Gβγ regulates mitotic Golgi fragmentation and G2/M cell cycle progression

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ABSTRACT **Heterotrimeric G proteins (αβγ) function at the cytoplasmic surface of a cell's plasma membrane to transduce extracellular signals into cellular responses. However, numerous studies indicate that G proteins also play noncanonical roles at unique intracellular locations. Previous work has established that G protein βγ subunits (Gβγ) regulate a signaling pathway on the cytoplasmic surface of Golgi membranes that controls the exit of select protein cargo. Now, we demonstrate a novel role for Gβγ in regulating mitotic Golgi fragmentation, a key checkpoint of the cell cycle that occurs in the late G2 phase. We show that small interfering RNA–mediated depletion of Gβ1 and Gβ2 in synchronized cells causes a decrease in the number of cells with fragmented Golgi in late G2 and a delay of entry into mitosis and progression through G2/M. We also demonstrate that during G2/M Gβγ acts upstream of protein kinase D and regulates the phosphorylation of the Golgi structural protein GRASP55. Expression of Golgi-targeted GRK2ct, a Gβγ-sequestering protein used to inhibit Gβγ signaling, also causes a decrease in Golgi fragmentation and a delay in mitotic progression. These results highlight a novel role for Gβγ in regulation of Golgi structure.**

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INTRODUCTION

Heterotrimeric G proteins, made up of α, β, and γ subunits, function classically at the cytoplasmic surface of the plasma membrane (PM) to link G protein–coupled receptors (GPCRs) to intracellular signaling pathways. Agonist binding to a GPCR promotes the exchange of bound GDP for GTP on the Gα subunit and subsequent dissociation of Gα and Gβγ subunits. Each of GTP-bound Gα and free Gβγ binds to and regulates a variety of effector proteins, and in classical G protein signaling Gα and Gβγ regulate PM-localized enzymes and ion channels. However, it is now clear that G proteins have essential functions at intracellular locations, and some of these noncanonical functions include regulation of protein transport, transcription, nuclear transport, mRNA processing, and oxidative phosphorylation at locations including the Golgi, endosomes, nuclei, and mitochondria (Hewavitharana and Wedegaertner, 2012; Khan *et al.*, 2016; Senarath *et al.*, 2018).

For Gβγ, one noncanonical function of great interest is signaling at the cytoplasmic surface of the Golgi. Previous work by our group and others has demonstrated that Gβγ signaling at the Golgi is necessary for the fission of transport vesicles with protein cargo destined for the PM (Jamora *et al.*, 1997; Diaz Anel and Malhotra, 2005; Irannejad and Wedegaertner, 2010; Saini *et al.*, 2010; Klayman and Wedegaertner, 2017) and that Golgi-localized Gβγ functions upstream of protein kinase D (PKD), a key regulator of this pathway (Liljedahl *et al.*, 2001; Baron and Malhotra, 2002; Godi *et al.*, 2004; Yeaman *et al.*, 2004; Hausser *et al.*, 2005; Diaz Anel, 2007; Fugmann *et al.*, 2007; Nhek *et al.*, 2010; Pusapati *et al.*, 2010; Malhotra and Campelo, 2011). In addition, a recent report showing activation of the MAPK pathway by Golgi-localized Gβγ highlights the potential for multiple functions for Gβγ at Golgi membranes (Khater *et al.*, 2021).

Fragmentation and dispersion of the Golgi occurs in pathophysiological states, such as cancer, infection, and neurodegeneration, and in normal cell functions, such as during mitosis (Gonatas *et al.*, 2006; Heuer *et al.*, 2009; Colanzi and Sutterlin, 2013; Farber-Katz *et al.*, 2014; Haase and Rabouille, 2015; Rabouille and Haase, 2015).

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Abbreviations used: DT, double thymidine; G protein, guanine-nucleotide binding protein; Gβγ, G protein beta-gamma subunits; GRK2ct, carboxy-terminal domain of G protein–coupled receptor kinase 2; PKD, protein kinase D; siRNA, small interfering RNA.

In the late G2 stage of the cell cycle, as the cell prepares to enter mitosis, fragmentation and dispersal of the Golgi serves as a key cell cycle checkpoint (Misteli and Warren, 1995; Warren *et al.*, 1995; Colanzi *et al.*, 2007; Corda *et al.*, 2012). Phosphorylation by multiple kinases on the Golgi re-assembly stacking proteins (GRASPs), specifically, GRASP55 and GRASP65, was shown to be necessary for the deoligomerization of Golgi stacks (Valente and Colanzi, 2015; Li *et al.*, 2019). MEK-dependent phosphorylation of GRASP55 by extracellular signal–regulated kinase (ERK) has been shown to be important for Golgi fragmentation and mitotic entry (Jesch *et al.*, 2001; Colanzi *et al.*, 2003; Feinstein and Linstedt, 2007). Roles for other kinases, such as Polo-like kinase3 (Plk3), Myt1, and partial phosphorylation of GRASP55 by cyclin-dependent kinase 1 (Cdk1) have also been implicated in the mitotic disassembly of Golgi (Cornwell *et al.*, 2002; Nakajima *et al.*, 2008; Xiang and Wang, 2010). GRASP65 was also reported to be phosphorylated at multiple residues by Plk1, c-Jun N-terminal kinase (JNK), and Cdk1 to mediate Golgi fragmentation at the onset of mitosis (Lin *et al.*, 2000; Wang *et al.*, 2003; Preisinger *et al.*, 2005; Tang *et al.*, 2012; Cervigni *et al.*, 2015). Even though many mediators of phosphorylation have been identified, much remains to be understood about the upstream regulators of this process. Interestingly, Golgi fragmentation is observed upon overexpression of components of the signaling pathway regulating the fission of Golgi transport vesicles, such as Gβγ, PKD, PKCη, or PAQR3 (Diaz Anel and Malhotra, 2005; Bossard *et al.*, 2007; Irannejad and Wedegaertner, 2010; Saini *et al.*, 2010; Hewavitharana and Wedegaertner, 2015). Moreover, a previous study showed that PKD functions upstream of the Raf1-MEK1/2-ERK1/2 signaling cascade in regulating mitotic Golgi fragmentation (Kienzle *et al.*, 2013). The involvement of PKD in mitotic Golgi fragmentation and the ability of expressed Gβγ to induce fragmentation of the Golgi prompted us to ask whether Gβγ played a role in mitotic Golgi fragmentation. Accordingly, in the current report, we have used small interfering RNA (siRNA)-mediated depletion of Gβ subunits and molecular inhibition by the Gβγ-binding protein GRK2ct to demonstrate that Gβγ is required for physiological Golgi fragmentation occurring during mitosis and for proper progression through G2/M of the cell cycle, thereby demonstrating a novel noncanonical role for Gβγ at the Golgi.

RESULTS AND DISCUSSION

Depletion of Gβ subunit causes a delay in mitotic Golgi fragmentation

To investigate a role for Gβγ in mitotic Golgi fragmentation, we used siRNA to deplete the most abundant Gβ isoforms, Gβ1 and Gβ2, in HeLa cells (Krumins and Gilman, 2006). Cells were transfected with control siRNA (Csi), Gβ1/β2 siRNA (βsi), or PKD siRNA and synchronized at the G1/S border by a double thymidine (DT) block followed by subsequent release. Golgi integrity was visualized by GM130 staining (Figure 1A), and immunoblotting confirmed efficient knockdown of Gβ1, Gβ2, and PKD (Figure 1B). At 0 h release from the DT block, Golgi fragmentation was observed in <10% of cells, regardless of siRNA treatment (Figure 1, A and C). At 8 h after release from the DT block, corresponding to late G2, Golgi fragmentation occurred in almost 70% of control cells. However, depletion of Gβ1/β2 resulted in a dramatic decrease in the percentage of cells (<20%) with fragmented Golgi at 8 h post–DT block. Likewise, depletion of PKD greatly decreased Golgi fragmentation in late G2, consistent with a previous report (Kienzle *et al.*, 2013).

Next, we used a phospho-histone H3 (pHis-H3) antibody to identify cells in late G2 after release from the DT block (Hendzel *et al.*, 1997). In control siRNA cells, Golgi fragmentation was observed in >80% of pHis-H3–positive late G2 cells, while knockdown of Gβ1/β2 resulted in an ~50% reduction in the number of cells with fragmented Golgi in late G2 (Figure 1, D–F). This experiment rules out the possibility that the observed decrease in Golgi fragmentation upon Gβ1/β2 depletion is due simply to a failure of cells to reach late G2; instead, Gβ1/β2-depleted cells transition to late G2, as confirmed by pHis-H3 staining, but display decreased Golgi fragmentation. The results in Figure 1 provide the first evidence that Gβγ regulates mitotic Golgi fragmentation.

Inhibition of Gβγ subunit causes a delay in mitotic Golgi fragmentation

To complement the siRNA depletion approaches and specifically target Golgi-localized Gβγ, we used a molecular inhibitor of Gβγ, Golgi-targeted GRK2ct, that was previously shown to block the signaling activity of endogenous Gβγ at the Golgi (Irannejad and Wedegaertner, 2010; Khater *et al.*, 2021). Cells were transfected with either pCDNA3 control, Golgi-GRK2ct, Golgi-GRK2ct-RQ, a mutant that is unable to sequester and inhibit Gβγ, or PM-GRK2ct constructs (Figure 2C) and synchronized at the G1/S border by a DT block followed by subsequent release. Golgi integrity was visualized by GM130 staining (Figure 2A). At 0 h release from the DT block, Golgi fragmentation was observed in <15% of cells (Figure 2, A and B). At 8 h after release from the DT block, corresponding to late G2, Golgi fragmentation occurred in almost 80% of control cells and in cells transfected with Golgi-GRK2ct-RQ or PM-GRK2ct constructs. However, expression of Golgi-GRK2ct resulted in a dramatic decrease in the percentage of cells (<35%) with fragmented Golgi at 8 h post–DT block (Figure 2, A and B). These results support a role for specifically Golgi-localized Gβγ in mediating mitotic Golgi fragmentation.

Depletion of Gβ subunit causes decreased activation of PKD

Because PKD can regulate mitotic Golgi fragmentation (Figure 1) (Kienzle *et al.*, 2013) and Gβγ functions upstream of PKD in the regulation of Golgi transport carrier formation (Jamora *et al.*, 1999; Diaz Anel and Malhotra, 2005; Irannejad and Wedegaertner, 2010; Malhotra and Campelo, 2011), we asked whether Gβγ regulates PKD activity during G2/M. HeLa cells were transfected with control and Gβ1/β2 siRNA and subjected to a DT block and release. Cells were harvested 0–14 h after release, and immunoblotting of cell lysates with a phospho-PKD (pPKD) antibody determined PKD activation as cells transitioned from G2 through mitosis (Figure 3, A and B). In control cells, we observed a trend toward increased pPKD as cells moved into late G2 and mitosis (8–14 h). Interestingly, earlier work implicated PKD in mitotic Golgi fragmentation (Kienzle *et al.*, 2013), but that report did not show increased G2/M phosphorylation of PKD. Nonetheless, upon knockdown of Gβ1/β2, a substantial decrease in pPKD levels was observed at all time points upon release from the DT block. These results are consistent with the idea that Gβγ acts upstream of PKD to regulate mitotic Golgi fragmentation.

Knockdown of Gβ subunit reduces the phosphorylation of GRASP55

Phosphorylation of the Golgi structural protein GRASP55 was shown to be necessary for the unlinking of Golgi ribbons and progression to mitosis (Feinstein and Linstedt, 2007; Duran *et al.*, 2008; Xiang and Wang, 2010). To delineate whether Gβγ subunits regulate the phosphorylation of GRASP55, we transfected HeLa cells with control or Gβ1/β2 siRNA and then synchronized with a DT block as described above. Cells were collected every 2 h between 6 and 14 h after release from the DT block and then lysed. Lysates were run on a Phostag gel to ensure the separation of phosphorylated proteins and then immunoblotted with anti-GRASP55 antibodies (Figure 3, C and D). In

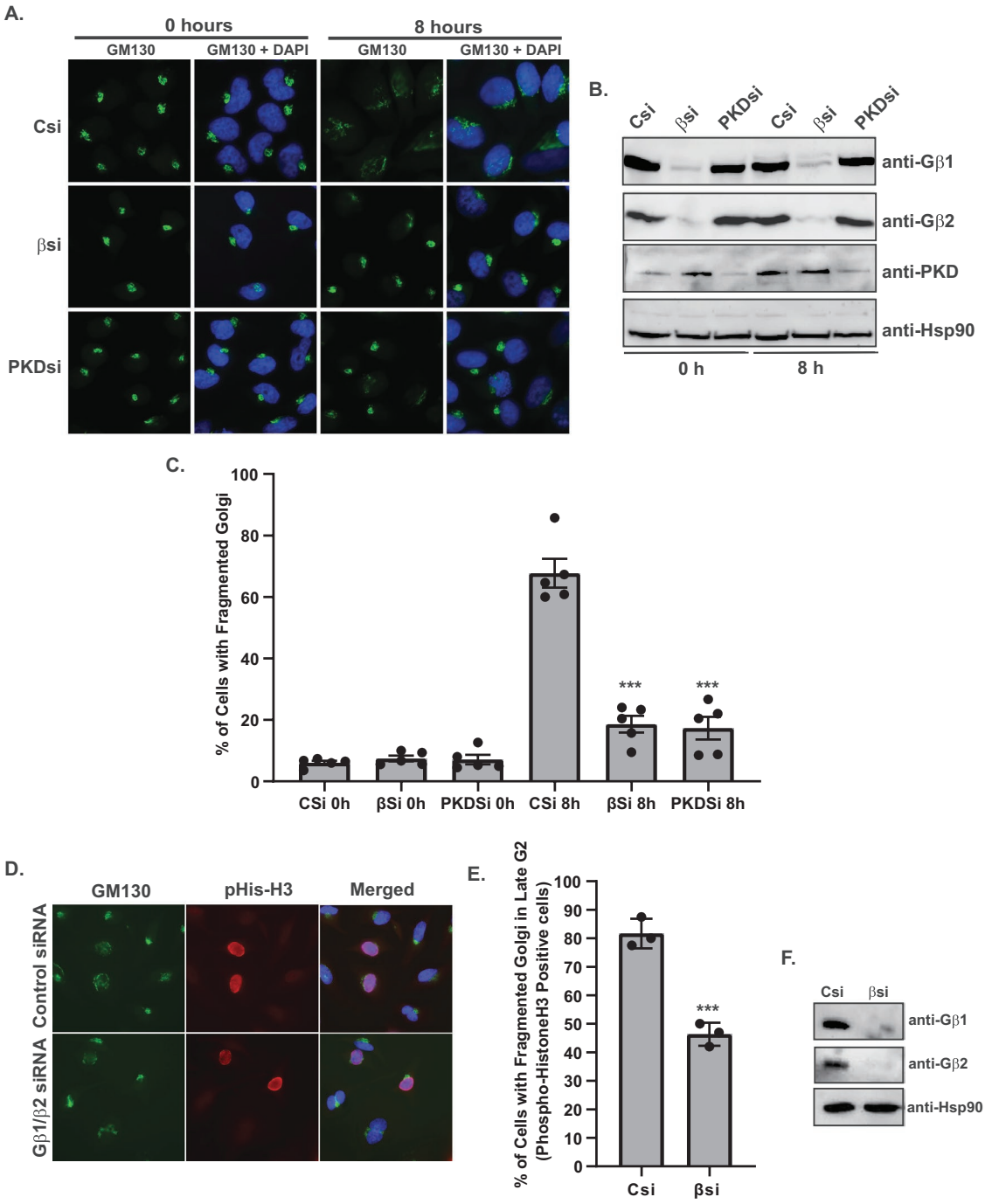

FIGURE 1: Depletion of Gβ causes a delay in mitotic Golgi fragmentation. (A) HeLa cells were transfected with control (Csi), Gβ1/β2 (βsi), or PKD (PKDsi) siRNA and, after the DT block, thymidine was washed out and the cells were harvested at 0 h or after 8 h (G2). Golgi integrity was observed with GM130 antibody. Representative cells depicting intact Golgi and fragmented Golgi are shown. (B) Immunoblot analysis of cell lysates showing the efficiency of knockdown. (C) Cells, as described above (A), were scored for the fragmented Golgi phenotype. Graph shows the average ± SD for five independent experiments (>150 cells for each experiment). (D) HeLa cells were transfected with the indicated siRNA, and after a DT block, thymidine was washed out and the cells were fixed on coverslips after 8 and 9 h (late G2) and stained with pHis-H3 and GM130 antibodies. (E) The pHis-H3–positive cells (late G2) were scored for the Golgi fragmentation phenotype (>40 cells for each experiment). Graph shows the average ± SD for three independent experiments. (F) Immunoblot analysis of cell lysates collected at late G2 stage verify the efficiency of knockdown.

control cells, low levels of phosphorylated GRASP55 were detected at the 0 time point, but phosphorylation increased as cells transitioned into late G2 and then returned toward basal levels as cells passed through mitosis (10–14 h). However, when Gβ1/β2 was depleted, phosphorylation of GRASP55 was decreased and delayed as cells moved through G2 and mitosis. These results indicate that Gβγ

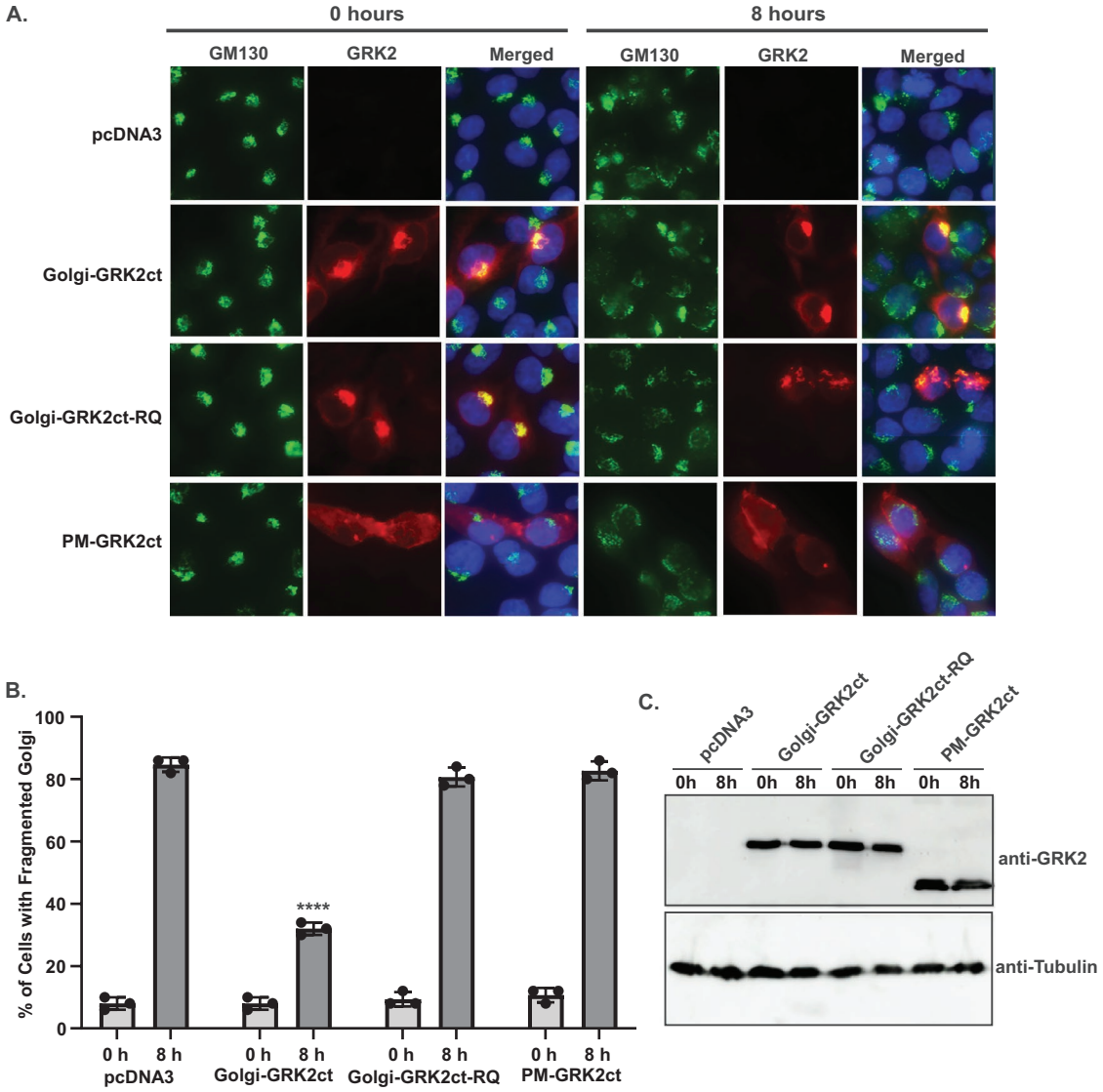

FIGURE 2: Inhibition of Gβγ causes a delay in mitotic Golgi fragmentation. (A) HeLa cells transfected with pcDNA3, Golgi-GRK2ct, Golgi-GRK2ct-RQ, or PM-GRK2ct constructs were synchronized using a DT block and harvested at 0 and 8 h post–release from the block. Golgi integrity was observed with GM130 antibody. Representative cells depicting intact Golgi and fragmented Golgi are shown. (B) Cells, as described above (A), were scored for the fragmented Golgi phenotype. Graph shows the average ± SD for three independent experiments (~50 uniformly transfected cells for each construct per experiment). (C) Immunoblot of the transfected cell lysates probed with GRK2 antibody showing the expression of Golgi-GRK2ct, Golgi-GRK2ct-RQ, and PM-GRK2ct constructs.

is involved in regulating the phosphorylation of Golgi structural proteins, specifically GRASP55, shown to mediate mitotic Golgi fragmentation, thus providing more evidence for a key role of Gβγ in promoting mitotic Golgi fragmentation.

Knockdown or inhibition of the Gβ subunit results in accumulation of cells at the G2 stage of the cell cycle

Golgi fragmentation is an important checkpoint for cells to enter mitosis, and any interruption of this process would affect the ability of cells to advance toward cell division. The results so far show that Gβγ regulates the mitotic Golgi fragmentation that occurs in late G2 and regulates key proteins, such as GRASP55, but it is important to test whether Gβγ is required for proper cell cycle progression. Hence, we first depleted Gβ1/β2 subunits in asynchronous HeLa cells (Figure 4C) and performed immunofluorescence microscopy by staining with pHis-H3 (Ser10) antibody and 4′,6-diamidino-2-phenylindole (DAPI) (Figure 4A). Cells with intact round nuclei that stained for pHis-H3 were scored as G2, and cells positive for pHis-H3 but with condensed chromatin were scored as having progressed to mitosis (Figure 4A). In cells transfected with control siRNA, pHis-H3–positive cells showed a G2 versus M distribution of 27% versus 73%, whereas in cells transfected with Gβ1/β2 siRNA, this distribution showed a shift to 41% of pHis-H3–positive cells in G2 and 58% in M (Figure 4B).

To examine the effect of depletion of Gβ1 or Gβ2 individually and to rule out off-target effects of the Gβ1/β2 siRNA, we used additional Gβ1 and Gβ2 siRNA. Whereas all the above experiments used an siRNA that targeted both Gβ1 and Gβ2, we also used individual siRNAs that targeted Gβ1 or Gβ2 (Figure 4, D and E). In asynchronously growing HeLa cells, individual siRNA knockdown of the Gβ1 or Gβ2 subunits revealed only a small increase in the G2:M ratio, suggesting that both Gβ1- and Gβ2-containing Gβγ contribute to the regulation of the cell cycle. On the other hand, a combination of

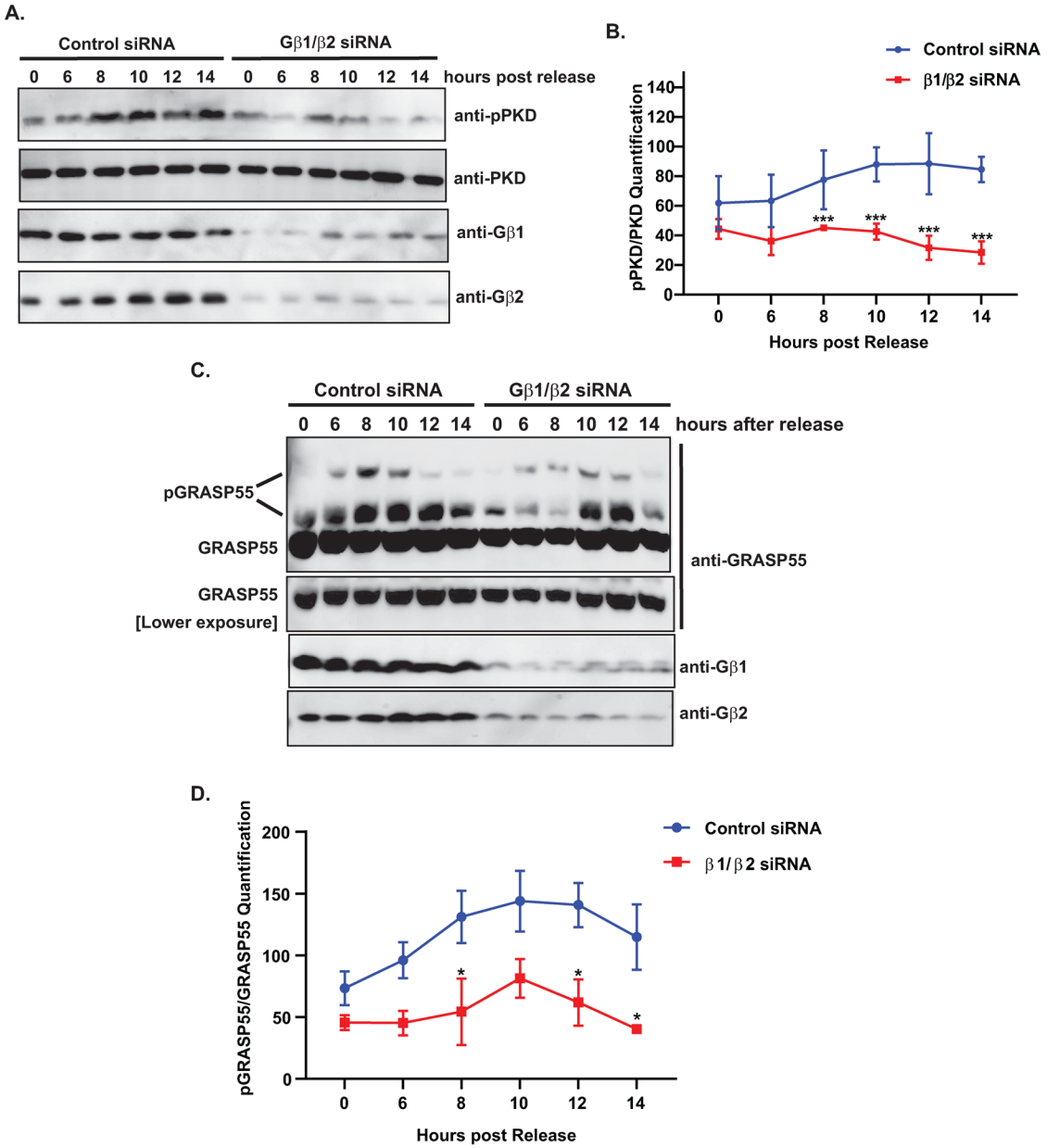

FIGURE 3: Depletion of Gβ causes decreased activation of PKD and reduced phosphorylation of Golgi structural protein GRASP55. (A) HeLa cells were transfected with control or Gβ1/β2 siRNA and synchronized using a DT block. Cell lysates collected at 0, 6, 8, 10, 12, and 14 h post–release from the block were immunoblotted with anti-pPKD (S910) and anti-PKD antibodies. Representative immunoblots are shown. (B) Intensities of pPKD signals from the blots were quantified using ImageJ software and normalized by dividing by total PKD signals. Graph shows average ± SEM for three independent experiments. (C) HeLa cells were transfected with control or Gβ1/β2 siRNA and synchronized using a DT block. Cell lysates collected at 0, 6, 8, 10, 12, and 14 h post–release from the block were run on Phos-tag gels and subsequently immunoblotted with GRASP55 or Gβ1/β2 antibodies. Phosphorylated, slower-mobility forms of GRASP55 are indicated in the top panel. (D) Intensities of total pGRASP55 signals were normalized by dividing by the GRASP55 signals. Graph shows average ± SEM for three independent experiments. Statistical significance compared with control siRNA was tested by two-way ANOVA followed by Bonferroni's multiple comparison test.

the individual Gβ1 or Gβ2 siRNAs resulted in a more substantial increase in the G2:M ratio, consistent with results with the single Gβ1/β2 siRNA, highlighting that depletion of both Gβ1 and Gβ2 is required to observe a delay in G2/M progression (Figure 4, A–E).

Golgi-GRK2ct was also used to define a role for Gβγ in cell cycle progression (Figure 4, F and G). In cells transfected with pCDNA3 control, pHis-H3–positive cells showed a G2 versus M distribution of 33% versus 66%, whereas in cells transfected with Golgi-GRK2ct, this distribution showed a shift to 53% of pHis-H3–positive cells in G2 and 47% in M. In cells transfected with Gβγ binding–deficient Golgi-GRK2ct-RQ, the G2 versus M distribution was similar to that of pCDNA3 control cells (Figure 4F). Taking the data together, the shift to a greater percentage of cells in G2 in Gβ1/β2-depleted or inhibited cells compared with control is consistent with the interpretation that loss of Gβ1/β2 delays the G2-to-M transition.

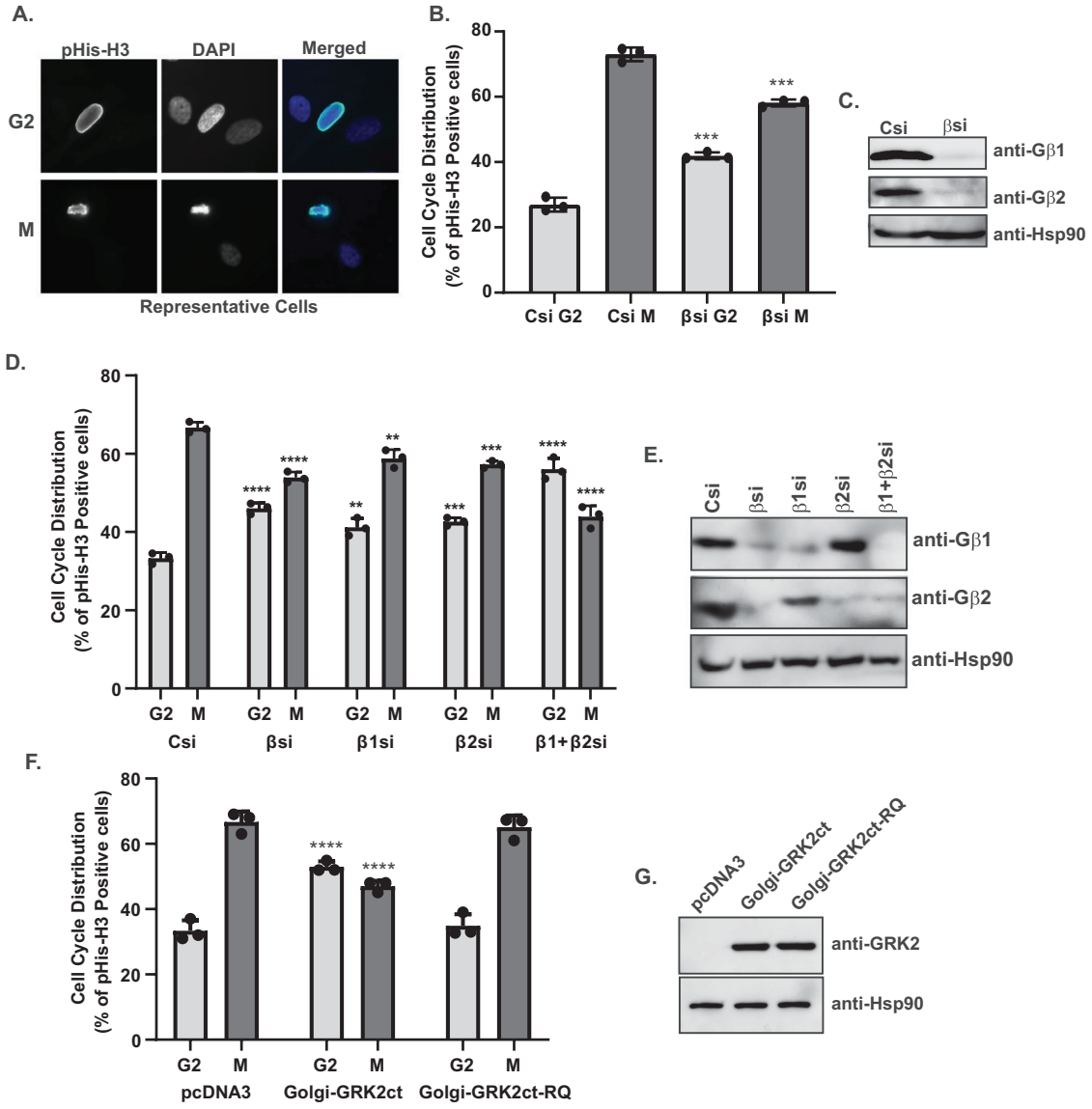

FIGURE 4: Knockdown of Gβ or inhibition of Gβγ results in accumulation of cells at the G2 stage of the cell cycle. HeLa cells were transfected with control (Csi) or Gβ1/β2 (βsi) siRNA, and 48 h later the cells were fixed and stained with anti–pHis-H3 antibody and DAPI. (A) Representative asynchronous pHis-H3–positive cells classified as G2 (intact nuclei) or M (containing broken nuclear envelope or condensed chromatin) are shown. (B) Cells were visually scored for G2 or M. The bar graphs show the average ± SD for three independent experiments (>100 cells were counted for each experiment). (C) Immunoblot analysis of cell lysates verifying the efficiency of knockdown. (D) HeLa cells were transfected with control, Gβ1/β2, Gβ1, or Gβ2 siRNA and, 48 h later, were fixed and stained with anti–pHis-H3 antibody and were visually scored for G2 or M, as in Figure 3. The bar graphs show the mean ± SD for three independent experiments (>100 cells were counted). (E) The cell lysates were probed with Gβ1 or Gβ2 antibodies to verify the efficiency of knockdown. (F) HeLa cells were transfected with pcDNA3, Golgi-GRK2ct, or Golgi-GRK2ct-RQ constructs, and 36 h posttransfection, the cells were stained with pHis-H3 antibody and visually scored for G2 or M. The graphs show the average ± SD for three independent experiments (>100 cells were counted for each experiment). (G) Immunoblot of the transfected cell lysates probed with GRK2 antibody, showing the expression of Golgi-GRK2ct and Golgi-GRK2ct-RQ constructs.

Knockdown or inhibition of the Gβ subunit causes a delay in mitotic progression

To additionally validate our findings, we tracked the progression of Gβ1/β2 knockdown cells through mitosis 6–14 h after release from the DT block. In control siRNA-treated cells, histone H3 phosphorylation was first detectable 6 h after release from the block, which peaked around 8 h, and started to diminish 10 h after release (Figure 5, A and B). However, in Gβ1/β2-depleted cells, histone H3 phosphorylation was still elevated even at 12–14 h after release (Figure 5, A and B), which signifies a delay in mitotic progression. Similarly, in cells transfected with Golgi-GRK2ct, inhibition of Gβγ signaling resulted in elevated histone H3 phosphorylation even at 12 h after release from the DT block. In contrast, cells transfected with either pCDNA3 or Golgi-GRK2ct-RQ started to exit from mitosis ~10 h, as evident from histone H3 phosphorylation (Figure 5, C and D). These results further support a role for Gβγ in mitotic progression.

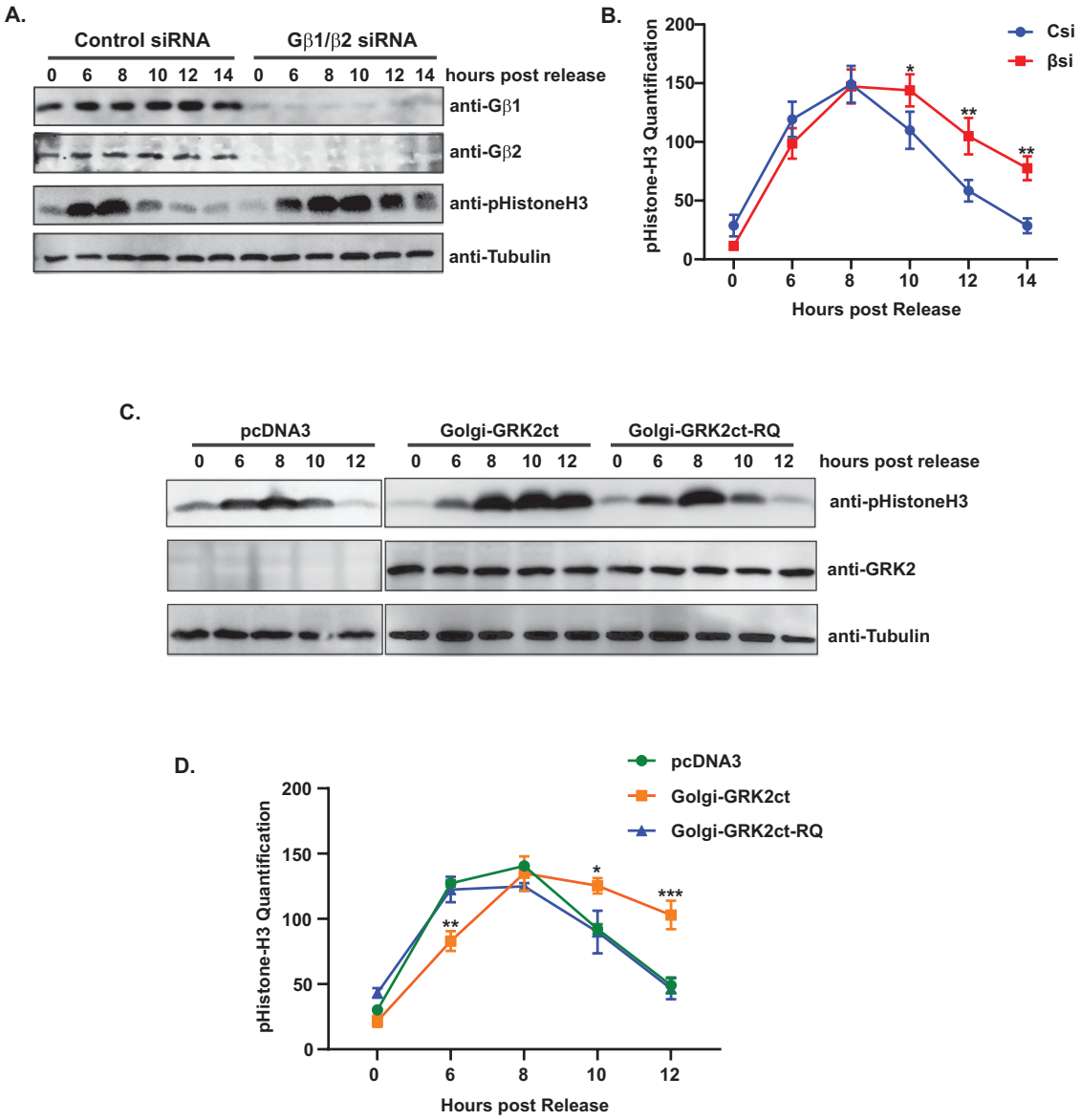

FIGURE 5: Knockdown or inhibition of the Gβ subunit causes a delay in mitotic progression. (A) HeLa cells were transfected with control (Csi) or Gβ1/β2 (βsi) siRNA and synchronized using a DT block. Cell lysates were collected at 0, 6, 8, 10, 12, and 14 h post–release from the block. Representative immunoblot analysis of cell lysates collected at the indicated time points after the DT block and probed with pHis-H3 antibody. Gβ1/β2 antibodies were used to validate the efficiency of knockdown. Tubulin was used as a loading control. (B) Intensities of pHis-H3 signals were quantified using ImageJ and normalized by dividing by total tubulin signals. Graph shows average ± SEM for three independent experiments. (C) HeLa cells were transfected with pcDNA3, Golgi-GRK2ct, or Golgi-GRK2ct-RQ constructs and synchronized using a DT block. Cell lysates were collected at 0, 6, 8, 10 and 12 h post–release from the block and probed with pHis-H3, GRK2, or tubulin antibodies. (D) Intensities of pHis-H3 signals were quantified using ImageJ. Graph shows average ± SEM for three independent experiments.

Taken together, the results reveal a novel role for Gβγ in promoting the characteristic Golgi fragmentation in late G2 of the cell cycle and consequently for proper G2/M mitotic progression. siRNA-mediated depletion of Gβ1 and Gβ2 or inhibition of Gβγ resulted in reduced Golgi fragmentation in late G2 (Figures 1 and 2), reduced phosphorylation of PKD and GRASP55 during progression through G2/M (Figure 3), and delayed cell cycle progression through G2/M (Figures 4 and 5). Moreover, expression of the Gβγ-binding protein, Golgi-GRK2ct, also resulted in delayed G2/M (Figures 4 and 5), highlighting that inhibition specifically of Golgi-localized Gβγ is causing the cell cycle delay rather than indirect effects of inhibition of canonical G protein signaling at the PM. These results describe a novel function for Gβγ and, together with previous studies demonstrating a role for Gβγ in regulation of Golgi transport vesicle generation, suggest potential overlapping mechanisms in the regulation of protein transport at the Golgi and in Golgi fragmentation as cells enter mitosis.

A major question for future investigation is how the Golgi functions of Gβγ are regulated. Gβγ can translocate from the PM to the Golgi in response to extracellular agonist stimulation of GPCRs, and this Golgi translocation of Gβγ can promote PKD-dependent secretory cargo transport from the Golgi to the PM (Saini *et al.*, 2010;

Jensen *et al.*, 2016; Zhao *et al.*, 2019). On the other hand, the Golgi role of Gβγ in regulating constitutive Golgi-to-PM secretory transport does not require cell-surface GPCR activation (Irannejad and Wedegaertner, 2010). The lack of an effect of PM-GRK2ct on mitotic Golgi fragmentation (Figure 2) argues against the involvement of cell-surface GPCR stimulation, although this cannot be ruled out. Other possibilities for activation of Gβγ for mitotic Golgi fragmentation include, but are not restricted to, activation of Golgi-localized G proteins by Golgi-localized GPCRs, activation of Golgi-localized G proteins by non-GPCR activators of heterotrimeric G proteins, and recruitment of free Gβγ to the Golgi by mechanisms independent of GPCR activation. Investigations of these and other questions regarding the role of Gβγ will shed new light on the cellular events that control mitotic Golgi fragmentation.

MATERIALS AND METHODS

Request a protocol through *Bio-protocol*.

Antibodies, reagents, plasmids, and siRNA

The anti-Gβ1 (Cat # 137635) and anti-Gβ2 (Cat # 108504) antibodies were obtained from Abcam. The antibodies for PKD (Cat # 8818), phospho-PKD/PKCμ (Ser916) (Cat # 2051), tubulin (Cat # 3873), and phospho-histone H3 (Ser10) (Cat # 9701) were purchased from Cell Signaling. The Hsp90 antibody (Cat # sc-7947) was obtained from Santa Cruz, and anti-GM130 (Cat # 11308-1-AP) and anti-GRASP55 antibodies (Cat # 10598-1-AP) were from Proteintech. The anti-GRK2 antibody (Sigma Cat # G7670) was a kind gift from Jeffrey Benovic. All the horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Promega. siRNA targeting both Gβ1 and Gβ2 (Gβ1/β2) (ACGACGACUUCAACUGCAA), Gβ1 individually (GGAUAACAUUUGCUCCAUU), and Gβ2 individually (ACUGGGUACCUGUCGUGUU) have been described previously (Irannejad and Wedegaertner, 2010). PKD siRNA consists of an ON-TARGET plus SMART pool targeting PKD1 and an ON-TARGET plus SMART pool targeting PKD2, as described (Kienzle *et al.*, 2013; Klayman and Wedegaertner, 2017). The ON-TARGET plus nontargeting siRNA (Thermo Scientific) was used for control siRNA transfections. Plasmids expressing Golgi-GRK2ct and Golgi-GRK2ct-RQ, in which the C-terminal Gβγ-binding region of GRK2 is targeted to the Golgi via fusion to KDELR-D193N, and a plasmid expressing PM-GRK2ct, in which GRK2ct is fused to the PM-targeting sequence from Rit GTPase, were described previously (Irannejad and Wedegaertner, 2010).

Cell culture and transfection

HeLa cells obtained from the American Type Culture Collection were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were grown and maintained in 100 mm dishes. The siRNA was transfected in cells grown on six-well plates using Lipofectamine RNAi Max (Invitrogen) as per the manufacturer's instructions.

Immunoblotting and Phos-tag gels

Immunoblots were developed using the indicated primary antibodies, secondary anti-mouse or rabbit antibodies conjugated to HRP, and chemiluminescence reagent (Thermo Scientific). Images were captured by AI 680 Imager (GE Healthcare). For the phospho-PKD blots, membranes were blocked with 5% bovine serum albumin followed by incubation with phospho-PKD/PKCμ (Ser916) antibody (Cell Signaling) and secondary IRDye 680 RD anti-rabbit antibody (Li-Cor) and visualized by the Li-Cor Odyssey Imaging system. Phos-tag gels (Cat # 195-17991) were purchased from Fujifilm Wako Chemicals and were run at 10–15 mA for 6–8 h at 4°C to ensure proper resolution.

Cell cycle synchronization by double thymidine block

The cells were synchronized at the G1/S border using a DT block. HeLa cells grown on six-well plates or coverslips were transfected with specific siRNAs, and 4 h later, medium containing thymidine (Sigma Aldrich) at a final concentration of 2 mM was added to the cells. After incubation for 19 h, the cells were released from the thymidine block by washing with phosphate-buffered saline (PBS) and adding normal growth media for 9 h. Subsequently, the cells were subjected to a second thymidine block for an additional 16 h. The cells were then harvested at distinct time points as mentioned.

Immunofluorescence microscopy

Cells on coverslips were collected at different time points, fixed with 3.7% formaldehyde in PBS for 15 min, and permeabilized by incubation in 1% Triton X-100 in Tris-buffered saline (TBST) for 5 min. Cells were blocked in blocking buffer (5% FBS in PBS) for 30 min. Coverslips were then incubated with the anti-mouse GM130 and anti-rabbit phospho-histone H3 antibodies in blocking buffer for 1 h. The cells were washed thrice with TBST and incubated with a 1:200 dilution of the goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594 secondary antibodies (Invitrogen) for 1 h. Subsequently, the cells were washed thrice with TBST and stained with DAPI solution for 5 min. The coverslips were then washed with PBS, rinsed in distilled water, and mounted on glass slides with Prolong Anti-fade reagent (Invitrogen). Images were acquired using an Olympus BX-61 microscope with a 60× oil immersion objective and an ORCA-ER camera (Hamamatsu) controlled by Slidebook version 4.0 (Intelligent Imaging Innovations, Denver, CO) or using an Olympus IX83 microscope with a 60× oil immersion objective and an ORCA Fusion sCMOS camera (Hamamatsu) controlled by cellSens (Olympus) software.

Statistical analysis

For all figures, analysis was done using GraphPad Prism. An unpaired *t* test (Figures 1, C and E, 3B, and 4B) or two-way analysis of variance (ANOVA) followed by either Tukey's multiple comparison test (Figures 2B, 4, D and F, and 5D) or Bonferroni's multiple comparison test (Figures 4B and 3D) or Fisher's least significant difference test (Figure 5B) was used to calculate significance (*, $p < 0.05$**; $p < 0.005$; ***; $p < 0.0005$; ****, $p < 0.0001$).

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