Phenotypic characterisation of RAB6A knockout mouse embryonic fibroblasts

Sabine Bardin*, Stéphanie Miserey-Lenkei*, Ilse Hurbain*, Daniela Garcia-Castillo†, Graça Raposo* and Bruno Goud*

*Institut Curie, PSL Research University and CNRS UMR 144, Paris 75248, France and †Institut Curie, PSL Research University, CNRS UMR 3666 and INSERM U1143, Paris 75248, France

Background Information. Rab6 is one of the most conserved Rab GTPases throughout evolution and the most abundant Rab protein associated with the Golgi complex. The two ubiquitous Rab isoforms, Rab6A and Rab6A', that are generated by alternative splicing of the RAB6A gene, regulate several transport steps at the Golgi level, including retrograde transport between endosomes and Golgi, anterograde transport between Golgi and the plasma membrane, and intra-Golgi and Golgi to endoplasmic reticulum transport.

Results. We have generated mice with a conditional null allele of RAB6A. Mice homozygous for the RAB6A null allele died at an early stage of embryonic development. Mouse embryonic fibroblasts (MEFs) were isolated from RAB6AloxP/loxP Rosa26-CreERT2 and incubated with 4-hydroxy tamoxifen, resulting in the efficient depletion of Rab6A and Rab6A'. We show that Rab6 depletion affects cell growth, alters Golgi morphology and decreases the Golgi-associated levels of some known Rab6 effectors such as Bicaudal-D and myosin II. We also show that Rab6 depletion protects MEFs against ricin toxin and delays VSV-G secretion.

Conclusions. Our study shows that RAB6 is an essential gene required for normal embryonic development. We confirm in MEF cells most of the functions previously attributed to the two ubiquitous Rab6 isoforms.

Introduction

Rab GTPases are now recognised as key regulators of intracellular transport in eukaryotic cells. By interacting with a wide variety of effectors, they virtually regulate all transport steps between cell organelles, including budding and fission of transport vesicles from donor membranes, their movement along cytoskeletal tracks and their docking/fusion to acceptor membranes (Hutagalung and Novick, 2011).

Most of our knowledge on Rab function in mammals comes from studies on cultured cells. Only a few mouse models have been developed but the ones that are available have been instrumental for clarifying the function of several Rab proteins in a physiological context. For instance, the generation of RAB8A conditional knockout mice has revealed that Rab8a is necessary for the proper localisation of apical proteins in the small intestine (Sato et al., 2007), whereas in vitro studies had pointed out a role for Rab8a mainly in basolateral transport (Huber et al., 1993a; Ang et al., 2003). A recent study on RAB8A and RAB8B double-knockout mice has confirmed the role of Rab8 in apical transport and challenged the hypothesis, based on in vitro studies, that Rab8 is essential for ciliogenesis (Sato et al., 2014). The generation of single, double, triple and quadruple RAB3 knockout mice allowed full understanding of the function of Rab3 in synaptic vesicle exocytosis (Schütze et al., 2004).
Rab3 does not appear to be directly involved in the core machinery of docking and fusion of synaptic vesicles with the presynaptic membrane, as suggested by many in vitro studies, but rather acts as a modulator of this machinery.

The Rab6 family comprises four proteins, named Rab6A, Rab6A', Rab6B and Rab6C. Rab6A' is generated by alternative splicing of the Rab6A gene and differs from Rab6A by only three amino acids (Echard et al., 2000). Both proteins are ubiquitously expressed. Rab6B is encoded by a separate gene and is mostly expressed in neurons and neuroendocrine cells (Opdam et al., 2000). Rab6C is a primate-specific retrogene transcribed in a limited number of human tissues. It encodes a protein with altered biochemical properties compared with other Rab6 isoforms that localises to centrosome and is involved in cell cycle progression (Young et al., 2010).

Although the exact function of the neuronal isoform Rab6B is unknown, numerous studies have established the essential role played by Rab6A/A' in the regulation of several transport steps at the level of the Golgi complex, including retrograde transport between endosomes and the endoplasmic reticulum via the Golgi complex, anterograde transport between Golgi and the plasma membrane, as well as in the homeostasis of Golgi membranes (Martinez et al., 1994; White et al., 1999; Mallard et al., 2002; Goud and Akhmanova, 2012). A study has also highlighted a role for Rab6 during mitosis (Miserey-Lenkei et al., 2006).

Here, we report the phenotypic characterisation of mouse embryonic fibroblasts (MEF) derived from conditional Rab6A knockout mice that do not express the ubiquitous isoforms Rab6A and Rab6A'.

Results and discussion

We have generated mice with a conditional null allele of Rab6A by flanking exon 4 with LoxP sites (Figure 1A). Homozygous Rab6A<sup>loxP</sup> mice, in which the Neo cassette was removed through FRT-mediated recombination, were viable and fertile, indicating that these conditional Rab6A alleles are functional. Rab6B<sup>loxP</sup> mice were first crossed with PGK-Cre mice (the Cre expression being ubiquitous and constitutive) to generate Rab6A-knockout animals. Mice homozygous for the Rab6A null allele died at an early stage of embryonic development (before day 7.5; data not shown). Rab6A<sup>loxP/loxP</sup> mice were then crossed with Rosa26-CreERT2 mice to establish inducible Rab6A-knockout animals. This resulted in the ubiquitous and inducible depletion of Rab6A and Rab6A', both isoforms being generated by alternative splicing of exon 4 (Echard et al., 2000).

We next isolated MEFs from Rab6A<sup>loxP/loxP</sup> Rosa26-CreERT2 embryos taken at E12.5. Rab6 A/<sup>k/o</sup> MEFs were generated by incubation for 4 days with 4-hydroxy tamoxifen (4-OHT). Cre-recombinase activity resulted in the efficient depletion of Rab6A/A' (referred below as Rab6), as shown by immunoblotting and immunofluorescence (Figures 1C and 1D).

We first investigated the effects of Rab6 depletion on cell growth. After the 4 days treatment with 4-OHT, we observed a 8% decrease in cell viability as compared with control cells (incubated with ethanol) but this difference was not significant (P = 0.57). 4-OHT was then removed and MEFs kept in culture for up to 6 days. MEFs previously treated with 4-OHT grown slower than controls (Figure 2A). Time-lapse videomicroscopy experiments showed cells that rounded up but failed to divide and eventually died (Figure S1, Supp. movies S1 and S2). This observation is consistent with our previous finding that the alteration of Rab6A' function leads to a block in metaphase (Miserey-Lenkei et al., 2006). We also observed in the videos cells that failed cytokinesis, giving rise to bi-nucleated cells (Figure S1, Supp movies S1 and S2). A significant increase in the number of bi-nucleated cells was indeed found in the Rab6-depleted cell population (Figure 2B). Such an effect has not been previously observed. However, cells depleted in Rab6IP1, a Rab6 effector that also interacts with Rab11, display cytokinesis defects (Miserey-Lenkei et al., 2007). It will be important in future experiments to investigate in more detail the role of Rab6-mediated trafficking pathways in the completion of cytokinesis.

Next, we investigated the effect(s) of Rab6 depletion on Golgi morphology. The overall Golgi/TGN (Trans-Golgi Network) organisation visualised with antibodies against the cis-Golgi marker GMAP-210, the Golgi matrix protein GM130, and TGN38 remained unaffected at the immunofluorescence level (Figure 3A). However, at the electron microscopy level, a significant increase (about twofold) in the maximum cisternal length was observed after Rab6 depletion (Figure 4). A similar result was obtained.
**Figure 1 | Generating MEF cells from inducible RAB6 k/o mice**
(A) Targetting strategy: the upper panel shows the RAB6 genomic locus; the middle panel depicts the targeted locus and the lower panel depicts the locus after deletion of exon 4 through the action of the Cre-recombinase; (B, C and D) MEFs derived from RAB6 loxP/loxP or RAB6 loxP/KO Rosa26-CreERT2 mice were incubated for 96 h with ethanol (control) or 4-OHT. RT-PCR analysis of mRNAs extracted from these cells is shown in (B). Rab6 expression was detected by Western blotting (C) or immunofluorescence (D).
Figure 2 | Effects of Rab6 depletion on cell growth
MEFs were plated and treated for 96 h with or without 4-OHT. After 4 days of treatment, the cells were washed three times with PBS and incubated in fresh medium without 4-OHT. (A) Cells were counted at different time points after trypsinization and addition of Trypan blue to estimate the cell viability. The results are from four independent experiments; (B) the number of bi-nucleated cells was counted on fixed cells after 96 h with or without 4-OHT. For control MEFs, the results are from \( n = 498 \) (ethanol) and \( n = 498 \) (4-OHT) cells. For RAB6 KO MEFs, the results are from \( n = 857 \) (ethanol) and \( n = 803 \) (4-OHT) cells. Student’s \( t \)-test: **\( P = 0.0167 \). Results are from three independent experiments.

following Rab6A/A’ depletion by siRNA in HeLa cells (Storrie et al., 2012). In contrast, we did not observe an increase in the number of cisternae as reported in this study. Another striking feature of Rab6 depletion in MEFs is the increase number of budding profiles on Golgi membranes. A significant accumulation of budding profiles was also observed in Rab6A/A’-depleted HeLa cells (Storrie et al., 2012). Altogether these results support the hypothesis that Rab6 is involved in the fission of Golgi-derived vesicles as well as in the homeoeostasis of Golgi membranes (Miserey-Lenkei et al., 2010, Storrie et al., 2012; Majeed et al., 2014). We also investigated the effect of Rab6
**Figure 3 | Organelle morphology at the immunofluorescence level**

MEFs treated with or without 4-OHT for 96 h were fixed and stained for (A) GMAP 210 (cis-Golgi marker), GM130 (Golgi matrix), TGN38; (B) M6PR; (C) Lamp1; (D) beta-COP and (E) clathrin heavy-chain (HC). The Golgi organisation was not affected following Rab6 depletion. No significant changes in the morphology or distribution of M6PR- and Lamp1-positive structures were observed. Scale bars: 10 μm.
Figure 4 | Golgi morphology at the electron microscope level
MEFs treated with (B, C and D) or without (A) 4-OHT were high pressure frozen and thin sectioned. Arrows denote free vesicles near the Golgi apparatus (GA), arrowheads membrane-linked vesicles and asterisks budding vesicles. The scale bar represents 1 μm in A and B and 2 μm in C and D; (E) quantification of Golgi cisternae in control and Rab6-depleted cells. While the average width (nanometer, nm) of Golgi stacks was comparable in control and Rab6-depleted cells, the length of cisternae significantly increased (Student’s t-test, **P < 0.01) following Rab6 depletion; (F) quantification of vesicle profiles in control and Rab6-depleted cells. The number of free or membrane-linked vesicles was similar in the two groups. Significantly (Student’s t-test, ***P < 0.001) more budding profiles from Golgi membranes were observed following Rab6 depletion.
depletion on the morphology of various organelles. As illustrated in Figure 3, B-D, no significant alteration of the morphology or the localization of the mannose-6 phosphate receptor (M6P-R)-positive structures or of Lamp1-positive lysosomes was observed in Rab6-depleted MEFs. Golgi-associated clathrin and COPI-coated vesicles (labelled with an antibody against β-COP) appeared also similar to controls.

About 20 putative Rab6 effectors have been identified (Goud and Akhmanova, 2012). Rab6 effectors include a lipid phosphatase (OCRL), several motor proteins or motor-binding proteins (myosin II, myosin Va and Vb, KIF20A, Bicaudal, p150Glued), scaffolding or adaptor proteins (Mint/X11), Golgi matrix proteins (giantin) and tethering factors (COG, Vps52) (Barnekow et al., 2009; Goud and Akhmanova, 2012; Lindsay et al., 2013). We investigated the expression levels of some of these proteins in Rab6-depleted MEFs. The expression of Rab6IP1, GAPCenA/TBC1D11, Bicaudal, Myosin Va, p150Glued, Myosin II, KIF20A/Rabkinesin-6, or OCRL was not significantly affected by Rab6 depletion (Figure 5A and data not shown). The expression levels of two Rab proteins that share common partners or effectors with Rab6, Rab1 (Giantin, Rosing et al., 2007) and Rab11 (Rab6IP1, Miserey-Lenkei et al., 2007), as well as the levels of two endocytic Rab proteins, Rab5 and Rab21, remained also the same following Rab6 depletion (Figure 5B and data not shown).

A generic function of Rab GTPases is to recruit effectors to specific membranes. Bicaudal D was shown to be associated to Golgi membranes in a Rab6A/A′-dependent manner (Matanis et al., 2002). Accordingly, about 65% of Golgi-associated endogenous Bicaudal D staining was lost in MEFs following treatment with 4-OHT (Figures 6A and 6B). We have previously shown Rab6 also participates in the recruitment of myosin II to Golgi/TGN membranes (Miserey-Lenkei et al., 2010). We confirmed in this study such a role for Rab6 (Figures 6A and 6B). The loss of endogenous Myosin II staining in Rab6-depleted MEFs was comparable (about 40%) to that observed in NRK cells depleted in Rab6A/A′ by siRNA (Miserey-Lenkei et al., 2010).
Figure 6 | Effects of Rab6 depletion on the localisation of endogenous GM130, Bicaudal-D, myosin II and GCC185
(A) MEFs treated with or without 4-OHT for 96 h were processed for immunofluorescence and stained with primary antibodies against Rab6:GTP, GM130, Bicaudal-D or myosin II; Scale bars: 10 μm. (B) Quantification (means ± SEM) of GM130 staining in control (n = 30) and 4-OHT-treated cells (n = 30), of Golgi-associated Bicaudal in control (n = 50) and 4-OHT-treated cells (n = 50), and of Golgi-associated myosin II in control (n = 87) and 4-OHT-treated cells (n = 108). The differences in Bicaudal-D and myosin II signals after Rab6 depletion were significant (Student’s t-test: ***P < 0.001). (C) MEFs treated with or without 4-OHT for 96 h were incubated with two siRNAs against Arl1 for 3 days (Arl1#1 and Arl1#4). Cells were then processed for immunofluorescence and stained with primary antibodies against Rab6:GTP, GM130 or GCC185. (D) Quantification (means ± SEM, n = 81–112 cells of Golgi-associated GCC185 fluorescence intensity in cells treated or not with 4-OHT and incubated or not with the two siRNAs against Arl1 Student’s t-test: *P < 0.05 in both cases; scale bars: 10 μm.
A controversial issue concerns the role of Rab6 in targeting the Golgi GCC185 to TGN membranes. Although Burguete et al. (2008) proposed that Rab6 cooperates with Arl1 to recruit GCC185 onto Golgi membranes, a further study suggested that depletion of both Rab6 and Arl1 does not affect the localisation of endogenous GCC185 (Houghton et al., 2009). We re-investigated this issue here using MEFs depleted of Rab6. Rab6 depletion did not significantly affect the level of Golgi-associated endogenous GCC185 (Figures 6C and 6D). Further depletion of Arl1 by siRNA (Figure S3) resulted in a small but significant decrease of endogenous GCC185 immunofluorescence signal. In the absence of both Rab6 and Arl1, the GCC185 immunofluorescence signal was about 65% of control level (Figures 6C and 6D). This suggests that Arl1 in conjunction with Rab6 could play some role in GCC185 targeting and/or its stabilisation of TGN membranes. However, as recently documented for Drosophila GCC185, targeting of GCC185 to the Golgi likely requires other interactions than with Arl1 and Rab6 (Torres et al., 2014).

Rab6 was shown to regulate several transport routes, including a retrograde pathway between endosomes and the endoplasmic reticulum via the Golgi complex and a post-Golgi anterograde pathway towards the plasma membrane (Goud and Akhmanova, 2012). The role of Rab6 in the retrograde pathway was deciphered using the B fragment of Shiga toxin (STxB) (Johannes and Goud, 1998). STxB binds to the glycolipid Gb3 prior to its internalisation. Unfortunately, no immunofluorescence signal could be
detected when MEFs were incubated with fluorescently-labelled STXb. In addition, MEFs were resistant to intoxication by the holotoxin, suggesting that they do not express (or at very low levels) Gb3 molecules on their plasma membrane. We therefore performed experiments with ricin toxin that follows, as Shiga toxin, a Rab6-dependent retrograde transport route between endosomes and the Golgi complex (Utskarpen et al., 2006). As shown in Figures 7A and 7B, Rab6 depletion led to a fivefold reduced sensitivity of MEFs to ricin toxin, confirming the regulatory role of Rab6 in the retrograde pathway of the toxin. Of note, transport to the Golgi of cholera toxin B fragment, which remains poorly characterised but could involve a Rab7b-dependent pathway (Progida et al., 2010), was not affected by Rab6 depletion in MEFs (Figure S2). To investigate post-Golgi anterograde transport, MEFs were transfected with a plasmid coding for the thermo-sensitive vesicular stomatitis virus glycoprotein (tsO-45 VSVG) labelled with GFP and the arrival of VSV-G at the plasma was quantified. In agreement with previous studies, the transport of VSV-G was delayed but not abolished in Rab6-depleted MEFs. This delay was comparable to published results using the same transport marker (Figure 7C) (Grigoriev et al., 2007; Miserey-Lenkei et al., 2010; Storrie et al., 2012).

In conclusion, we confirmed in this study most of the functions previously attributed to the two ubiquitous isoforms of Rab6 (Rab6A and Rab6A’), that is regulation of retrograde transport between endosomes and Golgi and anterograde transport between Golgi and the plasma membrane, as well as control of Golgi homoeostasis. We found that Rab6 is an essential gene required for normal embryonic development. Studies are underway to understand the reason(s) of this embryonic lethality. It will be also important to deplete selectively Rab6 in various tissues to determine whether Rab6 has tissue-specific function(s). In particular, it will be interesting to deplete Rab6 in the brain to better investigate the function of the neuronal isoform Rab6B whose exact function is not understood.

Material and methods

Generation of Rab6A/A’-knockout mice

The Rab6 conditional KO mutant mouse line was established at the MCI/ICS (Institut Clinique de la Souris). The targeting vector was constructed as follows. A 5 kb fragment corresponding to the 5′ homology arm and a 2.6 kb fragment corresponding to the 3′ homology arm were amplified by PCR and subcloned in an MCI proprietary vector, resulting in step 1 plasmid. This MCI vector has a floxed Neomycin resistance cassette. A 0.6 kb fragment encompassing exons 4 or 4′ was amplified by PCR and subcloned in step 1 plasmid to generate the final targeting construct. The linearised construct was electroporated in 129/SvPas mouse embryonic stem (ES) cells. After selection, targeted clones were identified by PCR using external primers and further confirmed by Southern blot with a 5′ external probe. One positive ES clone was injected into Balb/CN blastocysts, and male chimerae derived were crossed with Flp deleter mice and gave germ line transmission.

The care and use of these mice having a non-harmful phenotype was strictly applying European and National regulation for the Protection of vertebrate animals (approval number of the establishment Trouillet C75-05-18). It complies also with internationally established principles of replacement, reproduction and refinement in accordance with the Guide for the care and use of laboratory animals (NRC 2011).

Antibodies

For the immunofluorescence experiments, the following antibodies were used: human anti-Rab6GTP (AA2) (1:100); rabbit antibody against KIF20A/Rabkinesin-6 (1:2000) (Echard et al., 1998); rabbit antibody against Bicaudal-D (1:300) (a kind gift of Anna Akhmanova); mouse antibody against GM-130 (BD Biosciences) (1:1000); mouse antibody (AD7) against myosin II (a kind gift of Jenny Stow) (1:200); rabbit antibody against Arl1 (ProteinTech) (1:200); rabbit antibody against GMAP-210 (a kind gift of Rosa Rios) (1:5000); rabbit antibody against M6P-R (a kind gift of Juan Bonifacino; Perez-Victoria et al., 2010) (1:200); rat antibody against CD107/Lamp1 (CliniSciences) (1:200); rabbit antibody against TGN38 (a gift of George Banting) (1:200); mouse antibody against β-COP (Sigma–Aldrich) (1:100); goat antibody against clathrin heavy chain (Santa Cruz Biotechnology) (1:100); Alexa- and HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

For the immunoblotting experiments, the following antibodies were used: rabbit antibody against Rab6 (Santa Cruz) (1:1000); rabbit antibody against KIF20A/Rabkinesin-6 (1:2000) (Echard et al., 1998); rabbit antibody against Bicaudal-D (1:2000) (a kind gift of Anna Akhmanova); rabbit antibody against Rab6IP1 (1:1000) (Miserey-Lenkei et al., 2007); rabbit antibody against GAPCenA (1:500) (Cuif et al., 1999); mouse antibody against p150glued (BD Biosciences) (1:1000); mAD7 mouse monoclonal antibody against myosin II (a kind gift of Jenny Stow) (1:2000); rabbit antibody against myosin Va (Sigma) (1:2000); rabbit antibody against OCRL (1:1000); rabbit antibody against Rab1 (1:1000) (Saraste et al., 1995); rabbit antibody against Rab5 (BD Biosciences) (1:500); rabbit antibody against Rab11 (1:1000); and rabbit antibody against Rab21 (a kind gift of Johanna Ivask). HRP-coupled secondary antibodies were obtained from Jackson Laboratories.

Mouse embryonic fibroblasts

MEFs were prepared from E12.5 Rab6loxP/loxP (control) or Rab6loxP/KO Rosa26CreERT2-TG embryos and...
**RAB6A KO MEFs**

cultured in a 5% CO2 humidified air in DMEM (Gibco) supplemented with 10% (v/v) fetal calf serum (Eurobio) and 100 U ml\(^{-1}\) penicillin/streptomycin. Cells were used for experiments until passage 5. For the induction of Rab6 depletion, cells were treated with 4-OHT (1 μM) for 96 h. 4-OHT was replaced by ethanol (0.004%) in control conditions.

**RNA extraction**

RNAs were extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich).

**Reverse transcription PCR**

cDNAs were synthesised using the High capacity cDNA reverse transcription kit (Sigma–Aldrich) and analysed by PCR using the following primers: 5′-ATGGTGAAGGCTGGTGTGAA-3′ and 5′-TTACTCCTGGAGGCCATGT-3′ that target the sequence of the GAPDH gene and primers 5′-ATGTCCGGGGCGGAGACTT-3′ and 5′-CCCTAATTTGAATCAGCCG-3′ that target the sequence of the RAB6A gene.

**Live cell imaging**

MEFs were plated on 35-mm glass dishes (Ibidi) and put in an open chamber equilibrated in 5% CO2 and maintained at 37°C. Time-lapse sequences were recorded at 30 min intervals for 72 h on a TiE Nikon videomicroscope using a 10× objective controlled by the Metamorph software (Molecular Devices). This microscope was equipped with a cooled CCD camera.

**Transfection**

MEFs treated or not with 4-OHT were seeded in six-well plates on 12-mm glass coverslips. Transfection was carried out using either lipofection (XtremeGENE 9; Roche) or electroporation, following the manufacturer’s instructions. For siRNA experiments, MEFs were transfected with the corresponding siRNA (Luc or Arl1) for 72 h, following the manufacturer’s instructions.

**RNA interference**

For silencing mouse ARL1, specific ON-TARGET plus Set of 4 siRNA were chemically synthesised by Thermo Scientific. Only the results obtained with the sequences GCCAAUUGCUUCUUGGGUUA and CGUUGACAGUUGUGACCGA are shown. SiRNA targeting luciferase (CGUAACCGGAUAUCUUGC) (Proligo-Sigma) was used as a control.

**Electron microscopy**

MEFs were grown on carbonated sapphire discs at 70% confluence and were high pressure frozen with a HPM100 apparatus (Leica Microsystems), freeze substituted in a Leica AFS (Leica Equipment Ltd.) as described (Hurbain et al., 2008). Sections were observed under an electron microscope (Philips CM120; FEI Company) and digital acquisitions were made with a numeric camera (Keen View; Soft Imaging System). The number of cisternae per Golgi stack, lateral budding structures and membrane-linked vesicles Golgi stacks, size of vesicles, average Golgi stack width, circumference of Golgi stack and maximum cisternae length were evaluated by EM analysis of randomly selected cell profiles from two distinct experiments using Analysis (Soft Imaging System). The definition of the distinct compartments was based on their morphology.

**Immunofluorescence microscopy and Western blotting**

For immunofluorescence, MEFs grown on coverslips were fixed either in 4% paraformaldehyde for 15 min at room temperature or in methanol (2 min, –20°C), depending on the antibody. Cells were then processed for immunofluorescence as described previously (Miseray-Lenkei et al., 2006). For Western blotting, cells were processed as described previously (Miseray-Lenkei et al., 2001).

**Quantification of GM130, Bicaudal-D, myosin II and GCC185 Golgi-associated fluorescence intensity**

The quantification of Golgi-associated GM130 and Bicaudal-D was performed as follows: images of control or Rab6-depleted cells were acquired using the same parameters, without automatic scaling and gain adjustment, avoiding saturated pixels. The absolute intensity of Golgi-associated GM130 and Bicaudal-D fluorescence intensity in the Golgi area was measured using Image J software (NIH Image). In the case of Bicaudal-D, Golgi area was defined by the GM130 staining. The quantification of Golgi-associated myosin II fluorescence intensity was performed as described in Miseray-Lenkei et al. (2010). Briefly, images of control or Rab6-depleted cells were acquired using the same parameters, without automatic scaling and gain adjustment, avoiding saturated pixels. The absolute intensity of Golgi-associated mAD7 fluorescence intensity in the Golgi area was measured using Image J software (NIH Image). Golgi area was defined by the remaining Myosin II staining. The quantification of Golgi-associated GCC185 fluorescence intensity was performed as follows: Images of control or Rab6-depleted cells treated with ethanol or with 4-OHT were acquired using the same parameters, without automatic scaling and gain adjustment, avoiding saturated pixels. For each cell, 3–5 round area of about 600–800 pixels were drawn on the Golgi area (defined by the GM130 labelling), and the mean intensity of Golgi-associated GCC185 fluorescence intensity in this area was measured using Image J software (NIH Image). The number of cells quantified in each case is indicated in the figure legends.

**Intoxication assay**

MEF cells treated or not with 4-OHT were seeded at 20,000 cells per well in 96-well plates and grown overnight. Cells were challenged with increasing doses of ricin toxin for 4 h. Protein biosynthesis was determined 1 h later by measuring the incorporation of radiolabelled methionine into acid-precipitable material, as previously described (Amessou and al., 2007). The mean percentage of protein biosynthesis was determined and normalised from duplicate wells. All values are expressed as mean ± SEM. Data were fitted with Prism v5 software (Graphpad) to obtain the 50% effective toxin concentration (EC\(_{50}\)). EC\(_{50}\) values and protection factor (i.e., EC\(_{50}\) ratio = EC\(_{50}\) treatment/EC\(_{50}\) toxin) were determined by the nonlinear regression dose–response EC\(_{50}\) shift equation. The goodness of fit for toxin and treatment was assessed by R\(^2\) and confidence intervals.

**VSV-G transport assay**

Cells were transfected with an expression plasmid containing GPP-VSV-G tsO45 cDNA using either lipofection transfection (XtremeGENE 9; Roche) or electroporation and incubated overnight at 40°C. Under these conditions, the misfolded
proteins accumulate in the ER. VSV-G folding and subsequent transport of the folded proteins to the Golgi apparatus and plasma membrane was initiated by shifting cells to 32°C. Expression at the plasma membrane was quantified several times later after fixation using an antibody directed against the extra-
cellular domain of VSV-G as described in Misery-Lenkei et al. (2010).

Transport of cholera toxin B fragment
MEFs were seeded in six-well plates on 12-mm glass coverslips and treated for 4 days with ethanol or 4-OHT. After treatment, the cells were washed three times with PBS and incubated in DMEM supplemented with 10% (v/v) of fetal calf serum, 100 U ml\(^{-1}\) penicillin/streptomycin, 20 mM Hepes and cholera 
 toxin B fragment labelled with Alexa 488 for 1 h at 4°C. Af-
ter washings with PBS, half of the coverslips were fixed in 4% paraformaldehyde and processed for immunofluorescence. The other half were further incubated for 90 min at 37°C in fresh medium, fixed in 4% paraformaldehyde and processed for immuno-
fluorescence.

Author contribution
S.B., S.M.-L., I.H. and D.G.-C. performed the experiments; G.R. and B.G. designed the research and S.B., S.M.-L. and B.G. wrote the manuscript.

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Conflict of interest statement
The authors have declare no conflict of interest.

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