CARP2 a novel regulator of Golgi dynamics and promising target of chemotherapy against drug resistant cancer

Introduction

Organelle dynamics, defined by their shape, size, number, and cellular localization, affect their function, essential to regulate cellular processes. These dynamics are regulated in a spatio-temporal manner by a coordinated network of complex cellular machinery[1,2]. The dynamic nature under physiological or pathological conditions of mitochondria, endoplasmic reticulum, lysosomes, endosomes, is relatively more investigated than Golgi bodies, organelle pivotal to protein and lipid modifications, and secretion of biomolecules.

In mammalian cells, Golgi bodies exhibit a high degree of complexity with a ribbon-like structure, mostly reported to be restricted to the perinuclear or pericentriolar region. Golgi ribbon consists of multiple stacks of a tightly packed flat-disc-shaped membranous structure (called cisterna) fused or joined laterally by tubular structure or proteinaceous matrix[3]. The stack consists of 5-7 polarized cisternae, divided into distinct regions like cis, medial, and trans-Golgi. The cis-Golgi region is located towards the ER and receives ER output. Cis-, medial-, and trans-Golgi are involved in cargo modifications and sorting of the proteins. The modified cargo destined to intracellular or extracellular locations exit from the trans-Golgi[4]. Notwithstanding, recent advancements establish that architecture of Golgi bodies changes between ribbon to dispersed/mini-stacks structures, to cater the needs of the cell (viz. cell division and migration)[5–7] . Moreover, Golgi also remodels in a cell type-specific manner. In neurons, Golgi bodies are present as outposts near to dendritic outgrowth besides the perinuclear Golgi body. Golgi outpost is thought to regulate the microtubule dynamics and local cargo delivery at dendrites. In muscle cells, Golgi mini stacks are regularly interspaced in the myofibril networks and act as a microtubule nucleation site, formed as a result of cell differentiation[6,8]. Molecular mechanisms that regulate Golgi dynamics under different physiological and pathological conditions are not quite clear.

Understanding of cancer development and cure is limited due to its complexity and multifaceted nature. Cancer cells demonstrated to reprogram the metabolism, organelle morphologies and localization, have altered cytosolic pH, heterogeneity among the cancer cell population etc. make them more invasive and malignant. Chemotherapy remains to be effective medical procedure to treat the cancer. But cancer cells acquired the resistance against the drugs either before or during the chemotherapy, like antibiotic drug resistant in bacteria. Drug resistant cancer development found to be contributed by various factors viz. by drug efflux or inactivation, inhibiting the cell death pathways, undruggable genomic drivers (Ras, Myc, TP53), tumour micro-environment etc. Though, clear mechanisms behind the development of drug resistant cancer are unknown. Strikingly, many tumour types are associated with fragmented Golgi structure phenotype. Surprisingly, drug resistant tumours are positive for fragmented Golgi. Fragmentation of Golgi is linked with altered protein glycosylation of the cancer cells like increased of sialylation, associated with enhanced metastatic potential.

We have identified couple of Golgi structure modulators having implications in development drug resistant tumour. Here, we report an endosomal-associated ubiquitin ligase, CARP2 as a novel regulator of Golgi structure in mammalian cells. CARP2 levels are high in many tumour types () and displayed drug resistance. Further, it has been shown to promote cell migration and lung fibrosis. Over-expression of CARP2 leads to Golgi fragmentation and ligase activity is required. In addition, we have elucidated the mechanism that CARP2 targets and degrade the Golgin45, a Golgi structural protein confers structural support, and leads to Golgi fragmentation. Inhibition of CARP2 could be promising approach to prevent the Golgi fragmentation thereby inhibit the development of drug resistant tumour formation.

Objectives

1. Identification and characterization of ubiquitin ligase(s) as a regulator of Golgi structural and functional dynamics.

2. Elucidation of mechanism underlying the Golgi fragmentation.

3. Utilize the regulator knowledge to decipher the regulation of Golgi dynamics to develop better therapeutic strategies towards the cure of chemotherapy resistant cancer.

Material and Methods

Cell culture

Cell lines used in this study was obtained from ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM with 4.5 g/l glucose, L-glutamine, 3.7 g/l sodium bicarbonate and sodium pyruvate) (SH30243.01, Hyclone GE) supplemented with 10% FBS (10270-106, Gibco-Thermo Fisher Scientific) and 1% penicillin-streptomycin (A001A, HiMedia). Cells were kept in incubator at 37°C supplied with humid 5% CO2. Periodically, cells were tested for contamination. Cells were plated 24 hours prior to transfection and Lipofectamine 3000 was used to transfect the plasmid as per the manufacture's instruction (L3000-015, Invitrogen). CARP2 variants stable cell lines (A549, HEK293T) were generated using retroviral method followed by selection with puromycin(1ug/ml) as described earlier[20].

Plasmids and Reagents

Human CARP2 cDNA (NCBI reference sequence: NM_001017368.2:141-1232) was cloned in pFlagCMV2 vector and pMYs-IP. Golgin45-V5 (Harvard plasmid repository), GFP-Golgin 97(Chia-Jung Yu, Chang Gung University, Taiwan), GRASP65-GFP (Yanzhuang Wang, University of Michigan, USA) and SiaT-RFP (Jack Rohrer, ZHAW, Switzerland) constructs received as generous gift.

Antibodies GM130 (G7295, Sigma), GFP (632375, Mouse Living Colours Clonetek), Tubulin (T6199, Sigma-Aldrich), Actin (4967S, CST), Flag (F3165-1MG, Sigma), V5 (R96125, Invitrogen), Anti mouse HRP (712-035-150, Jackson Immuno Research), Anti-rabbit HRP (65-6120, Invitrogen) were purchased. Polyclonal CARP2 antibody raised in Rabbit (generated in our laboratory).

Immunoblotting

After 30 hours of transfection cells were lysed in lysis buffer (20mM Tris-cl (pH 7.5), 50mM NaCl, 1mM EGTA, 0.5% Triton X-100, 1X-protease inhibitor cocktail, 1X-phosphatase inhibitor 2 and 3) for 30 mins on ice. Followed by spin at 13,500×g for 10 min and supernatant was collected, samples were prepared using Laemmli buffer and boiled at 95°C for 10 minutes. Samples were separated by SDS-PAGE and later transferred to PVDF membrane.

Immunoblotting was performed with specific antibodies as mentioned in the figures and detection was done with chemiluminescent HRP substrate (WBKLS0500, Merck).

Immunostaining and Imaging

Cells were grown on coverslips and briefly washed with PBS, followed by fixation with 4% paraformaldehyde for 20 minutes at room temperature. Washed thrice with PBS before permeabilization with 0.1% triton-X100 for 10 minutes. Then washed again, blocked with 3% bovine serum albumin prepared in PBS for 1 hour, followed by incubation with primary antibody and washing. Secondary antibodies conjugated with fluorophore against primary antibody was incubated for 1 hour and washed with PBS and nucleus was stained with DAPI. Then the coverslip was mounted on a glass slide with antifade mounting media.

Next day images were aquired using 63X oil immersion objective of Leica TCS 508 SP5 II laser scanning confocal microscope. For live cell imaging, cells were grown on glass bottom dish (MatTek 254 Corporation, Cat#: P35G-1.0-20-C) followed by imaging using the same microscope mentioned earlier. Here, Hoechst stain used to label nucleus.Golgi dispersal quantification was carried out manually with criteria like if signals are scattered dots in perinuclear region or not connected, or isolated dots considered as dispersed Golgi.

For Nile red staining, cells were plated 24 hours before live imaging in glass bottom dish. Then, stained with Nile red dye (to stain lipid) and Hoechst dye for nucleus.

Pulse width analysis

Cells were transfected with plasmids in mentioned cell lines (see legends), after 24 hours cells were collected with PBS supplemented with 5mM EDTA and analyzed at medium flow rate in flow cytometer FACS Aria III (BDBiosciences, San Jose, CA) and FlowJo software was used for analysis. Flow cytometer was equipped with lasers 405, 488 and 561nm. Forward scattered threshold of 5000 was used to collect more than 10,000 events. For each channel pulse height, width, and area parameters were recorded and analyzed the pulse shape as described earlier[17].

Statistical Analysis

Experimental data is represented as mean ± s.d or sem. Student's t test or ANOVA test was performed to obtain p-value, with α=0.05 and significance levels demonstrated as ns, not significant, *P<0.05, **P<0.01, *** P<0.001.

Results

CARP2 expression leads to Golgi dispersal

CARP2 upregulation at the transcription level by EGF promotes cell migration[22]. Since cell polarity/migration requires remodelling of cell organelles and cytoskeleton[14], we investigated the effect of exogenous CARP2 on different organelle like mitochondria, ER, Golgi, and cytoskeleton proteins. For this we have used lung carcinoma A549 cells stably expressing CARP2 without any tag. These cells were imaged after staining with MitoTracker Red CMXRos to visualize the mitochondria, or after transfecting with an endoplasmic marker (GFP-b5 ER) or after immunostaining with anti GM 130 antibody, to visualize the Golgi. No obvious changes were observed in the morphology of either the mitochondria or the ER network in control or CARP2 expressing cells (Figure 1B & C). While staining with a well-known Golgi marker GM130 displayed a compact structure in the perinuclear region in the vector control cells. Importantly, in CARP2 expressing cells no such GM130-positive compact structure was visible. However, in these cells GM130 appeared as dispersed intracellular vesicles (Figure 1A). In these cells the organization of cytoskeleton proteins tubulin and actin were assessed by immuno-staining with tubulin antibody or phalloidin. No obvious differences between control and CARP2 expressing cells were seen (Figure 1D & E). These results suggest that CARP2 can regulate Golgi structure, and hence for further studies we focused on CARP2 mediated Golgi dynamics.

Quantification of GM130 staining revealed that more than 80% of cells exhibited such dispersed Golgi phenotype in CARP2 expressing cells compared to 20% that was observed in control cells (Figure 2 A & B). In these cells, as reported earlier CARP2 appeared as endocytic vesicles and the protein level of exogenous CARP2 as assessed by western blot.

As in A549 cells, similar Golgi dispersal was observed in 293T cells as well indicating that CARP2 effects on the Golgi architecture is independent of the cell type. Dispersal of the Golgi was further evaluated using another assay procedure involving SiaT-RFP (another Golgi marker). SiaT is a sialyase transferase enzyme known to localize to trans-Golgi region, and glycosylate numerous proteins within the Golgi. SiaT-RFP is a construct consisting of human Golgi localization sequence (nucleotide sequence corresponding to 1-110 amino acids) cloned in frame with RFP. Usage of this construct as a marker to monitor Golgi dynamics in cells has been used earlier[16]. When SiaT-RFP was used, unlike in control cells where RFP signal was limited to the perinuclear region, dispersed RFP fluorescence, reflecting dispersed Golgi phenotype, was observed upon CARP2 expression in both A549 and 293T cells (Figure 2C & D). To investigate the Golgi dispersal observed in CARP2 stably expressing cells is indeed because of CARP2 expression, we have used siRNA specific to CARP2 to reduce the expression of CARP2 in these cells and monitored effect on Golgi dispersal using SiaT-RFP as a marker. As expected, transfection with control siRNA did not reduce CARP2 level and also showed no effect on the Golgi dispersal. However, transfection with CARP2 specific siRNA not only substantially reduced CARP2 protein level, but also resulted in compact Golgi structure, indicating reversal of the Golgi dispersal (Figure 2E & F). Next, to monitor distribution of the Golgi signal at different intracellular locations we employed pulse width analysis (PulSA) approach which measures the width of the fluorescent signal of individual cells in a population by flow cytometry[17–19]. For this analysis we used CARP2 stably expressing 293T cells transfected with SiaT RFP. Shift in pulse width of SiaT-RFP was observed in CARP2 expressing cells compared to control, confirming that CARP2 promotes Golgi dispersal (Figure 2G). These data collectively demonstrate that CARP2 ubiquitin ligase regulates Golgi dynamics.

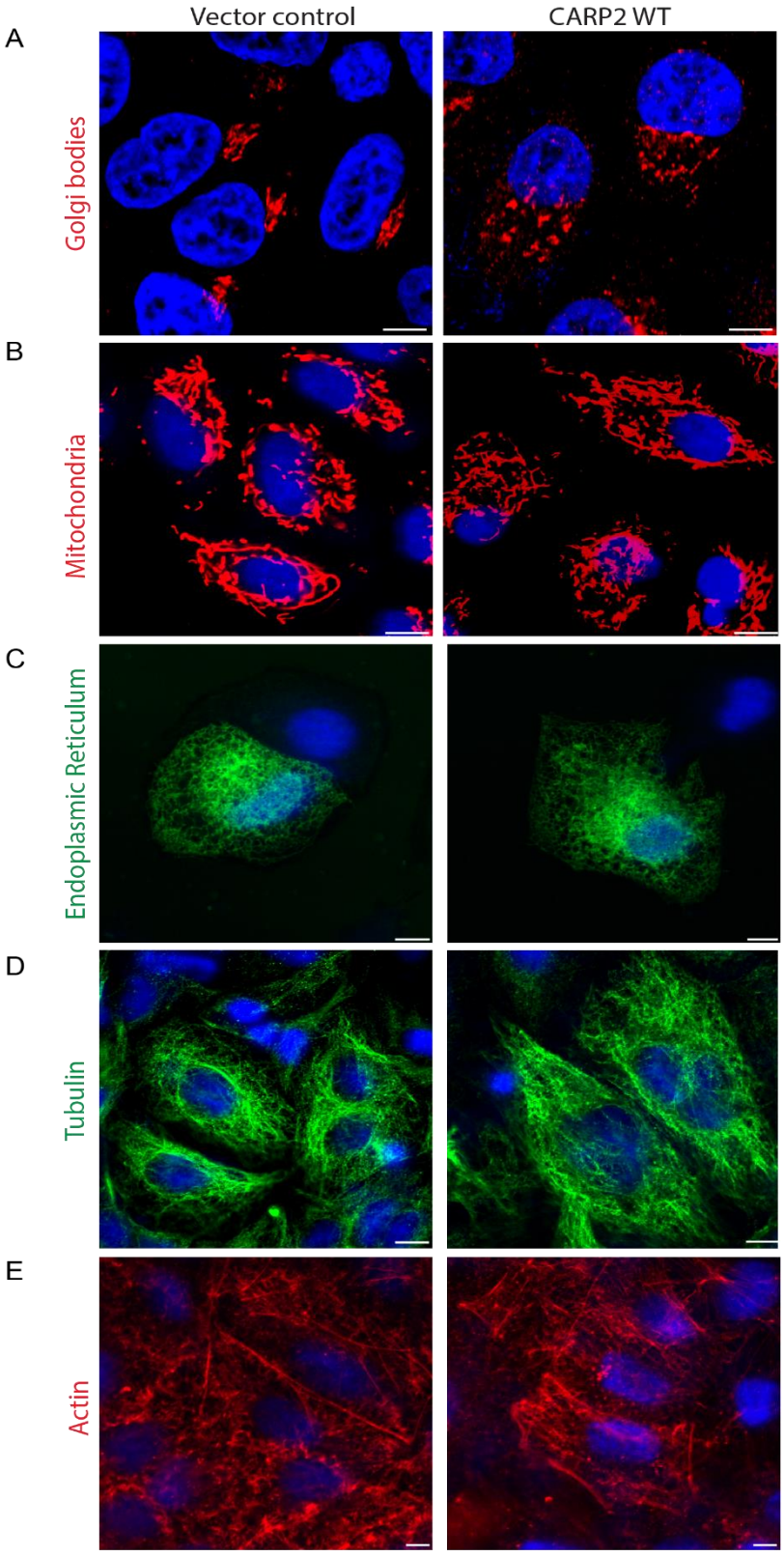

Figure 1. CARP2 expression does not affect morphology or localization of other organelles and cytoskeleton. A. A549 cells expressing control vector or CARP2 WT were immunostained with anti-GM130 (for Golgi) and DAPI stained the nucleus. **B.** Cells described in A were stained with MitoTracker Red CMXRos (for mitochondria) and Hoechst stain (nucleus) and imaged live. **C.** Cells described in A were transfected with GFP-b5 ER (for endoplasmic reticulum) and cells were imaged live. Nucleus was stained with a hoechst stain. **D.** Cells described in A were immunostained with anti-tubulin antibody (for cytoskeleton) and the nucleus with DAPI. **E.** Cells described in A were stained with phalloidin (actin marker) and hoechst stain (nucleus) and imaged live. Scale bar 10µm (A), 10µm (B), 5µm (C), 5µm (D), 5µm (E)

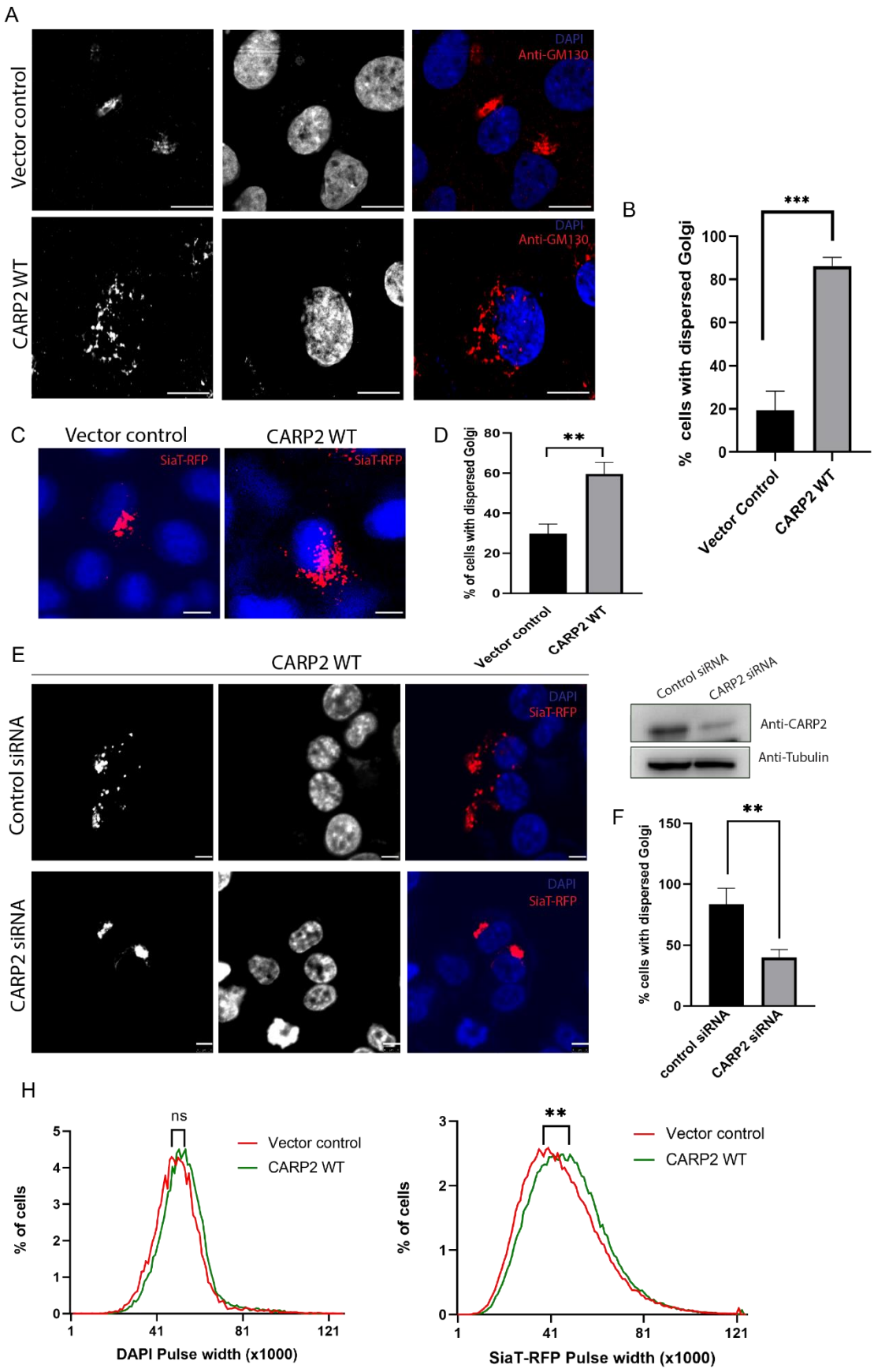

Figure 2. Expression of an endosomal-associated ubiquitin ligase CARP2 results in the dispersal of Golgi structure A. A549 cells stably expressing either vector control or CARP2 without any tag were cells immunostained with GM130 antibody (red). Nucleus was stained with DAPI (nucleus). Scale 10µm. **B.** The dispersed Golgi in cells described in A was quantified. More than 200 cells were imaged per experiment. Statistical significance was computed by Student's *t*-test, ***P<0.001. Error bars represent mean ± SEM from three independent experiments. **C&D**. A549 cells stably expressing either control vector or CARP2 without any tag were imaged live with SiaT-RFP marker. Nucleus was stained with DAPI (nucleus). At least 50 cells were imaged per experiment. Statistical significance was computed by Student's *t*-test, **P<0.001. Error bars

represent mean ± SEM from three independent experiments. Scale 10µm. **E&F.** HEK293T cells stably expressing CARP2 were transfected with control or CARP2-specific siRNA for 48 hours followed by SiaT-RFP (Golgi marker) live imaging. Nucleus was stained with a hoechst stain. At least 200 cells were imaged per experiment. Statistical significance was computed by Student's *t*-test, **P<0.001. Error bars represent mean ± SEM from three independent experiments. Scale 5µm. **G**. HEK293T cells stably expressing either control vector or CARP2 stable were transfected with SiaT-RFP. After 24h of transfection the cells were analysed by FACS and pulse width histograms were represented. Nucleus staining (hoechst stain) was used as negative control. Statistical significance was computed by Student's *t*-test, **P<0.001. Error bars represent mean ± SEM from three independent experiments.

CARP2 ubiquitin ligase activity is essential for Golgi dispersal

Since, CARP2 is a RING domain protein, which confers ubiquitin ligase function, we were curious to know whether CARP2 ligase activity is required for Golgi dispersal. For this A549 cells stably expressing CARP2 ligase inactive mutant (CARP2 H333A) were generated. Immunostaining with CARP2 antibody demonstrates that the mutation H333A did not affect CARP2 localization to endosomal vesicles and the expression at the protein level remained the same as the wildtype (Figure S1B). Immunostaining of H333A mutant expressing cells for GM130 showed compact and perinuclear Golgi, as that in vector control cells, suggesting that CARP2 ligase activity is indeed essential for dispersal (Figure 3A & B). In addition to the RING domain, CARP2 contains a FYVE-like motif. This FYVE-like motif is believed to facilitate anchoring of CARP2 on phospholipids with the help of modifications like palmitoylation in the N-terminus. Amino acids cysteine at positions 5 and 6 are the residues predicted to be involved in palmitoylation. Our lab along with other groups showed that mutation of C5,6 to A disrupts the CARP2 localization to intracellular vesicles[20]. We have used this variant of CARP2 to assess the importance of CARP2 endosomal localization for Golgi dispersal. GM130 distribution in C5,6A expressing cells appeared like vector control cells (compact and perinuclear) than CARP2 wild type expressing cells, indicating that CARP2 endosomal localization is required for Golgi dispersal (Figure 3A & B). Like GM130, SiaT-RFP distribution in A549 control, H333A and C5,6A mutant appeared compact than CARP2 WT expressing cells

(Figure 3C & D). These observations indicate that CARP2 ubiquitin ligase activity and endosomal localization play important role in Golgi structure modulation.

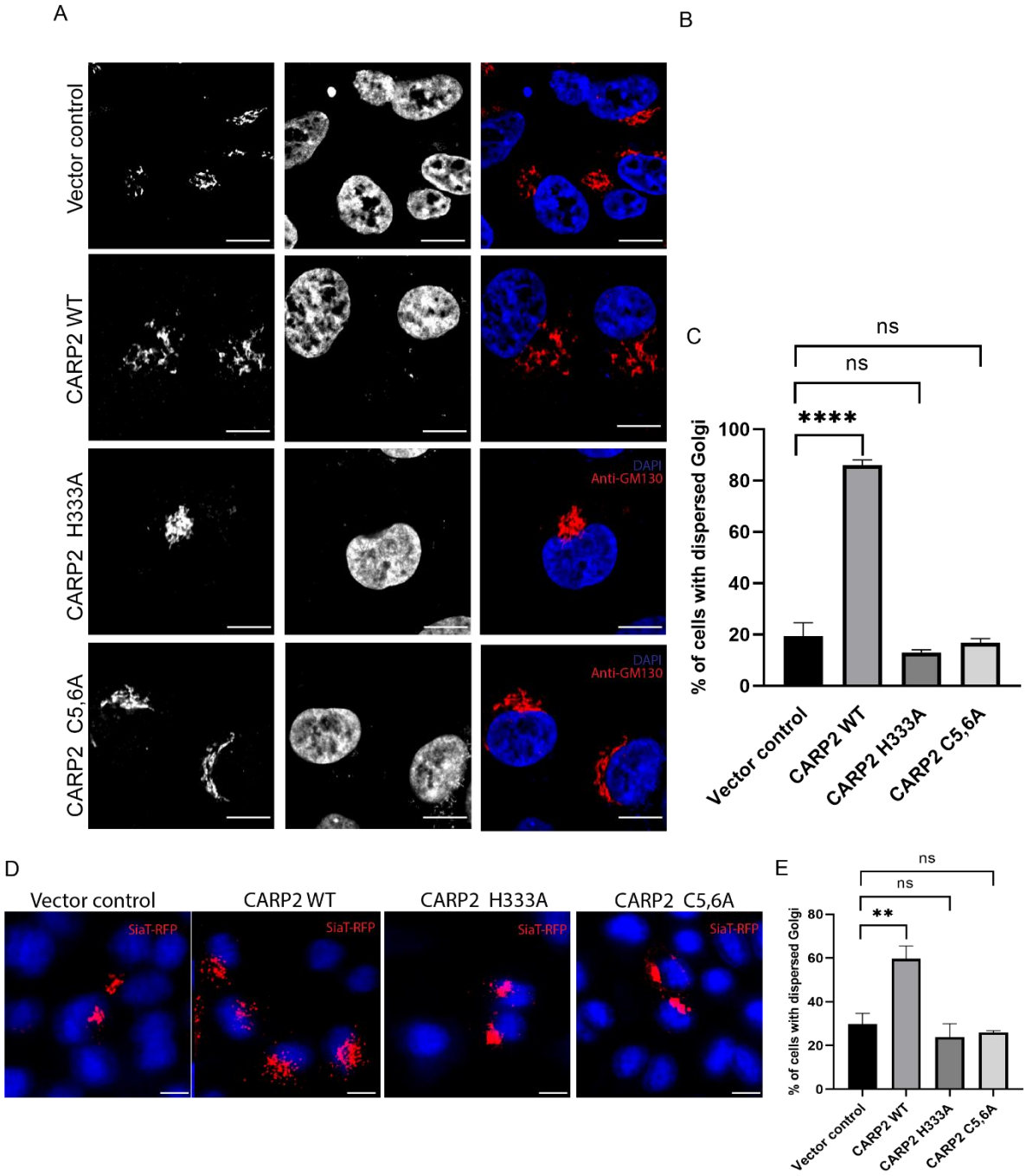

Figure 3. CARP2 ubiquitin ligase activity is required for Golgi dispersal. A. A549 cells stably expressing either vector control or CARP2WT or CARP2 H333A or CARP2 C5,6A without any tag were immunostained anti-GM130 antibodies (red). Nucleus is stained with DAPI (blue). Scale 10µm. **B.** The dispersed Golgi in cells described in A was quantified. More than 200 cells were imaged per experiment. Statistical significance was computed by ANOVA, ****P<0.001. Error bars represent mean ± SEM from three independent experiments. **C.** A549 cells stably expressing either vector control or CARP2WT or CARP2 H333A or CARP2 C5,6A without any tag were transfected with SiaT-RFP and imaged live (red). Nucleus is stained with DAPI (blue). Scale 10µm. **D.** The dispersed Golgi in cells described in C was quantified. More than 200 cells were imaged per experiment. Statistical significance was computed by ANOVA, **P<0.001. Error bars represent mean ± SEM from three independent experiments.

Effect of CARP2 on protein and lipid trafficking

Golgi is central to the secretory system where packaging, post translational modifications and targeting of lipids and proteins from the endoplasmic reticulum (ER) to the plasma membrane occurs[3]. Having demonstrated that CARP2 expression leads to Golgi dispersal, we wanted to know whether this dispersal affects the secretion of proteins from the ER. To address the same, we used soluble SS-HRP (SS-HRP oxidase) protein as a surrogate marker. Having a specific signal sequence (SS) that enables Horseradish peroxidase (HRP) to reach the ER, SS-HRP translocate to the ER where the signal sequence is cleaved and the HRP protein is transported outside the plasma membrane through the Golgi bodies. Secreted HRP activity from the media is quantified using luminol as a substrate[21]. To evaluate the effect of CARP2 expression on the secretion of HRP, HEK 293T cells were transfected with CARP2 variants along with SS-HRP constructs and after 24 hours of transfection the amount of HRP secreted was measured. Increased HRP activity was noted in the media of cells expressing CARP2 WT compared to that of the vector expressing control cells (Figure 4A). Moreover, we could observe dose-dependent enhancement in the HRP activity with an increase in CARP2 expression (Figure 4B). Since CARP2 ubiquitin ligase inactive mutant H333A is unable to affect Golgi integrity, we next measured HRP activity from the media from cells expressing CARP2 H333A mutant. Unlike CARP2 WT, H333A expression did not show any enhanced HRP activity, suggesting that under these experimental conditions, CARP2-induced dispersal of the Golgi results in enhanced secretion of the HRP and this requires the ubiquitin ligase activity (Figure 4C). In fact, expression of CARP2 H333A mutant was reported to facilitate accumulation of vesicular structures, which could explain a reduction below the basal level of HRP in these cells[22].

To understand whether CARP2 regulates lipid trafficking or localization we used the Nile red stain[23]. The Nile red staining of A549 cells stably expressing the control vector or CARP2 H333A revealed staining of intracellular structures appeared as punctate, and present juxtaposed to plasma membrane uniformly. Whereas cells expressing CARP2 WT, staining was more prominently polarized to some areas near the plasma membrane. Nile red stained polarized pattern was quantified and presented (Figure 4D & E). These data suggest the expression of CARP2 WT results in polarized Nile red positive lipid distribution. This polarization requires CARP2 ligase activity.

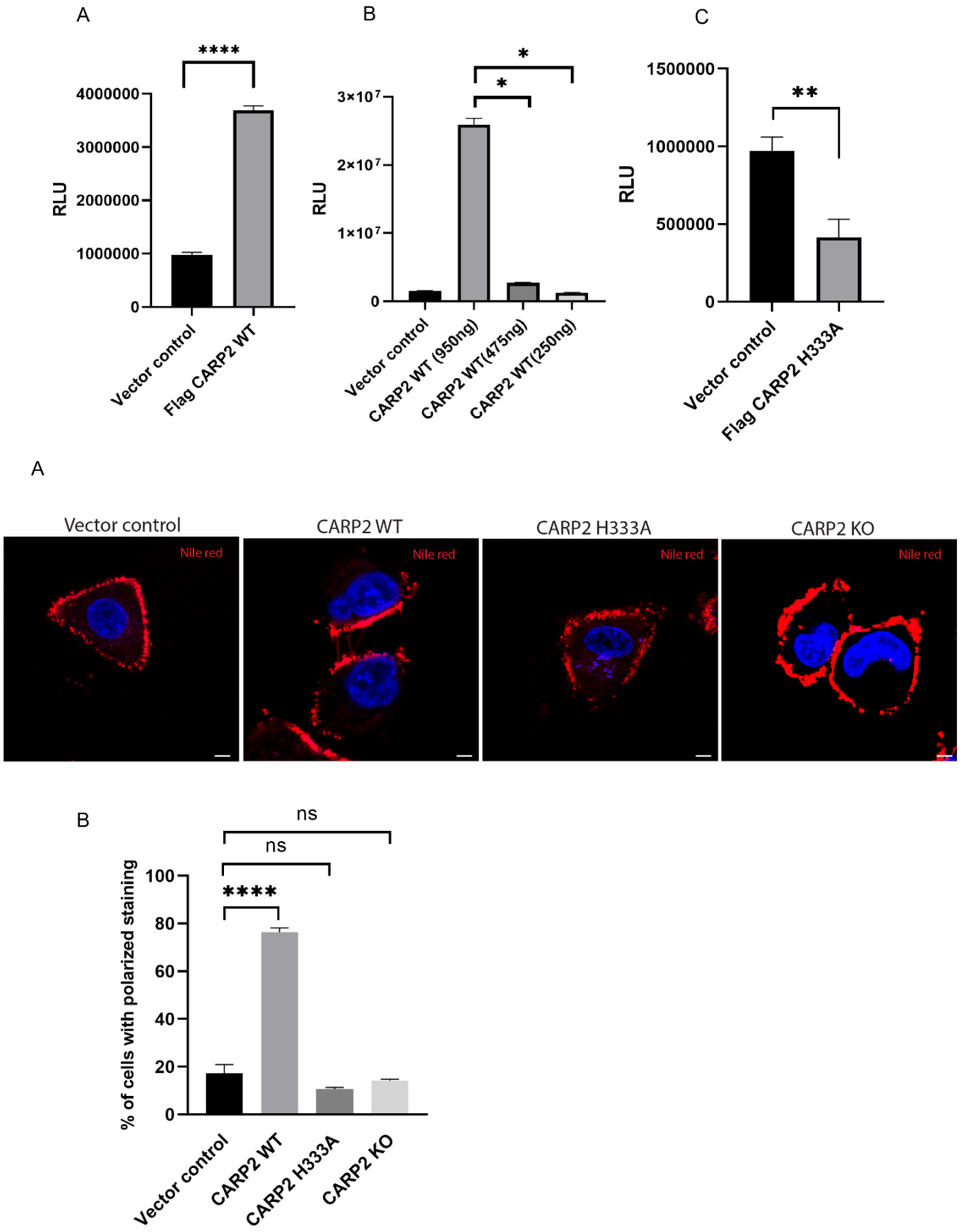

Figure 4. CARP2 affects lipid and protein trafficking. A. HRP activity in extracellular media from HEK293T cells transfected with SS-Flag-HRP along with either control vector or Flag CARP2 WT was measured using ECL kit, after 24 hours of transfection. Graph shows the HRP activity from media from cells transfected with different concentrations of Flag CARP2 WT **(B)** and Flag CARP2 H333A **(C)**. Quantification of HRP activity described in A, B, C was computed by ANOVA or Student's *t*-test. ****,**,*P<0.001. Error bars represent mean ± SEM from three independent experiments. **D.** A549 cells stably expressing control vector or CARP2 WT or H333A without tag and CARP2 KO cells were stained for neutral lipids with Nile red (red) and nucleus with Hoechst stain, and imaged live. Scale is 5µm. **E.** Quantification of Nile red staining described in D. Statistical analysis was computed by ANOVA, ****P<0.001. Error bars represent mean ± SEM from three independent experiments.

Golgin45, a structural protein of Golgi interacts with CARP2

To identify the mechanism by which CARP2 can affect Golgi dynamics, we looked out for CARP2 associated proteins using the Tap-tag system[24]. For this we have expressed CARP2 in 293T cells and the endogenous interaction partners were identified by mass spec analysis. We found one of the CARP2 associated proteins as Golgin45, also known as BLZF1. Golgin45 is a Golgi tethering molecule with a central coiled coil region that is reported to localize to medial Golgi. Golgin45 is also known to interact with other Golgi membrane adhesion factors like GM130, GRASP55, Syntaxin5 and regulate the Golgi structural dynamics [11,25].

To confirm whether the Golgin45 indeed is an interacting partner of CARP2, we have performed immunoprecipitation experiments. We transfected 293T cells with Flag-CARP2 and Golgin45-V5 constructs and cell lysates were immunoprecipitated with Flag antibody and the precipitates were probed with anti-V5 antibody. The immunoblot results showed the presence of Golgin45-V5 in the precipitates of CARP2, suggesting that Golgin45 is associated with CARP2. We also found that Golgin45 also associates strongly with CARP2 ligase inactive H333A mutant (Figure 5A). Given that Golgin45 is associated with CARP2, which is a ubiquitin ligase and is known to modify its substrates with ubiquitin linkages, and subsequent degradation, we explored whether the interaction with CARP2 could result in Golgin45 degradation. As expected, co-expression of Golgin45-V5 with Flag CARP2 wildtype (WT), but not H333A mutant, resulted in reduced Golgin45 protein level (Figure 5B & D). Interestingly, while probing with V5 antibody, we also noticed the presence of an additional band just above the expected Golgin45 protein band both in the lysates and immunoprecipitates. Then we tested the levels of Golgin45 with or without Flag-CARP2 WT in HEK293T cells and found that Golgin 45 levels (modified and unmodified bands) significantly reduced compared to control (Figure 5C). It has been reported that Golgi structural proteins get phosphorylated to promote Golgi dissassembly[7,26]. Based on this, we reasoned that the modified Golgin45 band could be phosphorylated. To confirm this Golgin45-V5 lysate was treated with alkaline phosphatase. Interestingly, modified Golgin45 band disappeared compared to control lanes (Figure 5E). Thus, we conclude that Golgin45 is a real substrate and gets downregulated by CARP2. To exclude the possibility of CARP2 not targeting other Golgi structural proteins. Levels of GRASP65 (cis Golgi structural protein) and Golgin97 (trans Golgi network structural protein)

were checked in presence of CARP2 WT or CARP2 H333A mutant. No visible difference in GARSP65 and Golgin97 levels was observed (Figure 5F & G)[7,11]. These experiments clearly demonstrated CARP2 specifically targets and downregulates the Golgin45. We sought that overexpression of Golgin45 in CARP2 WT stably expressing cells can reverse the dispersed Golgi phenotype. SiaT-RFP imaging of HEK293T cells stably expressing CARP2 transfected with Golgin45-V5, indicate that Golgin45 can rescue the phenotype up to some extent (Figure 5H & I).

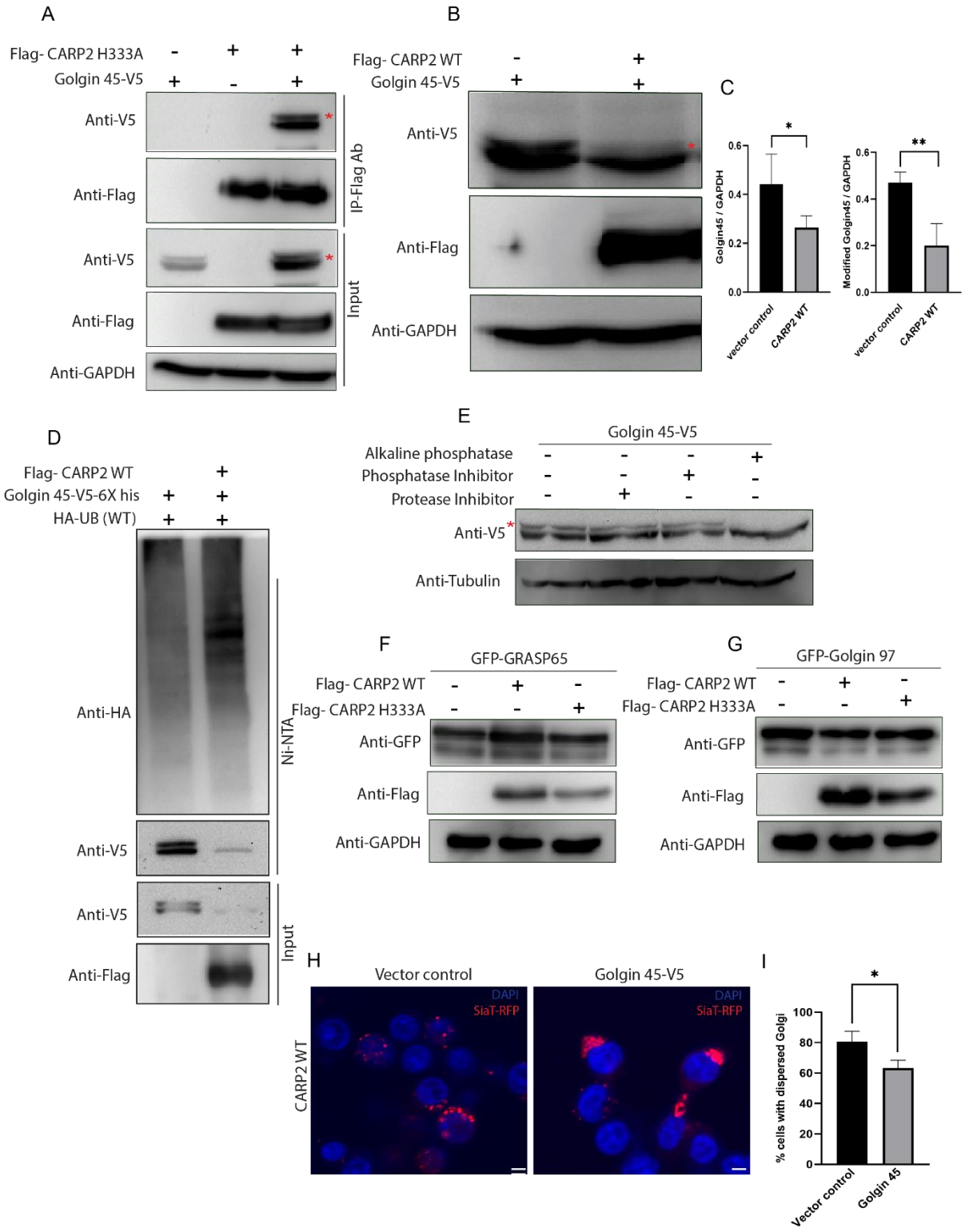

Figure 5. CARP2 interacts and downregulates Golgin45 protein level via ubiquitination. A. Cellular extracts from HEK293T cells transfected with indicated constructs were subjected to immunoprecipitation with anti Flag antibody and the precipitates (IP) along with extracts (input) were probed for Golgin45-V5 with anti-V5 antibody. **B.** Cellular extracts from HEK293T cells transfected with indicated constructs were probed with denoted antibodies. Modified Golgin45 band denoted with * (migrate slower than the unmodified). **C.** Quantification of Golgin45 levels after normalizing with GAPDH. Statistical analysis was computed by Student's *t*-test, *,**P<0.001. Error bars represent mean ± S.D from at least three independent experiments. **D.** Cellular extracts

from HEK293T cells transfected with indicated constructs were subjected to Ni-NTA pulldown and probed with denoted antibodies. **E.** Lysates from HEK293T cells transfected with Golgin45-V5, after treatment with alkaline phosphatase for 1 hour, at 37°C were probed with anti-V5 and anti-tubulin antibodies. Modified Golgin45 bands were denoted with*. **F&G.** Cellular extracts from HEK293T cells transfected with indicated constructs were probed with denoted antibodies. **H.** HEK293T cells stably expressing CARP2 WT were transfected with control vector or Golgin45-V5 along with SiaT-RFP (Golgi marker) and imaged live cell after 24 hrs. Nucleus was stained with a Hoechst stain. Scale is 5µm. **I.** Quantification of dispersed Golgi morphology from three independent experiments described in H were scored. More than 200 cells imaged per experiment. Statistical analysis was computed by Student's *t*-test, *P<0.001. Error bars represent mean ± SEM.

EGF stimulated CARP2 upregulation leads to Golgi dispersal

Stimulation with hEGF (Epidermal growth factor) results in the activation of ERK/MEK (Extracellular-signal-regulated kinase / Mitogen-activated protein kinase) pathway and Golgi remodelling[27]. We have earlier reported that activation of ERK/MEK transcriptionally upregulates CARP2[28,29]. Hence, we hypothesized that EGF-dependent Golgi remodeling is mediated by CARP2. To investigate this, we treated A549 cells with human EGF for different time points and found that CARP2 protein levels were significantly high compared to untreated cells (Figure 6A). Brightfield imaging of A549 cells with EGF treatment induced changes in the morphology of cells suggesting the activation of ERK/MEK pathway as reported earlier (data not shown)[30]. Next, we sought to know under the same experimental conditions the effect of EGF treatment on the Golgi structure. Towards this end, A549 cells transiently expressing SiaT-RFP (Golgi marker) were imaged live at different time intervals after EGF treatment. We observed that the localization of SiaT-RFP was more dispersed compared to untreated cells and the difference is more pronounced at later time points, i.e., 3 hr. (Figure 6B). Given that exogenous expression of CARP2 alone results in the dispersal of the Golgi, these results collectively led us to believe that CARP2 contributes to EGF-induced Golgi dispersal.

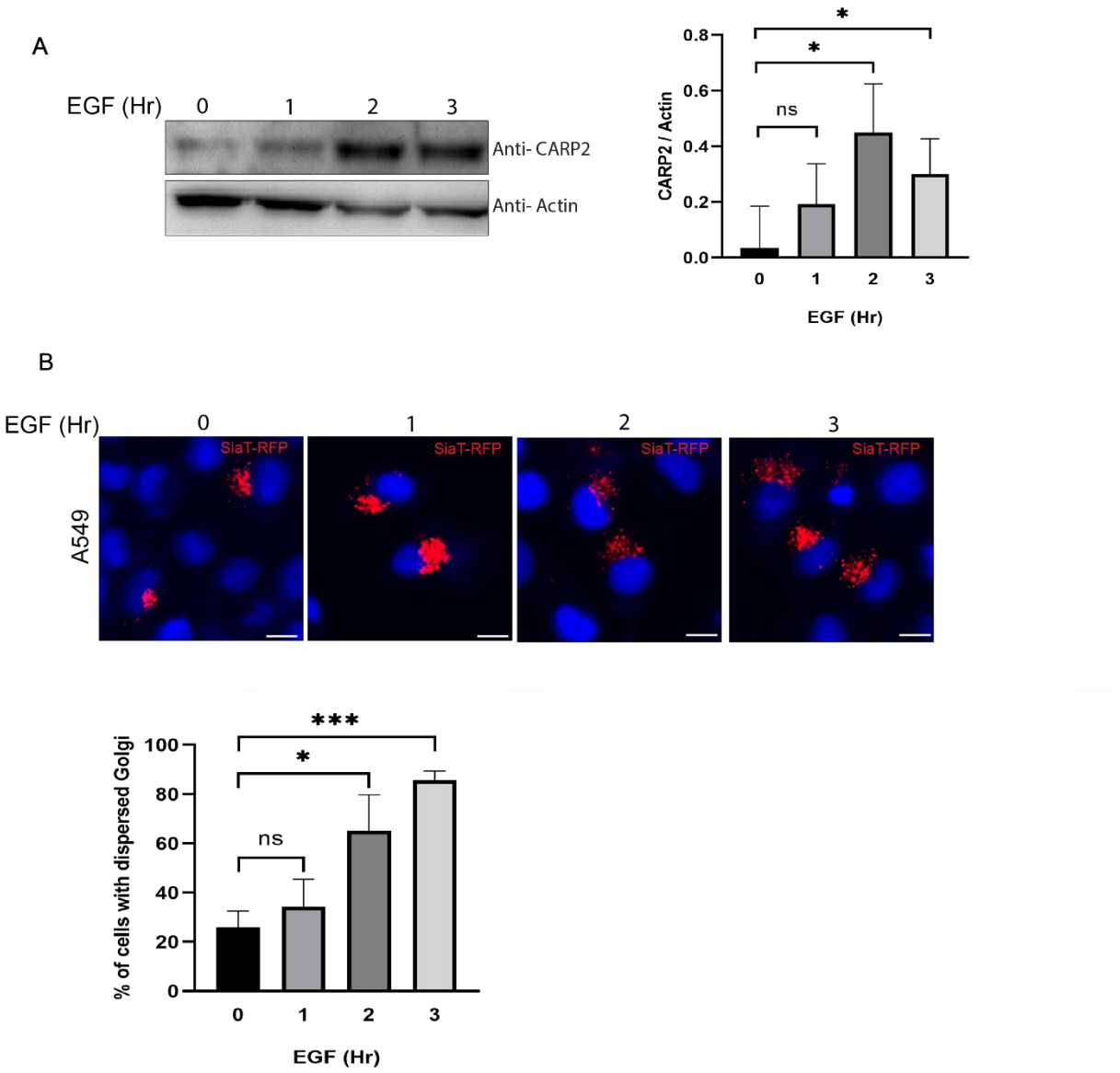

Figure 6. Effect of EGF on Golgi dispersal. A. A549 cells were treated with EGF for indicated time periods and extracts were probed with indicated antibodies (left panel) and quantification of CARP2 levels after normalization with actin shown (right panel). Statistical analysis was computed by ANOVA, *P<0.001. Error bars represent mean ± S.D from three independent experiments. **B.** A549 cells transiently expressing SiaT-RFP were treated with EGF and imaged live at different time points. Nucleus was stained with a Hoechst stain (upper panel). Scale is 10µm. Quantification of Golgi dispersal was shown in the lower panel. Statistical analysis was computed by ANOVA, ***,*P<0.001. Error bars represent mean ± SEM from three independent experiments. At least 50 cells were imaged for each biological replicate.

Discussion

Delicate balance of dynamic vesicle fission and fusion events govern the Golgi structural and functional integrity[31]. Converging evidence indicates that Golgi structural remodeling occurs in several physiological events including cell division and migration. In dividing cells, the Golgi ribbon undergo disassembly by sequential unlinking and unstacking followed by reassembly after cytokinesis, which is essential for segregation of Golgi in daughter cells[3]. This is ensured with the help of cell cycle dependent kinases, phosphatases, and ubiquitin ligases. In migrating cells, unlinked Golgi bodies redistribute to perinuclear sites towards the leading edge of the cell[6,14]. Dysregulation of Golgi dynamics and fragmentation is reported in several pathological conditions like cancer and neurological disorders[32]. The knowledge of the regulator(s) that control Golgi dynamics is limited and in this project we presented evidence that supported that CARP2, an endosomal ubiquitin ligase is a novel regulator of the dynamics of Golgi.

Increased levels of CARP2 protein leads to dispersal of compact Golgi into mini-stacks as observed by GM130 (cis-Golgi marker) and SiaT-RFP (trans Golgi marker) distribution. Depletion of exogenously supplied CARP2 by CARP2 specific siRNA reversed the dispersed Golgi phenotype suggesting the specificity of CARP2 effect on Golgi structural integrity. Further, CARP2 mediated Golgi dispersal phenotype is not restricted to A549 but similar distribution was observed upon exogenous expression of CARP2 in other cell types like HEK293T cells and N2A. Moreover, CARP2 ubiquitin ligase activity is essential for Golgi dispersal because CARP2 ligase inactive mutant is unable to cause Golgi dispersal. Given that CARP2 expression is reportedly high in brain and gonadal tissues[33], it would be interesting to investigate whether differential expression of CARP2 in a given cell or tissue type could be linked to cell or tissue specific variations in Golgi morphologies.

While deciphering the molecular mechanism underlying CARP2 mediated Golgi dispersal, we identified Golgin45 as an interacting partner by Tandem affinity purification tag approach. We demonstrated CARP2 interacts with Golgin45, and ubiquitinates and downregulates its protein level in ubiquitin ligase-dependent manner. Interestingly we also found a modified Golgin45 variant that is sensitive to alkaline phosphatase and susceptible to CARP2-mediated degradation. These observations are consistent with earlier reports that suggested

downregulation of Golgin45 by siRNA leads to Golgi dispersal. Depletion of Golgin45 results in increase in cisternae luminal width and reduction in average number of cisternae per stack as observed by electron microscopy in HeLa cells[25,34]. Importantly, the phenotype of the dispersal of Golgi in CARP2 WT stably expressing cells could partly be reversed upon Golgin45 overexpression indicating signalling between Golgin45 and CARP2 determines Golgi architecture. These results collectively suggest that CARP2 mediated Golgi dispersal is a consequence of Golgin45 downregulation

The ER-Golgi system plays a central role in modification of proteins with moieties like glycosylation, acetylation and their sorting to different destinations through vesicular trafficking and secretion. This process is complex and is regulated at multiple levels depending on the biochemical nature of the cargo such as proteins or lipids and their size, solubility and topology. Some of the structural proteins like Golgins also facilitate cargo transport in addition to giving structural integrity to the Golgi. For example Golgin97 promotes transportation of E-cadherin while Golgin245 facilitates that of TNF-alpha suggesting the existence of diverse transportation routes[35,36].

While maintenance of the integrity of the Golgi is required for cellular homeostasis as the dispersal of the Golgi was reported under pathological conditions like neurodegeneration and cancer[32]. Loss of the Golgi ribbon structure alters intracellular trafficking of both proteins and lipids with reports suggesting either increase or decrease in the secretion. Increased secretion of tau protein or amyloid beta observed in case of Alzheimer's diseases, and GRASP proteins- essential for Golgi stacking, depletion associated with increased CD8 or Cathepsin D trafficking linked with the Golgi dispersal[37,38]. On the contrary, decrease in the secretion of extracellular components was observed upon loss of Golgi structure protein like GMAP-210 (cis- golgin) or Giantin (medial-golgin) in mouse model system associated with altered Golgi[39,26]. In consistency with these reports CARP2 expressing cells showed increased secretion of SS-HRP.

By and enlarge, CARP2 functions support the different phases of tumour progression. Like, CARP2 levels found to be high in different cancer types- demonstrated to be resistant towards chemotherapy, promotes cell migration of mouse embryonic fibroblasts (MEFs), and the lung fibrosis associated with secretion of extra matrix protein secretions. Drug resistant cancer

cells are positive for Golgi fragmentation and altered glycoproteome. Further, CARP2 is transcriptionally regulated by well-known oncogene c-myc. Here, our study proposes that CARP2 would be promising candidate for therapeutic intervention to treat drug resistant tumours. Further, this study opens up the new avenue to unravel how Golgi fragmentation confers the drug resistance. Interestingly, CARP1 is functionally redundant to CARP2 but its role in Golgi fragmentation needs to be tested yet.

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Impact of the research in the advancement of knowledge or benefit to mankind

Chemotherapy and radiation therapy remain to be reliable clinical method to treat various types of cancer. Either prior to or after drug therapy cancer cells acquire drug resistance by reprogramming the cellular processes like metabolism, cell death inhibition, drug inactivation etc. Reoccurrence of drug resistant cancer is big hurdle for medical oncology. Our work attempt to elucidate the molecule(s) and associated cellular reprogramming that help cancer cell to develop the drug resistance. CARP2 is a ubiquitin ligase, its increased protein levels are associated with drug resistant cancer development in various tumour types. Moreover, increased CARP2 protein levels lead to fragmentation of Golgi bodies and promote cell migration, probably, required for cancer metastasis. In line with evidences, drug resistant tumours found to have Golgi fragmentation, in contrast, healthy cells have compact Golgi bodies. Mechanistic understanding Golgi fragmentation mediated development of the drug resistance in tumour cells will bring deeper insight to customize the cancer chemotherapy. Development of specific chemical or genetic inhibitors of Golgi fragmentation could be promising approach to enhance the chemotherapy efficacy.

Literature references

References

1. Carlton, J. G., Jones, H. & Eggert, U. S. Membrane and organelle dynamics during cell division. *Nature Reviews Molecular Cell Biology* **21**, 151–166 (2020).

2. Jongsma, M. L. M., Berlin, I. & Neefjes, J. On the move: Organelle dynamics during mitosis. *Trends in Cell Biology* **25**, 112–124 (2015).

3. Huang, S. & Wang, Y. Golgi structure formation, function, and post-translational modifications in mammalian cells. *F1000Research* **6**, 2050 (2017).

4. Klumperman, J. Architecture of the mammalian Golgi. *Cold Spring Harb. Perspect. Biol.* **3**, 1–19 (2011).

5. Farber-Katz, S. E. *et al.* DNA damage triggers golgi dispersal via DNA-PK and GOLPH3. *Cell* **156**, 413–427 (2014).

6. Saraste, J. & Prydz, K. A New Look at the Functional Organization of the Golgi Ribbon. *Front. Cell Dev. Biol.* **7**, 171 (2019).

7. Tang, D. & Wang, Y. Cell cycle regulation of Golgi membrane dynamics. *Trends Cell Biol.* **23**, 296–304 (2013).

8. Liu, C. *et al.* Loss of the golgin GM130 causes Golgi disruption, Purkinje neuron loss, and ataxia in mice. *Proc. Natl. Acad. Sci.* **114**, 346–351 (2017).

9. Gonatas, N. K., Stieber, A. & Gonatas, J. O. Fragmentation of the Golgi

apparatus in neurodegenerative diseases and cell death. *J. Neurol. Sci.* **246**, 21–30 (2006).

10. Joshi, G., Chi, Y., Huang, Z. & Wang, Y. Aβ-induced Golgi fragmentation in Alzheimer's disease enhances Aβ production. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E1230–E1239 (2014).

11. Short, B., Haas, A. & Barr, F. A. Golgins and GTPases, giving identity and structure to the Golgi apparatus. *Biochimica et Biophysica Acta - Molecular Cell Research* **1744**, 383–395 (2005).

12. Zhang, X. & Wang, Y. GRASPs in Golgi Structure and Function. *Front. Cell Dev. Biol.* **3**, 84 (2016).

13. Barinaga-Rementeria Ramirez, I. & Lowe, M. Golgins and GRASPs: Holding the Golgi together. *Seminars in Cell and Developmental Biology* **20**, 770–779 (2009).

14. Ravichandran, Y., Goud, B. & Manneville, J. B. The Golgi apparatus and cell polarity: Roles of the cytoskeleton, the Golgi matrix, and Golgi membranes. *Current Opinion in Cell Biology* **62**, 104–113 (2020).

15. Kopp, Y. *et al.* CHIP as a membrane-shuttling proteostasis sensor. *Elife* (2017). doi:10.7554/eLife.29388

16. Tie, H. C. *et al.* A novel imaging method for quantitative Golgi localization reveals differential intra-Golgi trafficking of secretory cargoes. *Mol. Biol. Cell* **27**, 848–861 (2016).

17. Gosavi, P., Houghton, F. J., McMillan, P. J., Hanssen, E. & Gleeson, P. A. The Golgi ribbon in mammalian cells negatively regulates autophagy by

modulating mTOR activity. *J. Cell Sci.* **131**, jcs211987 (2018).

18. Chia, P. Z. C., Ramdzan, Y. M., Houghton, F. J., Hatters, D. M. & Gleeson, P. A. High-throughput quantitation of intracellular trafficking and organelle disruption by flow cytometry. *Traffic* **15**, 572–582 (2014).

19. Toh, W. H. *et al.* Application of flow cytometry to analyze intracellular location and trafficking of cargo in cell populations. *Methods Mol. Biol.* **1270**, 227–238 (2015).

20. Ravindran, R. *et al.* Endosomes facilitate mitochondrial clearance by enhancing Mfn2 degradation and subsequent Parkin recruitment. (2020). doi:10.1101/2020.02.19.955880

21. Duran, J. M. *et al.* The role of GRASP55 in Golgi fragmentation and entry of cells into mitosis. *Mol. Biol. Cell* **19**, 2579–87 (2008).

22. Sakai, R. *et al.* The integral function of the endocytic recycling compartment is regulated by RFFL-mediated ubiquitylation of Rab11 effectors. *J. Cell Sci.* **132**, (2019).

23. Greenspan, P., Mayer, E. P. & Fowler, S. D. Nile red: A selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* **100**, 965–973 (1985).

24. Rigaut, G. *et al.* A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol. 1999 1710* **17**, 1030–1032 (1999).

25. Tiwari, N. *et al.* Golgin45-Syntaxin5 Interaction Contributes to Structural Integrity of the Golgi Stack. *Sci. Rep.* **9**, (2019).

26. Lowe, M. The physiological functions of the Golgin vesicle tethering proteins. *Frontiers in Cell and Developmental Biology* **7**, 94 (2019).

27. Bisel, B. *et al.* ERK regulates Golgi and centrosome orientation towards the leading edge through GRASP65. *J. Cell Biol.* **182**, 837–843 (2008).

28. Gan, X. *et al.* Different RAF protein kinases mediate different signaling pathways to stimulate E3 ligase RFFL gene expression in cell migration regulation. *J. Biol. Chem.* (2013). doi:10.1074/jbc.M113.477406

29. Gan, X. *et al.* PRR5L degradation promotes mTORC2-mediated PKC-δ phosphorylation and cell migration downstream of Gα12. *Nat. Cell Biol.* **14**, 686–696 (2012).

30. Peter, Y., Comellas, A., Levantini, E., Ingenito, E. P. & Shapiro, S. D. Epidermal growth factor receptor and claudin-2 participate in A549 permeability and remodeling: Implications for non-small cell lung cancer tumor colonization. *Mol. Carcinog.* **48**, 488–497 (2009).

31. Bonifacino, J. S. & Glick, B. S. The Mechanisms of Vesicle Budding and Fusion. *Cell* **116**, 153–166 (2004).

32. Makhoul, C., Gosavi, P. & Gleeson, P. A. Golgi Dynamics: The Morphology of the Mammalian Golgi Apparatus in Health and Disease. *Front. Cell Dev. Biol.* **7**, 112 (2019).

33. Uhlén, M. *et al.* Tissue-based map of the human proteome. *Science (80-.).* **347**, (2015).

34. Lee, I. *et al.* Membrane adhesion dictates Golgi stacking and cisternal morphology. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 1849–54 (2014).

35. JG, L., LA, H., F, H., PA, G. & JL, S. E-cadherin transport from the trans-Golgi network in tubulovesicular carriers is selectively regulated by golgin-97. *Traffic* **6**, 1142–1156 (2005).

36. Boncompain, G. & Perez, F. The many routes of Golgi-dependent trafficking. *Histochem. Cell Biol. 2013 1403* **140**, 251–260 (2013).

37. Joshi, G., Bekier, M. I. & Wang, Y. Golgi Fragmentation in Alzheimer's Disease. *Front. Neurosci.* **0**, 340 (2015).

38. Thayer, D. A., Jan, Y. N. & Jan, L. Y. Increased neuronal activity fragments the Golgi complex. *Proc. Natl. Acad. Sci.* **110**, 1482–1487 (2013).

39. Munro, S. The golgin coiled-coil proteins of the Golgi apparatus. *Cold Spring Harb. Perspect. Biol.* **3**, 1–14 (2011).

40. Tate, J. G. *et al.* COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res.* **47**, D941–D947 (2019).