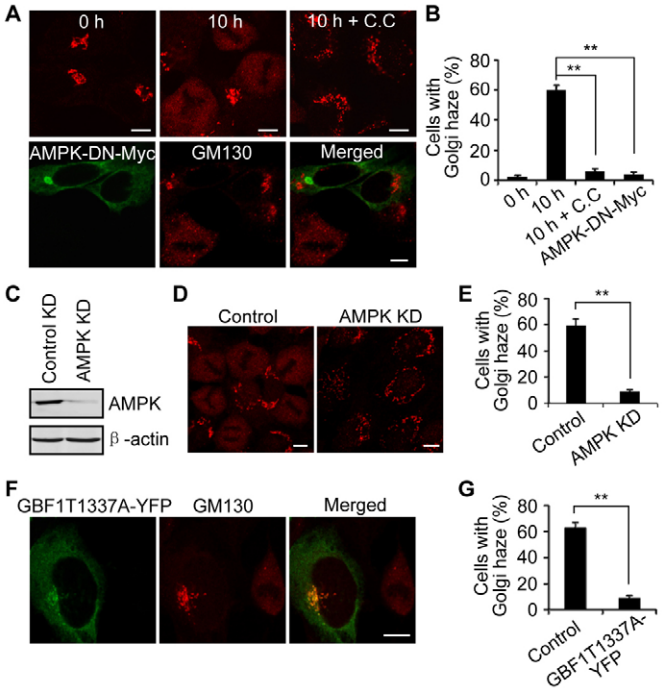

Fig. 3. Activation of AMPK and phosphorylation of GBF1 are essential to mitotic Golgi disassembly. (**A**) HEK293 cells with or without AMPK-DN–GFP expression were synchronized at the G1/S boundary by a double-thymidine block. Then the cells were released from G1/S phase in the absence or presence of Compound C for 10 hours. The cells were fixed, stained with anti-GM130 or anti-Myc, and imaged by confocal microscopy. (**C**) HEK293 cells were transfected with AMPK siRNA or control siRNA for 48 hours. Then the cells were lysed and analyzed by western blotting using anti-AMPK. (**D**) HEK293 cells were transfected with AMPK siRNA or control siRNA. Cells were synchronized by a double-thymidine block 6 hours after transfection. Cells were then released and 10 hours later were fixed, stained with anti-GM130 and imaged. (**F**) HEK293 cells expressing GBF1T1337A–YFP were synchronized and released as in D, then stained with anti-GM130 and imaged. Scale bars: 10 μm. (**B**,**E**,**G**) Statistical analysis of cells with Golgi haze, stained with anti-GM130 in A, D and F. The values shown are means ± s.d. from three individual experiments; $^{**}P<0.01$.

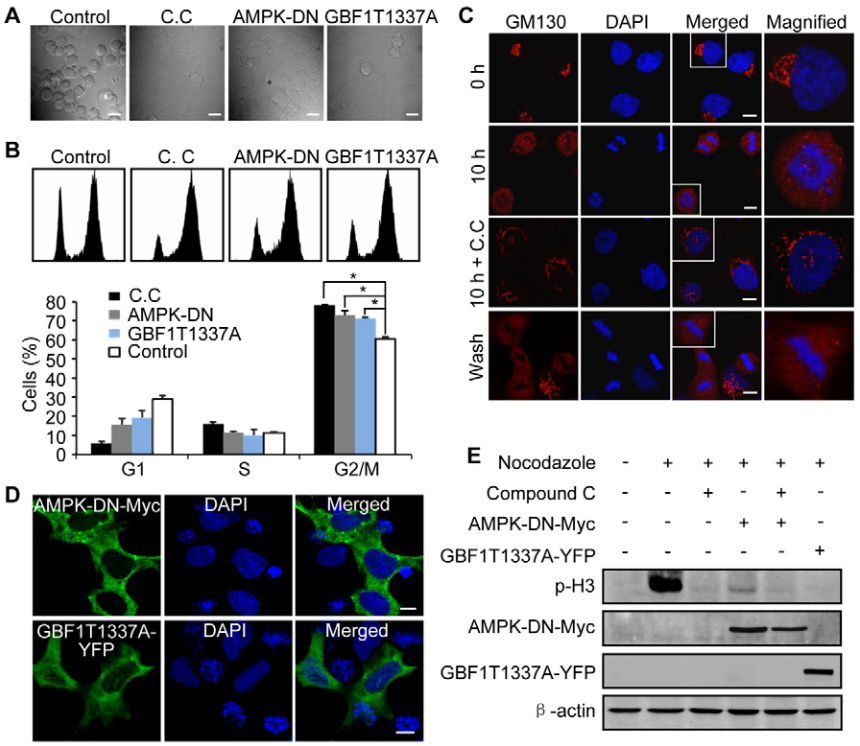

Fig. 4. Activation of AMPK and phosphorylation of GBF1 are required for mitosis. (**A**,**B**) HEK293 cells with or without AMPK-DN–GFP or GBF1T1337A–YFP expression were synchronized by a double-thymidine block. Cells were released from G1/S in the absence or presence of Compound C, and 10 hours later were imaged using DIC microscopy (A) or stained with propidium iodide and analyzed by flow cytometry (B). Scale bars in A: 20 μm. In B, $*P<0.05$. (**C**) HEK293 cells were synchronized by a double-thymidine block. Ten hours after release from G1/S in the absence or presence of Compound C or for an additional 4 hours after elimination of Compound C, cells were fixed, stained with anti-GM130 and DAPI, and imaged by confocal microscopy. Scale bars: 10 μm. (**D**) HEK293 cells transfected with AMPK-DN-Myc or GBF1T1337A-YFP were synchronized by a double-thymidine block. Cells were released, then 10 hours later they were fixed, stained with anti-Myc and DAPI and imaged. Scale bars: 10 μm. (**E**) HEK293 cells transfected with or without AMPK-DN-Myc or GBF1T1337A-YFP were either untreated or treated with nocodazole with or without Compound C for 16 hours. Then the cells were lysed and analyzed by western blotting.

expression apparently arrested the cells in G2/M (Fig. 4B). When the cells were stained with DAPI, we found that, 10 hours after release from G1/S, fragmentation of the Golgi was accompanied by chromatin condensation which was prevented not only by Compound C (Fig. 4C) but also by expression of AMPK-DN–YFP or GBF1T1337A–YFP (Fig. 4D). When Compound C was washed out, the cells entered mitosis, confirming temporal regulation of the process by AMPK (Fig. 4C).

Finally, we examined the phosphorylation of histone-3, a typical marker for cells in mitosis. Nocodazole treatment led to strong histone-3 phosphorylation. Clearly, treatment of cells with Compound C, or expression of AMPK-DN–GFP or GBF1T1337A–YFP abolished the phosphorylation of histone-3 (Fig. 4E), indicating a strong blockade of mitotic entry. Taken together, these results suggest a requirement for AMPK activation and subsequent GBF1 phosphorylation at Thr1337 for cells to pass through the G2/M checkpoint and enter mitosis.

Phosphorylation at Thr1337 attenuates membrane association of GBF1

In view of the necessity for active Arf1 in the maintenance of Golgi structure and the mediation of GBF1 in Arf1 activation, the requirement for GBF1 phosphorylation at Thr1337 for mitotic Golgi disassembly prompted us to investigate the influence of phosphorylation on the Arf-GEF activity of GBF1. First, using GBF1T1337A, we checked whether this mutant is resistant to brefeldin A (BFA), a non-competitive inhibitor of the exchange reaction that binds to an Arf-GDP–Arf-GEF complex (Peyroche et al., 1999). We found, in HEK 293 cells, that expression of GBF1T1337A–YFP impaired the BFA-induced disassociation of Arf1 from the Golgi (Fig. 5A), indicating the prevention of Arf1 from inactivation by BFA. We then created a GBF1 mutant changing the Thr1337 to phosphomimetic glutamate (GBF1T1337E). Surprisingly, compared to the strong association of GBF1T1337A–YFP with the Golgi, in HEK293 cells, GBF1T1337E–YFP showed a cytosol-dominant distribution (Fig. 5A,B). Expression of GBF1T1337E–YFP caused Arf1 and GM130, but not a GTP-bound-locked form of Arf1, ArfQ71L (Dascher and Balch, 1994), to be redistributed from the Golgi to the cytosol (Fig. 5B,C), indicating inactivation of Arf1 by GBF1T1337E–YFP expression. These results suggest that phosphorylation of GBF1 at Thr1337 disassociates GBF1 from the Golgi membranes thereby abolishing the activity of GBF1 as an Arf1-GEF in cells.

Membrane disassociation of GBF1 and inactivation of Arf1 during mitosis

The Golgi membrane-associated protein GBF1 cycles between the Golgi pool and the cytosolic pool, and only the Golgi-bound GBF1 encounters and activates Arf1 (Niu et al., 2005). Our results suggested that phosphorylation at Thr1337 interferes with the membrane association of GBF1. To test whether this occurs in mitotic cells, we first checked the level of membrane-associated GBF1 in nocodazole-arrested mitotic cells. Clearly, compared to interphase cells in which GBF1 was located in both the cytosol and the membrane, in mitotic cells GBF1 was strictly localized to the cytosol (Fig. 6A). We also carried out an *in vitro* assay by treating Golgi membranes isolated from rat liver with the cytosol extracted from mitotic HEK293 cells. We found that culture with nocodazole-arrested mitotic cytosol caused an evident decrease of the Golgi-bound GBF1 (Fig. 6B). Deletion of AMPK from or addition of Compound C to the extracted mitotic cytosol evidently prevented the disassociation of GBF1 from the Golgi membranes, indicating dependence of the process on AMPK activity (Fig. 6C).

Finally, we asked whether the activation of AMPK and phosphorylation of GBF1 during mitosis are indeed followed by

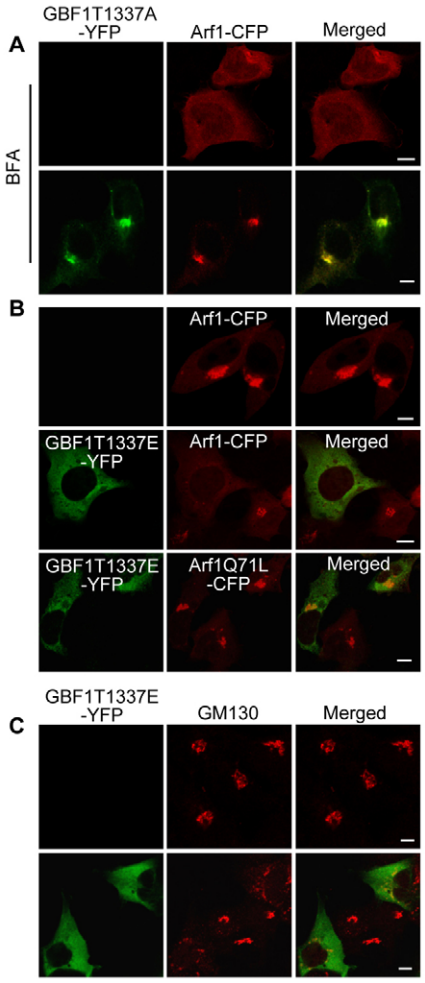

Fig. 5. Phosphorylation at Thr1337 attenuates membrane association of GBF1. (**A**) HEK293 cells transiently expressing Arf1–CFP or Arf1–CFP and GBF1T1337A–YFP were treated with BFA for 30 minutes. Then the cells were fixed and imaged by confocal microscopy. (**B**) HEK293 cells were transfected with Arf1-CFP, Arf1-CFP and GBF1T1337E-YFP or Arf1Q71L-CFP and GBF1T1337E-YFP. Cells were imaged by confocal microscopy 20 hours after transfection. (**C**) HEK293 cells transfected with or without GBF1T1337E-YFP were fixed, immunostained with anti-GM130 and imaged by confocal microscopy. Scale bars: 10 μm.

inactivation of Arf1. We introduced the GST-GGA3-GAT domain fusion protein to monitor the amount of Arf1-GTP protein in cell lysates of Arf1-HA-expressing cells (Niu et al., 2005). GGA3 is an effector of Arf1, and the GAT domain of GGA3 binds with high affinity specifically to Arf1-GTP (Boman et al., 2000). We found that, compared with that in interphase cells, the Arf-GTP levels in BFA-treated and nocodazole-arrested mitotic cells decreased dramatically (Fig. 6D). The mitotic decrease in Arf1-GTP was prevented by Compound C addition or GBF1T1337A–GFP overexpression (Fig. 6D), clearly indicating a dependence on AMPK activity and GBF1 phosphorylation.

Discussion

Using biochemical and cell biological approaches, we have discovered in this study that phosphorylation of GBF1 at Thr1773 by AMPK is essential for the inactivation of Arf1 which is required for Golgi fragmentation and mitosis entry.

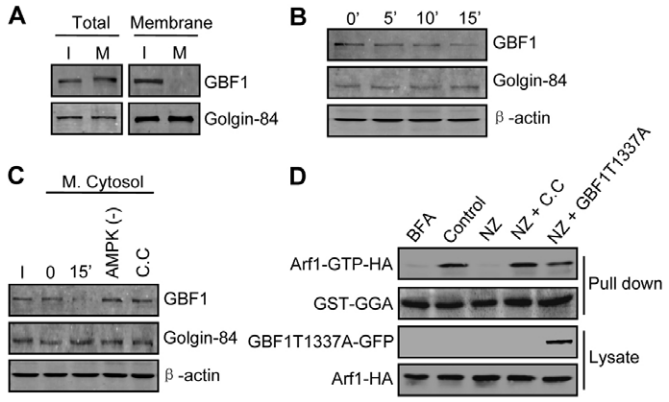

Fig. 6. Membrane dissociation of GBF1 and inactivation of Arf1 during mitosis. (**A**) Total cell lysates or the membrane fractions from interphase (I) or mitotic (M) HEK293 cells were subjected to western blotting using anti-GBF1 and anti-Golgin-84. (**B**) Rat liver Golgi membranes were incubated with mitotic cytosol for the indicated times at 30℃. Then the membranes were analyzed by western blotting using GBF1 antibody. (**C**) Rat liver Golgi membranes were incubated with interphase cytosol, mitotic cytosol or mitotic cytosol that had been depleted of AMPK or treated with Compound C for 15 minutes at 30℃. Then the membranes were analyzed by western blotting. (**D**) HEK293 cells transfected with Arf1-HA or Arf1-HA and GBF1T1337A-YFP were left untreated, or treated with BFA for 30 minutes, nocodazole (NZ) for 16 hours, or nocodazole and Compound C (NZ+C.C) for 16 hours. Arf-GTP pull-down by GST–GGA3–GAT bound to glutathione–Sepharose was then performed as described in the Materials and Methods.

Genetic studies have identified AMPK as an important regulator of cell division which displays its role through mediating the function of its upstream LKB1 and controlling its targets, including protein phosphatase 1 regulatory subunit 12C (PPP1R12C) and myosin regulatory light chain (MRLC) (Banko et al., 2011; Thaiparambil et al., 2012). However, the exact nature of the role of AMPK in mitotic progression and the mechanisms by which it might control mitosis are far from clear. By identifying GBF1 as a new substrate of AMPK for mitotic Golgi fragmentation, our data indicate that activation of AMPK is a very early event for mitosis. This is different from the AMPK-regulated phosphorylation of PPP1R12C or MRLC, which functions mainly in the execution phase required for the completion of cell division (Banko et al., 2011; Thaiparambil et al., 2012). A possible interpretation is that AMPK regulates the mitotic process by targeting different molecules that act at specific steps of mitosis, and each of the substrates has distinct sensitivity to activated AMPK. Consistent with this, it has recently been reported that the active form of AMPK transiently associates with many mitotic structures (Vazquez-Martin et al., 2009b), and a network of substrates of AMPK involved in mitosis has been found (Banko et al., 2011).

Because AMPK has the dual functions of energy-sensing and cell structure control, it has been proposed to play a connecting role between energy status and mitosis-related regulation of cell structure (Koh and Chung, 2007). However, recent studies also suggested that the role of AMPK in mitosis may be independent of its function as an energy biosensor (Banko et al., 2011; Vazquez-Martin et al., 2009a). Here, our data indicate that by phosphorylation of Golgi-associated GBF1, AMPK regulates the organization of the Golgi complex, one of the mitotic apparatuses. This is consistent with the findings that glucose-starvation triggers

polarity change in yeast (Cullen and Sprague, 2000; Kuchin et al., 2002), and AMPK is required for cell division induced by glucose deprivation (Honigberg and Lee, 1998). Phosphorylation of GBF1 by activated AMPK at Thr1337, the same site as in ATP-depleted cells (Miyamoto et al., 2008) and cells in mitosis, further confirmed a connecting role of AMPK in energy sensing and the control of mitosis. Nevertheless, our data suggest that rather than rearrangement of the cell skeleton, the disassembly of the Golgi structure caused by AMPK activation during mitosis is mediated by inactivation of the small GTPase Arf1.

So far, the major dissension between the two models of fragmentation of the Golgi during mitosis is whether the mitotic Golgi haze represents small and dispersed vesicles in the cytosol or Golgi proteins relocates into the ER. The core of the debate is whether Arf1 remains active during mitosis. While most of the reports that propose a requirement of active Arf1 for mitotic Golgi fragmentation come from cell-free systems or *in vitro* reconstitution assays (Misteli and Warren, 1994; Xiang et al., 2007; Tang et al., 2008), evidence that supports an inactivation of Arf1 during mitosis is mainly based on live cell imaging (Altan-Bonnet et al., 2003). By targeting phosphorylation events of GBF1, we found in intact cells that it was associated with decreased Arf-GEF activity, because of dissociation of the protein from the Golgi membrane. Apparently, dissociation of GBF1 from the membrane was accompanied by dissociation of Arf1 and disassembly of the Golgi. Importantly, we provided evidence that the same phenomenon occurred in mitotic cells. Using AMPK inactivators and GBF1 mutants, both in cellular and cell-free systems, our data clearly showed that the activation of AMPK and phosphorylation of GBF1 were connected with a dramatic decrease in GTP-bound Arf1 in mitotic cells. Thereby, our data provide corroborative evidence supporting the idea that Arf1 is inactive during mitosis and that this inactivation is required for mitotic Golgi fragmentation.

Materials and Methods

Vectors, reagents and antibodies

Myc-tagged dominant-negative-AMPK (AMPK-DN) was a gift from David Carling (Imperial College School of Medicine, London, UK). GBF1-GFP, GBF1-YFP, Arf1-HA, Arf1-CFP, Arf1Q71L-CFP and pGST-GGA3$_{VHS-GAT}$ (encoding the VHS and ARF binding domains of GGA3 fused to GST) were obtained from Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD). GBF1T1337A-YFP and GBF1T1337E-YFP were constructed by site-directed mutagenesis based on GBF1-YFP. Nocodazole, Compound C, and thymidine were from Sigma (St Louis, MO); brefeldin A was from Epicentre Technologies (Madison, WI). The following antibodies were used: rabbit polyclonal antibodies against AMPKα, phospho-AMPKα (Thr172), Sepharose-bead-conjugated AMPKα, phospho-acetyl-CoA carboxylase (Ser79; p-ACC) and phospho-Akt substrate (RXXS*/T*; PAS), from Cell Signaling Technology (Danvers, MA); mouse monoclonal antibodies against GBF1, GM130 and GFP, from BD Biosciences (San Jose, CA); mouse monoclonal antibodies against Myc, HA and GST, from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-β-actin antibody, from Sigma; polyclonal anti-phospho-histone-3 antibody, from Upstate Biotechnology (Buffalo, NY); and monoclonal anti-Golgin-84 antibody, from Abcam (Cambridge, MA). Alexa-Fluor-488- and Alexa-Fluor-545-tagged second antibodies were from Molecular Probes (Eugene, OR). Secondary antibodies goat anti-rabbit IRDye 800CW and goat anti-mouse IRDye 680 were from LI-COR Biosciences (Lincoln, NE).

In all the related experiments, nocodazole was used at 400 ng/ml, thymidine at 2 mM, Compound C at 20 μM, and BFA at 25 μg/ml.

Cell culture, transfection and synchronization

HEK293 cells were grown in DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2 at 37℃. Transient transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Cell synchronization at the G1/S boundary was based on double-thymidine block (Merrill, 1998). In short, HEK293 cells were treated with 2 mM thymidine for 14 hours, washed twice with PBS, grown for 8 hours in regular medium, and then treated again with 2 mM thymidine for 16 hours. To accumulate cells in mitosis, they were incubated in a complete medium containing 400 ng/ml nocodazole for 16–18 hours.

Immunofluorescence staining and fluorescence microscopy

For immunostaining, cells were fixed in 4% formaldehyde. After washing twice in PBS, cells were incubated in PBS with FBS (PBS, pH 7.4, 10% FBS) to block nonspecific sites of antibody adsorption. The cells were then incubated with appropriate primary and secondary antibodies in 0.1% saponin (Sigma) as indicated in the legends.

Images were captured in multi-tracking mode on a Zeiss LSM510 Meta laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) with a 63× Plan Apochromat 1.4 NA objective. Live-cell imaging was performed in LabTek chambers (Nalge Nunc International, Rochester, NY), which were maintained at 37℃ with 5% CO_2.

For quantification of the cells with Golgi haze, a total of 100 cells were recorded and analyzed.

Immunoprecipitation and western blotting

For immunoprecipitation, cells were lysed in immunoprecipitation (IP) buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM EGTA, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 100 mM NaF and complete protease inhibitor cocktail (Roche)]. Lysates were centrifuged at 15,000 ***g*** for 15 minutes at 4℃. The supernatants were incubated with primary antibodies overnight followed by protein-A–Sepharose (Pierce Biotechnology) for a further 1 hour at 4℃. After washing five times with IP buffer, the bound proteins were eluted by boiling in SDS sample buffer and analyzed by western blotting with appropriate antibodies.

Western blotting was performed as described previously (Guo et al., 2012). In brief, sample aliquots of proteins were denatured and loaded on sodium dodecyl sulfate polyacrylamide gels. Afterwards, the proteins were transferred to PVDF membrane and subjected to western blotting. Membranes were blocked in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 0.1% Tween 20) containing 5% (w/v) BSA, then incubated with the corresponding primary and secondary antibodies. The specific bands were analyzed using an Odyssey infrared imaging system (LI-COR Biosciences) after incubation with the corresponding secondary antibodies.

Flow cytometry

For FACS analysis, cells were trypsinized, washed with PBS and fixed in 70% ice-cold ethanol overnight at 4℃. The samples were then centrifuged (500 ***g*** for 2 minutes), resuspended in 0.5 ml of staining solution (100 μg/ml RNase A and 50 ng/ml propidium iodide in PBS) and incubated for 30 minutes at room temperature. The cells were analyzed by FACsort (Beckman Coulter), and the percentage of cells at different stages was calculated using CellQuest software (BD Biosciences).

Preparation of cytosolic and cell membrane fractions

Interphase or mitotic cell cytosol was extracted as described by Stuart (Stuart et al., 1993). Briefly, cells were washed in EBS buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 100 mM sucrose, 1 mM dithiothreitol and 1 mM PMSF, pH 7.2), then the cell pellet was suspended in the same buffer and broken using a Dounce homogenizer with a clearance of 0.025 mm. After centrifugation at 400,000 ***g*** for 30 minutes at 4℃, the supernatants were collected, frozen in liquid nitrogen and stored at −80℃. For isolation of membrane fractions, the cell homogenates were cleared by centrifugation at 1000 ***g*** for 10 minutes to generate a post-nuclear supernatant. This was subsequently layered onto EBS buffer containing 0.4 M sucrose, and the membrane fraction was generated by centrifugation at 50,000 rpm for 15 minutes in a TLS55 rotor (Beckman).

Immunodepletion of AMPK from mitotic cytosol and AMPK RNAi

To delete AMPK from the mitotic cytosol, 300 μl mitotic HEK293 cytosol (10 mg/ml) was incubated with 50 μl AMPK antibody-coupled beads for 1 hour at 4℃. After removing the beads by centrifugation, another 50 μl beads were added to the cytosol and incubated for an additional 1 hour. Then, the beads were removed and the supernatants were collected.

For RNA interference, siRNA duplexes designed against conserved targeting sequences of AMPKα1 and AMPKα2 were transfected into HEK293 cells using Lipofectamine 2000 as specified by the manufacturer. The following siRNA duplexes were used: 5′-CCCUCAAUAUUUAAAUCCUUCUGUGTT-3′ for AMPKα1; 5′-GCAUACCAUCUUCGUGUAAGATT-3′ for AMPKα2; 5′-AAGACCAAUUUCAGCAGACAGTT-3′ for control siRNA. All the siRNA duplexes were from GenePharma (Shanghai, China).

Isolation of rat liver Golgi membranes and the membrane incubation assay

The Golgi membranes from rat liver were purified as described previously (Slusarewicz et al., 1994). Fresh rat livers were homogenized by passage through a 150-μm mesh sieve into ice-cold 0.5 M sucrose-PM buffer (0.1 M sodium

phosphate, pH 6.7, 5 mM $MgCl_2$) to 0.6 g liver/ml buffer. This was applied to a two-step gradient comprising 1.3 and 0.86 M sucrose-PM buffer. After centrifugation at 28,000 rpm using an SW28 Ti rotor (Beckman) for 1 hour at 4℃, the Golgi fraction was collected from the 0.5/0.86 M sucrose interface. After dilution to 0.25 M sucrose using PM buffer, the membranes were pelleted at 7000 rpm for 30 minutes at 4℃, washed in 0.25 M sucrose-PM buffer, re-pelleted and stored at −80℃ in the same buffer.

For Golgi membrane incubation assays, interphase and mitotic cytosols were desalted into buffer A (20 mM β-glycerophosphate, 15 mM EGTA, 50 mM potassium acetate, 10 mM magnesium acetate, 2 mM ATP, 1 mM dithiothreitol, 0.2 M sucrose), then the rat Golgi membranes (30 μg) were incubated with desalted cytosol (10 mg/ml) in a final volume of 300 μl in the presence of an ATP regenerating system (10 mM creatine phosphate, 20 μg/ml creatine kinase) for various times at 30℃. Reactions were terminated by placing on ice. Membranes were re-isolated by spinning through a layer of 0.4 M sucrose (in buffer A) at 100,000 ***g*** for 15 minutes at 4℃, then solubilized in sample buffer and analyzed by western blotting.

Arf-GTP pull-down assay

To assess the amount of activated Arf-GTP in cells, we performed a pull-down assay by using a GST-GGA3-GAT domain construct as described previously (Niu et al., 2005). Briefly, HEK293 cells expressing Arf1-HA with or without GBF1–T1337A–YFP were left untreated or treated with BFA or nocodazole. Cell lysates were prepared and incubated with glutathione–Sepharose beads containing GST–GGA3–GAT fusion protein. Bound proteins were eluted from beads and analyzed by western blotting.

Statistics

Values are expressed as means ± s.d. and were compared using the Student's *t*-test. *P*-values <0.05 were considered statistically significant.

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Author contributions

W.L., N.L. and L.M. designed the research; N.L. and L.M. performed most of the experiments; Y.G. and L.G. conducted the Arf-GTP pull-down assay; X.X. and L.Z. purified GST-GGA; X.X. and Y.X. analyzed data; N.L., L.M. and W.L. wrote the paper.

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