JCB

New factors for protein transport identified by a genome-wide CRISPRi screen in mammalian cells

Laia Bassaganyas, Stephanie Popa, Max Horlbeck, Claudia Puri, Sarah Stewart, Felix Campelo, Anupama Ashok, Cristian Butnaru, Nathalie Brouwers, Kartoosh Heydari, Jean Ripoche, Jonathan Weissman, David Rubinsztein, Randy Schekman, Vivek Malhotra, Kevin Moreau, and Julien Villeneuve

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March 25, 2019

Re: JCB manuscript #201902028

Dr. Julien Villeneuve
University of Cambridge
Metabolic Research Laboratories
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Cambridge CB2 0QQ
United Kingdom

Dear Dr. Villeneuve,

Thank you for submitting your manuscript entitled "New factors for protein transport identified by a genome-wide CRISPRi screen in mammalian cells". The manuscript was assessed by three expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that two of the reviewers indicate that the manuscript could be improved by more discussion about the proteins identified in the screen as well as greater insight into how this screen advances the field. Such suggestions should largely be addressable via changes to the text and potentially some additional analysis of screen results. Reviewer 1's comment regarding the characterization of knockdown, as well as all minor comments from this reviewer, should be addressed as well. Reviewer 3 also indicates several instances where additional information is needed in the text for readers to understand the approach and results, and also recommends some edits to figures to aid interpretation. This reviewer also raises concerns regarding the characterization of phenotypes, as mentioned by Reviewer 1 (Rev 1 comment about Figure 3 and knockdown characterization). It is important that for key controls and phenotypic characterization the method of classification is clear, objective, and quantified. The further suggestions from this reviewer are interesting points but are not required for resubmission. Please ensure that these important concerns detailed here are addressed in revision, via text changes, additional analysis and -- if needed -- new data.

Although submitted as a short Report, given the feedback from the reviewers and our editorial assessment of this resource we feel that this manuscript would be more appropriate as a Tool. This change in format would allow more space to fully explain the methods and results, as well as greater discussion of the advance for the field. Please ensure that the methodology is comprehensively described, such that readers could reproduce this work. The Methods section is not included in our character count.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for a Tool is < 40,000, not including spaces. Count includes title page,

abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Tools may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Tools may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ira Mellman, Ph.D.
Editor
The Journal of Cell Biology

Rebecca Alvania, Ph.D.
Executive Editor
The Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Review: New factors for protein transport identified by a genome-wide CRISPRi screen in mammalian cells

Summary:
This study uses a pooled sgRNA library and FACS-based CRISPRi approach to examine how

systematic gene knockdown effects secretory trafficking in a human cell line (HeLa). As a reporter for secretory trafficking to the cell surface, the authors develop a ratiometric approach which compares expression of a fluorescent fusion protein-the single pass protein TAC-to the ability to detect surface delivery of that protein with an antibody. The authors validate a subset of these hits with an HRP secretion assay, and then focus on two novel factors: CCDC157 and TTC17.

Major Criticisms:
•The screen and supporting data appear generally sound, and the novel reporter and genome-wide CRISPRi represent some technical progress. However, neither of the new hits validated is followed up in significant mechanistic detail. It is not clear-in its current state-if there is major novelty or advance from this work. There have already been many screens along these lines and much is already known, as the authors note. Given these points, at the very least could the authors include a figure identifying which hits from their screen are novel secretory factors (not previously identified as such), and what fraction of previously known factors were identified in the present screen? In other words, try to put this screen in context with previous work to fill in the gaps of how close to (or far from) saturation such approaches are in detailing basic protein export.

•The characterization of knockdown as reported is superficial and some of it, such as scoring for Golgi fragmentation (Figure 3), appears subjective and difficult for this reviewer to accept from looking at the example micrographs shown.

•The authors find many RNA related genes (Figure 1H). Given their screening approach (surface vs total), loss of expression (through general mechanisms reducing mRNA) alone should not score as a hit. Could the authors explore or discuss this finding further? Is there some non-synthetic function of the RNA related hits identified?

•The trafficking interpretation of TfR localization relative to Golgi (Figure 4) requires the assumption that TfR goes from early endosomes to the Golgi. The cited reference (Snider and Rogers 1985) did report such a route as stated, but it didn't claim this as the only route or even the major route. In fact, the kinetics of resialylation measured in that study were slow relative to receptor cycling. I think the literature overall supports transit through the Golgi as a minor pathway, accounting for the relatively slow kinetics of TfR resialylation, and transit bypassing Golgi (recycling endosomes) as major. If this is the case, I don't think the interpretation of steady state TfR localization is valid as claimed.

Minor Criticisms:
•In the materials and methods, the authors state that 10E6 cells were sorted for each quartile. Back of the envelope estimate suggests this would provide slightly less than 100-fold coverage of the pooled library. Field standard, as I understand it, is >500-fold (e.g., PMID: 28333914). I understand that sorting the >50 million cells is probably not practical using adherent cells. However, I am slightly concerned that the general reader might not be aware of this key point, or grasp the cost-benefit analysis that the authors decided upon when they chose a pooled, genome-wide, library approach with lower coverage. I think it would be useful to discuss this in the materials and methods section.

•The flow cytometry plots are pixelated and hard to read. a minimum, the text within the plots should be re-written to improve legibility and size (such as PE-1.36 in Figure 1D(i)). We have found that exporting FlowJo figures as .svg can help with this issue.

•For figure 1G, not clear that red/blue vs black dots on volcano plot represent. Presumably, the

highlighted categories from 1H. A quick line in Figure legend would help.

•No scale bars for any micrographs after Figure 1

•In introduction, "gaining continuous complexity" is a little hard for this reviewer to parse.

•In introduction, it appears this sentence may be missing citations, " [...] high throughput or flow cytometry systems."

•Following the pooled siRNA approach for Figure 2A, the authors' write "specific siRNA" in description of subsequent experiments. It was not clear if these experiments were still using the pooled siRNA from Dharmacon.

•It may be that the word 'knockdown' would be more appropriate than 'downregulation' in this sentence, "Flow cytometry analysis revealed that downregulation of the selected genes ..."

•As the authors state in their introduction, aspects of the machinery which mediate protein secretion have been mapped in yeast, biochemical studies, and with RNAi. It would be useful to see a figure in which the CRISPRi hits (passing a p-value and FC cutoffs) are novel.

Reviewer #2 (Comments to the Authors (Required)):

This is a straightforward study that uses CRISPR screening to identify proteins involved in exocytosis. The strategy is clever and based on a cell line expressing a TAC-GFP fusion protein that allows them to assess PM versus intracellular expression as wells the repressor CRISPR system that allows repression of gene expression. They identify several novel proteins and focus very superficially on two of them TTC17 and CCDC157 and show that each are GOLGI proteins that have distinct functions.

The analysis is interesting but not very intensive, but since I judge this mainly as a methods paper, this is probably sufficient to make their point. I have a few minor comments.

1. While there is a long list of proteins that are identified, it seems a bit surprising that known regulators of GOLGI morphology and of exocytic transport were not identified. The authors should discuss this.
2. Along with a list of proteins that appear to be critical for exocytic trafficking, there are also many proteins that appear to enhance secretion. There is no comment on this.
3. The description of TfR is that is is synthesized travels through the Golgi to the PM and then returns to the Golgi. This is surprising to me as my understanding is that TfR is the model protein for rapid recycling from the early endosome to the PM. The way the experiment is performed, isn't it possible that most of the protein detected is from newly synthesized protein that could be aberrantly trafficked in an ER/Golgi like compartment?

Reviewer #3 (Comments to the Authors (Required)):

Bassaganyas et al describe the characterisation of four genes they isolated from a genome wide CRISPRi screen on secretion in HeLa cells. They uncover several genes that either inhibit or increase the transport of their test protein TAC.

General comment: images are not quantified in this manuscript. This is important for the interpretation of the images and to allow others to reproduce and compare results (FAIR data). In my opinion the manuscript can only be published after quantification of all phenotypes reported.

The reporter system is not explained anywhere in the text. The reader is left figuring out how it works and how the authors can distinguish between total production and cell surface expression. Please add a sentence or two explaining the dual reporter system so that people from other fields might understand what was done. The demonstration of the reporter system with BFA, trypsin and the knock down of various genes is convincing.
The experiments of figure S1 are not explained in the text and the legend is difficult to understand and has no motivation for the experiments. The authors should either remove the data or explain it more thoroughly.
In Figure 1E lower panel, the couloring is puzzling as the boundaries between color and gray are not perpendicular to the x-axis. If the lower or upper quartiles are selected, the lines should be straight, please explain. The authors should explicitly state that the definition of quartiles is set on the sgGal4 population and applied to other experimental conditions, if this is indeed the case. Readers are left guessing.
In figure 1F, the subplot #4 is unclear to me. What is the scale bar? Why do the read counts of the lower quartile correlate with the read counts of the upper quartile? Should they not anticorrelate?
Screen:
Table S1 is not included in the manuscript.
How were 63 genes out of 200 selected for the secondary assay?
Figure 2D, why is MHC expression downregulated in the siRNA experiments? There appears to be very little cytoplasmic fluorescence after silencing of the target genes. This should be quantified as this control is important. The lack of MHC expression could suggest that the genes have an effect on expression, although the FACS analysis does not show the same reduction in total signal.

Figure 3: The changes in morphology should be quantified in the images. Scoring cells containing fragmented Golgi or not is not good enough. The morphology and distribution of the ER and ERES look to be profoundly affected in the images in Figures 3B and S2B, yet the authors report no difference. Quantification of the structures would help resolve this. The localisation of CCDC151 next to centrin-3 is not shown image 3I. It is clear from the images that TTC17 localises to more structures than just TGN46 positive structures. A colocalisation with GM130 would be interesting to show. In general the colocalisation should be quantified.
Also the TfR should undergo image analysis. In the images, TfR vesicles appear smaller. A kinetic analysis of TfR uptake should be carried out to demonstrate that the endocytosis of TfR is normal, but that the recycling of the cargo is affected.
The colocalisation of GN46 and GM130 needs to be quantified, the experiment is not interpretable otherwise.
Other comment: when analysing 3 independent experiments, the SEM should be reported not the SD.

Further suggestions:
The author should consider doing EM to study the structure of the Golgi
The authors have identified several interacting genes. How did these genes score in the genome wide screen? The authors should do knock down of these genes and compare to the phenotypes of TCC17, CCDC151 and C10orf88. Epigenetic studies could be undertaken to reinforce that the interactors do indeed function together.

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Dear Editor

We thank the reviewers for their advice and assistance on our paper. We have revised the paper extensively and included new analysis and results to address the reviewer's concerns. As suggested, the revised manuscript is now formatted as a "Tool", allowing us to explain in more details key aspects of our study. We hope that our revised manuscript is suitable for publication in Journal of Cell Biology.

Best wishes,

Julien Villeneuve

In the following section we address the reviewers specific concerns.

Reviewer #1

This study uses a pooled sgRNA library and FACS-based CRISPRi approach to examine how systematic gene knockdown effects secretory trafficking in a human cell line (HeLa). As a reporter for secretory trafficking to the cell surface, the authors develop a ratiometric approach which compares expression of a fluorescent fusion protein-the single pass protein TAC-to the ability to detect surface delivery of that protein with an antibody. The authors validate a subset of these hits with an HRP secretion assay, and then focus on tow novel factors: CCDC157 and TTC17.

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We apologize for the lack of details provided in the original manuscript formatted as a "Brief report", on novelty and advances from our work. In the revised manuscript, formatted as a "Tool", an accurate description of results obtained as well as an extended discussion is included to address these concerns.

- First, a new **Table S2** provides the list of 62 interesting hit candidates with either unknown or poorly characterized function selected from the lower or upper quartiles of the pooled CRISPRi screen. They

represent potential new secretory factors that we tested using the arrayed secondary screen based on an HRP assay.

- Second, given the high degree of redundancy between paralog genes involved in protein trafficking and secretion, and taking into account the specificity of the transport machinery required following cargo proteins and cell type, it is not conceivable to identify the overall list of genes known to be involved along the secretory pathway, regardless of the approach used. In this context, an important added value of our study, as compared to other previously published screens, is that we developed a strategy allowing the efficient identification of genes involved in secretory pathway function and organization (See **Fig. 2B to 2D**), which can be easily adapted in different cell types, with various cargo proteins and environmental conditions. A detailed description of genes previously known to be involved in these processes, which we also identified with our approach is now included in the "Results" section. The following is mentioned: "[…] In the lower quartile, Gene Ontology (GO) term enrichment analysis of the 100 top ranked genes revealed that knockdown of genes encoding components of "Golgi vesicle transport" was the main functional category inhibiting TAC transport (**Fig. 2B and 2C**). Genes involved in "toxin transport", "antigen processing and presentation", "Golgi organization" were also highly enriched. More specifically, we identified as top hits most of the COPI subunits (COPA, COPB1, COPB2, COPG1, COPZ1 and ARCN1), as well as the COPII subunits Sec24A, Sec24B and Sec13. We also identified the SNARE Sec22B and the SNARE associated factors, SCFD1 and NBAS; several subunits of the TRAPP complex (TRAPPC3, TRAPPC8, TRAPPC11 and TRAPPC12) and the Conserved Oligomeric Golgi complex (COG1, COG2, COG3 and COG8); the small GTPase SAR1 and the Rab GTPase RAB1A, the Sec23-interacting protein Sec23IP and the exocyst complex component EXOC2, among others (**Table S1 and Fig. 2B**). Interestingly, factors belonging to small ARL and ARF GTPases, Golgins or additional SNAREs were not identified among the top hits. This is consistent with previous reports (Simpson et al., 2012; Wendler et al., 2010), highlighting the high degree of redundancy between paralog genes that could explain why the knockdown of these components did not alter robustly TAC transport."

- Third, the novelty and advances from our work are now clearly stated in the extended "Discussion" as described below:

"**Discussion**. Although a large number of gene products involved in protein transport were identified in recent decades (Novick et al., 1980; Braell et al., 1984; Bard et al., 2006; Wendler et al., 2010), the list is far from complete and several crucial issues are emerging. Indeed, in eukaryotic cells, evolution has given rise to an incredible diversity and complexity of cargo molecules, whose efficient and accurate transport along the secretory pathway is of paramount importance for organismal development, cell and tissue homeostasis, immunity, metabolic regulation, nerve transmission and healthy aging (Lee et al., 2004; Zanetti et al., 2012; Guo et al., 2014; Malhotra and Erlmann, 2015; Rothman and Orci, 1992; Söllner and Rothman, 1994). An increasing number of studies have demonstrated that cells are endowed with specific structural and functional machinery that can be tightly regulated to cope with the whole spectrum of cargo proteins (Cancino et al., 2014; Ma et al., 2018; Raote et al., 2018; Lopes-da-Silva et al., 2019). In this context, it is essential to develop versatile and high-throughput screening strategies to uncover how secretory pathways are adapted to and are regulated in response to intrinsic demands, environmental cues, and altered in diseases.

In this study, we implemented an unbiased pooled genome-wide CRISPRi screen allowing us to efficiently identify new components of the secretory pathway. Major benefits have already been associated with pooled CRISPR screens, establishing such approaches as powerful tools for systematically defining gene function in mammalian cells (Gilbert et al., 2014; Shalem et al., 2015; Canver et al., 2018). The efficiency of our strategy relies on the combination of key parameters that were optimized in our experimental approach. First, we utilized a complementary reporter system associated with cell selection based on the ratio of two fluorescent signals. This reporter system allowed monitoring surface/total TAC expression ratio variations and therefore differential FACS-based cell sorting, directly in relative to the transport of TAC protein to the cell surface. The strengths of this strategy were exemplified with Sec61A knockdown, where we could avoid following up undesirable phenotypes such as defective TAC protein

synthesis. Second, as the secretory pathway is of critical importance for cell homeostasis and viability, gene expression was systematically downregulated using the dCas9 fused to a KRAB effector domain for CRISPR interference, instead of the active Cas9 for CRISPR knockout (Kampmann, 2018; Shalem et al., 2015). The resulting transcriptional repression of target genes preserved minimal gene expression, preventing massive cell death after down regulation of key factors for protein and membrane trafficking. Third, the combination of the dual fluorescent reporter with the CRISPRi system allowed us to monitor TAC transport and perform FACS-based cell sorting at a relatively early time point (seven days after lentivirus transduction), which also would reduce excessive cell death after gene knockdown. Altogether, combining these technical parameters make our approach a noteworthy advance compared to previous RNA interference-based genome-wide screens, and resulted in a powerful platform allowing us to focus on the identification of genes involved in secretory pathway function and organization.

Our work highlighted several factors, whose knockdown inhibited or promoted the trafficking of both exogenous and endogenous cargo proteins. Various genes encoding proteins known to be involved either in calcium homeostasis, signaling pathways, or lysosomal activity, among others, (**Table S2**), strengthen the idea that there are functional links between these cellular processes and the secretory pathway. In addition, several proteins with unknown or poorly characterized function were also identified. In particular, TMEM167A, FAM46A and USP32, which promote TAC transport and HRP secretion upon knockdown, as well as C10orf88, WDR7, YPEL5, TMEM161, FAM162B, and GPR162, which inhibit TAC transport and HRP secretion upon knockdown, deserve greater attention. For example, USP32 is a membrane-bound ubiquitin protease localized in the Golgi apparatus and overexpressed in breast cancer (Akhavantabasi et al., 2010); and GPR162 an orphan GPCR. Interestingly, compelling evidence suggests that the activation of GPCRs on Golgi membranes is critical for protein and membrane trafficking but their identities remain elusive (Eichel and von Zastrow, 2018; Cancino et al., 2014; Diaz Anel et al., 2005). Altogether, our results provide a valuable resource to gain future important insights into fundamental mechanisms governing protein and membrane transport along the secretory pathway.

Our study further characterized two of the newly identified factors, TTC17 and CCDC157, as new actors of critical importance for the structure and function of Golgi membranes. Although additional studies will be required to fully decipher their roles, our results suggest that like other coiled-coil proteins (Wong and Munro, 2014; Cheung and Pfeffer, 2016), CCDC157 could be part of a tethering complex required for fusion events at the Golgi membranes. Furthermore, phenotypes observed in TTC17-depleted cells associated with TTC17 interacting partners involved in sphingomyelin metabolism (ENPP7 and NAAA, **Fig. 5A**) (Tsuboi et al., 2007; Duan et al., 2003), suggest that TTC17 could play a role in the production and distribution of particular lipids, a critical parameter for Golgi membrane organization (Campelo et al., 2017; van Galen et al., 2014). To decipher how CCDC157 and TTC17 are recruited to membranes and how their functions are coordinated with other structural factors and components of the tethering and fusion machinery will be of major interest.

In conclusion, while initial screen performed in yeast revealed the basic principles conserved across species (Novick et al., 1980), and that more recently, arrayed RNA interference screens revealed key players that function in metazoans (Bard et al., 2006; Saito et al., 2009; Von Blume et al., 2009), our pooled CRISPRi screen unveiled new components that further refine the steps along the secretory pathway. In addition, we anticipate that the adaptation of our screening platform to specific cargo proteins, different professional secretory cell types, and to particular intrinsic or environmental challenges, will open new and stimulating perspectives for a better understanding of the secretory pathway architecture in health and disease, for both conventional and unconventional secretion (Chiritoiu et al., 2019; Cruz-Garcia et al., 2018, Villeneuve et al., 2018; Zhang and Schekman, 2013)".

2. The characterization of knockdown as reported is superficial and some of it, such as scoring for Golgi fragmentation (Figure 3), appears subjective and difficult for this reviewer to accept from looking at the example micrographs shown.

We have now further quantified all experiments in order to ensure an unbiased and robust characterization of

reported phenotypes after knockdown of hit genes. In the corresponding "Material and methods" section and "Figures legends", the following is described:

- For the surface/total TAC expression ratio analyzed by flow cytometry in **Fig. 1G and 3C**, quantifications of the enrichment of cells in the lower quartile compared to the upper quartile are shown in **Fig. 1H and 3D**, respectively (mean ± SEM, n=4).
- To assess the surface MHC-I expression, in addition to results obtained by immunofluorescence microscopy (**Fig. 3G**), we have performed flow cytometry experiments and results are shown in **Fig. 3E**. Quantification of the mean of fluorescence of surface MHC-I expression for these additional experiments is presented on **Fig. 3F** (mean ± SEM, n=4).
- For unbiased analysis of Golgi membrane surface area (**Fig. 4D**), we analyzed cells (465 control cells and 388 TTC17-depleted cells) with the CellProfiler Analyst (Broad Institute) (Dao et al, 2016). For each image analyzed, area of Golgi membranes, identified as object, was extracted using the module "MeasureObjectSizeShape".
- For unbiased analysis of Golgi membrane morphology (**Fig. 4C and 4E**), we trained the classifier of the CellProfiler Analyst (Broad Institute) (Dao et al, 2016) using cells randomly chosen from the whole experiment. These were classified as having a ring shape structure, fragmented or intact Golgi. The classifier of the CellProfiler Analyst was then used to define for all cells, Golgi membrane morphology on object level. At least 200 cells were analyzed for each independent experiment (mean ± SEM, n=3).
- All co-localization experiments performed by immunofluorescence microscopy are now quantified (See **Fig. 5I, 6D, 6F, 6H and 7D**). Confocal images from ~30 cells per condition were acquired and co-localization quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Biptplane AG.

3. The authors find many RNA related genes (Figure 1H). Given their screening approach (surface vs total), loss of expression (through general mechanisms reducing mRNA) alone should not score as a hit. Could the authors explore or discuss this finding further? Is there some non-synthetic function of the RNA related hits identified?

The identification of false positives or genes with indirect effects is inherent to any screening approach. But as suggested by the reviewer, there is also the possibility that some of the RNA related genes that scored as hits have additional non-synthetic functions. We did not experimentally test this possibility but the following is now mentioned in the "Results" section: " […] Of note, genes involved in mRNA homeostasis and protein translation were also identified among hit genes. Most likely, their knockdown indirectly alters TAC transport, but a subset of them may also be part of transcriptional or translational programs required for the expression of components specifically involved in the secretory pathway. We can also not exclude the possibility that some of these genes may have additional non-synthetic functions. To test these possibilities will require further investigation". Yet, this class of genes did not represent the main functional category contributing to TAC transport, making our approach a noteworthy advance compared to previous RNA interference-based genome-wide screens (Bard et al., 2006; Simpson et al., 2012), where the list of hit genes after primary screening was to a large extent composed of genes with indirect effects.

4. The trafficking interpretation of TfR localization relative to Golgi (Figure 4) requires the assumption that TfR goes from early endosomes to the Golgi. The cited reference (Snider and Rogers 1985) did report such a route as stated, but it didn't claim this as the only route or even the major route. In fact, the kinetics of resialylation measured in that study was slow relative to receptor cycling. I think the literature overall supports transit through the Golgi as a minor pathway, accounting for the relatively slow kinetics of TfR resialylation, and transit bypassing Golgi (recycling endosomes) as major. If this is the case, I don't think the interpretation of steady state TfR localization is valid as claimed.

We agree with the reviewer that the transport of TfR through the Golgi complex after endocytosis is a minor pathway, whereas TfR recycling towards the plasma membrane is the main route. To avoid confusion, in the

"Results" section, it is now indicated: "[…] To further test a role in membrane fusion, we assessed the impact of CCDC157 knockdown on the distribution of transport carriers derived from the endocytic pathway that fuse with Golgi membranes (Johannes and Popoff, 2008). We performed immunofluorescence microscopy experiments using an antibody targeting the transferrin receptor (TfR), which, although being mainly recycled to the plasma membrane after endocytosis (Huebers and Finch, 1987), is also retrieved to Golgi membranes (Snider and Rogers, 1985; Jin and Snider, 1993; Woods et al., 1986), and an antibody targeting EEA1, a marker of early endosomes, critical for endosomal trafficking (Barysch et al., 2009). We hypothesized that while TfR-containing transport carriers do not mainly fuse with Golgi membranes (Snider and Rogers, 1985; Woods et al., 1986), alteration of these fusion events could lead over time to the accumulation of transport carriers in the vicinity of the Golgi apparatus."
As shown in **Fig. 6E to 6H and S3**, our results demonstrated that in CCDC157-depleted cells, the distribution of both TfR and EEA1 were strongly altered, most of the signal being restricted to the perinuclear area in the form of enlarged TfR- and EEA1-positive vesicles, surrounded by Golgi membranes. In addition, we also showed that in CCDC157-depleted cells, Golgi membrane reassembly after BFA washout was strongly inhibited (**Fig. 6A and 6B**), and we included additional immunofluorescence experiments demonstrating that the ER-Golgi intermediate compartment (ERGIC) was more dispersed thorough the cytoplasm upon CCDC157 knockdown compared to control cells (**Fig. 6C and 6D**). This compartment, which is a collection of tubulovesicular membrane clusters, allows delivering secretory cargo from ER-exit sites to the Golgi complex in a COPII vesicle-dependent manner via homotypic and heterotypic fusion events (Lorente-Rodríguez and Barlowe, 2011). Altogether, these results strongly support CCDC157 as being an important factor for the fusion of transport carriers with the Golgi complex.

Minor Criticisms:
5. In the materials and methods, the authors state that 10E6 cells were sorted for each quartile. Back of the envelope estimate suggests this would provide slightly less than 100-fold coverage of the pooled library. Field standard, as I understand it, is >500-fold (e.g., PMID: 28333914). I understand that sorting the >50 million cells is probably not practical using adherent cells. However, I am slightly concerned that the general reader might not be aware of this key point, or grasp the cost-benefit analysis that the authors decided upon when they chose a pooled, genome-wide, library approach with lower coverage. I think it would be useful to discuss this in the materials and methods section.

Today, in the vast majority of studies using pooled genome-wide CRISPR screening, the phenotypic selection is based on cell viability or proliferation, allowing the identification of genes essential for viability or genes that confer resistance or sensitivity to drugs, toxins and pathogen infections. For such applications, it is relatively straightforward to obtain a high coverage at > 500 cells per sgRNA during the screening selection. However for FACS-based CRISPR screens in which the phenotypic selection is based on the alteration of protein expression detected by fluorescence, a coverage of ~ 100 cells per sgRNA is generally accepted during FACS sorting. Concretely, to collect 10 millions of cells in each fraction by FACS, as performed in our study, ~ 400 cells per second must be sorted efficiently and specifically for each quartile during 8 hours. For this reason, to collect 50 millions of cells, the sorting speed must be increased, but this will inevitably reduce the accuracy of cell selection. The following references included in the "Material and methods" section, correspond to studies performed by leading laboratories using FACS-based CRISPR screens where a coverage of ~ 100 cells per sgRNA, or even less, was used: (Menzies S.A. et al., The sterol-responsive RNF145 E3 ubiquitin ligase mediates the degradation of HMG-CoA reductase together with gp78 and Hrd1, 2018, Elife), (Park R.J. et al, A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors, 2017, Nat Genet), (Parnas O. et al., A genome-wide CRISPR screen in primary immune cells to dissect regulatory networks, 2015, Cell). .

6. The flow cytometry plots are pixelated and hard to read. a minimum, the text within the plots should be re-written to improve legibility and size (such as PE-1.36 in Figure 1D(i)). We have found that exporting FlowJo figures as .svg can help with this issue.

The revised manuscript includes figures with higher resolution.

7. For figure 1G, not clear that red/blue vs black dots on volcano plot represent. Presumably, the highlighted categories from 1H. A quick line in Figure legend would help.

In the legend of **Fig. 2B**, it is now mentioned: "[…] Each gene targeted by the library of sgRNA is indicated with a black dot. Genes included in the 100 top ranked genes and belonging to functional categories of interest (as highlighted in **Fig. 2C and 2D**) are indicated with red dots for genes inhibiting TAC transport and with blue dots for genes stimulating TAC transport".

8. No scale bars for any micrographs after Figure 1.

Scale bars have been added on each micrograph.

9. In introduction, "gaining continuous complexity" is a little hard for this reviewer to parse.

In the introduction, we changed the sentence by: "Recent demonstrations indicate that cell compartments establish cross-regulatory mechanisms with numerous membrane contact sites (Wu et al., 2018), are endowed of tightly regulated dynamics (Valm et al., 2017), and stand at the crossroad of signaling pathways where inputs and outputs are integrated and coordinated (Luini and Parashuraman, 2016). Thus, protein transport and secretion processes are clearly more complex than previously thought".

10. In introduction, it appears this sentence may be missing citations, " [...] high throughput or flow cytometry systems."

Corresponding references have been added.

11. Following the pooled siRNA approach for Figure 2A, the authors' write "specific siRNA" in description of subsequent experiments. It was not clear if these experiments were still using the pooled siRNA from Dharmacon.

For experiments involving siRNA, it is now specified in the text if experiments have been performed using "specific smart pool siRNAs" or "specific individual siRNA", directed against targeted genes.

12. It may be that the word 'knockdown' would be more appropriate than 'downregulation' in this sentence, "Flow cytometry analysis revealed that downregulation of the selected genes..."

The word "downregulation" has been changed by "knockdown" as suggested.

13. As the authors state in their introduction, aspects of the machinery which mediate protein secretion have been mapped in yeast, biochemical studies, and with RNAi. It would be useful to see a figure in which the CRISPRi hits (passing a p-value and FC cutoffs) are novel.

As indicated in the comment #1, a new **Table S2** with 62 hit genes selected from the lower or upper quartiles of the pooled CRISPRi screen and tested in the arrayed secondary screen is included in the revised manuscript. It is now explained in the corresponding "Figure legends" and the "Material and methods" section that: "As we intended to validate hit genes with a secondary screen, interesting hit candidates with unknown or poorly characterized function were selected as potential new secretory factors among those with a p-value < 0.01 and a log2 fold change (lfc) < -0.5 for genes enriched in the lower quartile, and a lfc > 0.3 for genes enriched in the upper quartile. The difference of lfc cutoff applied for the selection of candidate

genes relied on the weaker phenotypes observed on TAC transport for genes enriched in the upper quartile compared to those enriched in the lower quartile".

Reviewer #2

This is a straightforward study that uses CRISPR screening to identify proteins involved in exocytosis. The strategy is clever and based on a cell line expressing a TAC-GFP fusion protein that allows them to assess PM versus intracellular expression as wells the repressor CRISPR system that allows repression of gene expression. They identify several novel proteins and focus very superficially on two of them TTC17 and CCDC157 and show that each are GOLGI proteins that have distinct functions. The analysis is interesting but not very intensive, but since I judge this mainly as a methods paper, this is probably sufficient to make their point. I have a few minor comments.

1. While there is a long list of proteins that are identified, it seems a bit surprising that known regulators of GOLGI morphology and of exocytic transport were not identified. The authors should discuss this.

As discussed above (reviewer #1 - comment #1), given the high degree of redundancy between paralog genes involved in protein trafficking and secretion, and taking into account the specificity of the transport machinery required following cargo proteins and cell type, it is not conceivable to identify the overall list of genes known to be involved along the secretory pathway, regardless the approach used. In this context, an important added value of our study, as compared to other previously published screens, is that we developed a strategy allowing the efficient identification of genes involved in secretory pathway function and organization (See **Fig. 2B to 2D**), which can be easily adapted in different cell types, with various cargo proteins and environmental conditions. A detailed description of genes previously known to be involved in these processes, which we also identified with our approach is now included in the "Results" section. It is mentioned: "[...] In the lower quartile, Gene Ontology (GO) term enrichment analysis of the 100 top ranked genes revealed that knockdown of genes encoding components of "Golgi vesicle transport" was the main functional category inhibiting TAC transport (**Fig. 2B and 2C**). Genes involved in "toxin transport", "antigen processing and presentation", "Golgi organization" were also highly enriched. More specifically, we identified as top hits most of the COPI subunits (COPA, COPB1, COPB2, COPG1, COPZ1 and ARCN1), as well as the COPII subunits Sec24A, Sec24B and Sec13. We also identified the SNARE Sec22B and the SNARE associated factors, SCFD1 and NBAS; several subunits of the TRAPP complex (TRAPPC3, TRAPPC8, TRAPPC11 and TRAPPC12) and the Conserved Oligomeric Golgi complex (COG1, COG2, COG3 and COG8); the small GTPase SAR1 and the Rab GTPase RAB1A, the Sec23-interacting protein Sec23IP and the exocyst complex component EXOC2, among others (**Table S1 and Fig. 2B**). Interestingly, factors belonging to small ARL and ARF GTPases, Golgins or additional SNAREs were not identified among the top hits. This is consistent with previous reports (Simpson et al., 2012; Wendler et al., 2010), highlighting the high degree of redundancy between paralog genes that could explain why the knockdown of these components did not alter robustly TAC transport."

2. Along with a list of proteins that appear to be critical for exocytic trafficking, there are also many proteins that appear to enhance secretion. There is no comment on this.

As shown in **Fig. 2B**, we identified many genes whose the knockdown stimulated TAC transport (genes identified from the upper quartile). In **Fig. 2D**, Gene Ontology (GO) term enrichment analysis of the 100 top ranked genes from the upper quartile, revealed that these genes encode mainly known components belonging to the following functional categories: "protein exit from the ER", "response to topologically incorrect protein", "ER to cytosol transport", "response to ER stress" and "post-translational modification". We mention in the revised manuscript that, "[…] This suggests that inhibition of the machinery required for the maintenance of ER homeostasis and quality control along the secretory pathway may favor transport and

secretion of cargo proteins that are most likely misfolded or incompletely processed." In addition, we indicate that (1) on a total of 63 genes selected for a secondary screen, 23 genes were selected from the upper quartile based on having unknown or poorly characterized function; (2) that for several of them such as FAM46A, TMEM167A, USP32 and C19orf33, we demonstrated that their knockdown promoted HRP secretion; and (3) that future analysis will help to dissect their role on secretory pathway function.

3. The description of TfR is that is is synthesized travels through the Golgi to the PM and then returns to the Golgi. This is surprising to me as my understanding is that TfR is the model protein for rapid recycling from the early endosome to the PM. The way the experiment is performed, isn't it possible that most of the protein detected is from newly synthesized protein that could be aberrantly trafficked in an ER/Golgi like compartment?

As mentioned previously (reviewer #1 - comment #3), we agree with the reviewer that the transport of TfR through the Golgi complex after endocytosis is a minor pathway, whereas TfR recycling towards the plasma membrane is the main route. To avoid confusion, in the "Results" section, it is now indicated:
"[...] To further test a role in membrane fusion, we assessed the impact of CCDC157 knockdown on the distribution of transport carriers derived from the endocytic pathway that fuse with Golgi membranes (Johannes and Popoff, 2008). We performed immunofluorescence microscopy experiments using an antibody targeting the transferrin receptor (TfR), which, although being mainly recycled to the plasma membrane after endocytosis (Huebers and Finch, 1987), is also retrieved to Golgi membranes (Snider and Rogers, 1985; Jin and Snider, 1993; Woods et al., 1986), and an antibody targeting EEA1, a marker of early endosomes, critical for endosomal trafficking (Barysch et al., 2009). We hypothesized that while TfR-containing transport carriers do not mainly fuse with Golgi membranes (Snider and Rogers, 1985; Woods et al., 1986), alteration of these fusion events could lead over time to the accumulation of transport carriers in the vicinity of the Golgi apparatus."
As shown in **Fig. 6E to 6H and S3**, results obtained demonstrated that in CCDC157-depleted cells, the distribution of both TfR and EEA1 were strongly altered, most of the signal being restricted to the perinuclear area in the form of enlarged TfR- and EEA1-positive vesicles, surrounded by Golgi membranes. In addition, we also showed that in CCDC157-depleted cells, Golgi membrane reassembly after BFA washout was strongly inhibited (**Fig. 6A and 6B**), and we included additional immunofluorescence experiments demonstrating that "the ER-Golgi intermediate compartment (ERGIC) was more dispersed thorough the cytoplasm upon CCDC157 knockdown compared to control cells (**Fig. 6C and 6D**). This compartment, which is a collection of tubulovesicular membrane clusters, allows delivering secretory cargo from ER-exit sites to the Golgi complex in a COPII vesicle-dependent manner via homotypic and heterotypic fusion events (Lorente-Rodríguez and Barlowe, 2011). Altogether, these results strongly strengthened CCDC157 as an important factor for the fusion of transport carriers with the Golgi complex".

Reviewer #3

Bassaganyas et al describe the characterisation of four genes they isolated from a genome wide CRISPRi screen on secretion in HeLa cells. They uncover several genes that either inhibit or increase the transport of their test protein TAC.

1. General comment: images are not quantified in this manuscript. This is important for the interpretation of the images and to allow others to reproduce and compare results (FAIR data). In my opinion the manuscript can only be published after quantification of all phenotypes reported.

As described previously (reviewer #1 - comment #1), we have now further quantified all experiments in order to ensure an unbiased and robust characterization of reported phenotypes after knockdown of hit genes.

- For the surface/total TAC expression ratio analyzed by flow cytometry in **Fig. 1G and 3C**, quantifications of

the enrichment of cells in the lower quartile compared to the upper quartile are shown in **Fig. 1H and 3D**, respectively (mean ± SEM, n=4).

- To assess the surface MHC-I expression, in addition of results obtained by immunofluorescence microscopy (**Fig. 3G**), we have performed flow cytometry experiments and results are shown in **Fig. 3E**. Quantification of the mean of fluorescence of surface MHC-I expression for these additional experiments is presented on **Fig. 3F** (mean ± SEM, n=4).
- For unbiased analysis of Golgi membrane surface area (**Fig. 4D**), we analyzed cells (465 control cells and 388 TTC17-depleted cells) with the CellProfiler Analyst (Broad Institute) (Dao et al, 2016). For each image analyzed, area of Golgi membranes, identified as object, was extracted using the module "MeasureObjectSizeShape".
- For unbiased analysis of Golgi membrane morphology (**Fig. 4C and 4E**), we trained the classifier of the CellProfiler Analyst (Broad Institute) (Dao et al, 2016) using cells randomly chosen from the whole experiment. These were classified as having a ring shape structure, fragmented or intact Golgi. The classifier of the CellProfiler Analyst was then used to define for all cells, Golgi membrane morphology on object level. At least 200 cells were analyzed for each independent experiment (mean ± SEM, n=3).
- All co-localization experiments performed by immunofluorescence microscopy are now quantified (See **Fig. 5I, 6D, 6F, 6H and 7D**). Confocal images from ~30 cells per condition were acquired and co-localization quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Bitplane AG.

2. The reporter system is not explained anywhere in the text. The reader is left figuring out how it works and how the authors can distinguish between total production and cell surface expression. Please add a sentence or two explaining the dual reporter system so that people from other fields might understand what was done. The demonstration of the reporter system with BFA, trypsin and the knock down of various genes is convincing.

In the "Results" section, the dual reporter system used is now described in more detail. We included the following sentence: "[...] A GFP signal allows monitoring of the total expression of the TAC protein, whereas its cell surface expression can be assessed by immunofluorescence microscopy and flow cytometry analysis using a phycoerythrin (PE)-conjugated antibody, which recognizes the extracellular domain of the TAC protein (**Fig. 1A to 1C and Fig. S1**)".

3. The experiments of figure S1 are not explained in the text and the legend is difficult to understand and has no motivation for the experiments. The authors should either remove the data or explain it more thoroughly.

Fig. S1 describes experiments characterizing optimal conditions to monitor TAC surface expression by flow cytometry using a phycoerythrin (PE)-conjugated antibody. As the dual reporter system is now described in more details in the "Results" section as mentioned above, it is now more relevant to refer to these results. The corresponding figure legend has been simplified to facilitate understanding.

4. In Figure 1E lower panel, the couloring is puzzling as the boundaries between color and gray are not perpendicular to the x-axis. If the lower or upper quartiles are selected, the lines should be straight, please explain. The authors should explicitly state that the definition of quartiles is set on the sgGal4 population and applied to other experimental conditions, if this is indeed the case. Readers are left guessing.

For these experiments shown now in **Fig. 1G**, a homogenous cell population was analyzed by flow cytometry, and as expected, the surface/total TAC expression ratio presented a Normal distribution for the control condition (sgGal4). The lower and upper quartiles were then selected on this control cell population and applied to other experimental conditions. This is now explicitly stated in the figure legends (**Fig. 1G and 3C**).

The strength of flow cytometry is that it allows a rapid analysis of large populations of cells with a typical throughput of several hundred cells per second, requiring a trade-off between the accuracy of signals detected and the flow cell speed. Given these reasons, when thresholds are selected as the lower and upper quartiles, slight deviations in comparison of parameters set up occur inevitably. Therefore, as observed on panels **Fig. 1G and 3C**, boundaries between color and gray are not exactly perpendicular to the x-axis, characterizing the Normal distribution of cell populations.

5. In figure 1F, the subplot #4 is unclear to me. What is the scale bar? Why do the read counts of the lower quartile correlate with the read counts of the upper quartile? Should they not anticorrelate?

In **Fig. 2A** (previously Fig. 1F) we have shown a schematic representation of the pooled CRISPRi screen workflow. The scatter plot in step 4 represents the sgRNA read counts derived from each cell fraction obtained after FACS sorting and Illumina DNA sequencing. The x- and y-axis indicate sgRNA read counts (log2) from the lower and upper quartile, respectively. For most of the sgRNAs, we expect that they do not affect TAC transport and no enrichment in one or the other fraction is detected. In this case, read counts of these sgRNAs correlate, defining a phenotype with a value of ~ 0. sgRNAs with a phenotype < 0 indicate an enrichment in the lower quartile compared to the upper quartile, and inversely, sgRNAs with a phenotype > 0 indicate an enrichment in the upper quartile compared to the lower quartile. The colored bar on the right of the scatter plot indicates the values of phenotype (fold change (log2)) obtained for each individual sgRNA. This explanation is now included in the Figure legend 2A.

6. Screen:
Table S1 is not included in the manuscript.

We apologize for this mistake. The **Table S1** is now associated with the revised manuscript.

7. How were 63 genes out of 200 selected for the secondary assay?

Given the efficiency of our dual fluorescent reporter and of our experimental procedure to identify genes involved in protein transport and secretion (see **Fig.1G, 2C and 2D**), we hypothesized that many genes enriched in our pooled genome-wide CRISPRi screen results might be new secretory factors. Thus, we restricted our selection of interesting hit candidates to 63 genes with unknown or poorly characterized function with a p-value < 0.01 and a log2 fold change (lfc) < -0.5 for genes enriched in the lower quartile, and a lfc > 0.3 for genes enriched in the upper quartile. The difference of lfc cutoff applied for the selection of candidate genes relied on the weaker phenotypes observed on TAC transport for genes enriched in the upper quartile compared to those enriched in the lower quartile. This is now explained in the corresponding figure legends and the "Material and methods" section.

8. Figure 2D, why is MHC expression downregulated in the siRNA experiments? There appears to be very little cytoplasmic fluorescence after silencing of the target genes. This should be quantified as this control is important. The lack of MHC expression could suggest that the genes have an effect on expression, although the FACS analysis does not show the same reduction in total signal.

In **Fig. 3G** (previously Fig. 2D), immunofluorescence experiments were performed on HeLa cells fixed with PFA without permeabilization, in order to detect exclusively the cell surface expression of MHC-I. The anti-MHC-I antibody used recognizes the extracellular domain of this transmembrane protein. In all conditions tested, although a faint signal corresponding to background can be visualized in the cytosol, a clear signal can be detected at the plasma membrane in control cells. However, in accordance with results showing that hit gene knockdown inhibited TAC transport and HRP secretion, hit gene knockdown also reduced MHC-I expression at the cell surface as demonstrated in **Fig. 3G**. To quantify these effects, we performed flow cytometry analysis on cells fixed with PFA without permeabilization. Flow cytometry profiles and

quantification of these additional experiments are shown on **Fig. 3E and 3F**, respectively, and they confirmed that hit gene knockdown reduced MHC-I expression at the cell surface.

9. Figure 3: The changes in morphology should be quantified in the images. Scoring cells containing fragmented Golgi or not is not good enough. The morphology and distribution of the ER and ERES look to be profoundly affected in the images in Figures 3B and S2B, yet the authors report no difference. Quantification of the structures would help resolve this.

As indicated above, for unbiased analysis of Golgi membrane morphology (now on **Fig. 4C and 4E**), we trained the classifier of the CellProfiler Analyst (Broad Institute) (Dao et al, 2016) using cells randomly chosen from the whole experiment. These were classified as having a ring shape structure, fragmented or intact Golgi. The classifier of the CellProfiler Analyst was then used to define for all cells, Golgi membrane morphology on an object level. At least 200 cells were analyzed for each independent experiment (mean ± SEM, n=3). Then, for unbiased analysis of Golgi membrane surface area (**Fig. 4D**), we analyzed cells (465 control cells and 388 TTC17-depleted cells) with the CellProfiler Analyst, but here, for each image analyzed, the area of Golgi membranes identified as object was extracted using the module "MeasureObjectSizeShape".
We agree with the reviewer that the distribution of the ER exit sites as shown in **Fig. S2A** is disturbed following hit gene knockdown. However, based on the overall data presented in **Fig. 4 to 7**, this phenotype is most likely the consequence of changes in Golgi membrane organization. Indeed, it is well known that ER exit sites are more concentrated in the perinuclear area in close proximity to Golgi membranes, and that Golgi stack dispersion upon genetic or chemical perturbations, concomitantly alter ER exit sites distribution. This tight structural relation between Golgi membranes and ER exit sites is demonstrated in SCFD1-depleted cells used as positive control on **Fig. 4A, 4B and S2A**. For these reasons, we think that the quantification of ER exit sites distribution would not provide additional information.
Finally, based on data presented in **Fig. S2B**, we believe that any attempt of quantification of ER organization upon hit gene knockdown would only show very slight or moderate effects and would also not provide additional information.
It is now mentioned in the "Results" section that: "[…] Along with the perturbed Golgi membrane architecture, the distributions of ER exit sites were also altered upon candidate gene knockdown (**Fig. S2A**), with no obvious changes in the distribution and morphology of the ER (**Fig. S2B**)."

10. The localisation of CCDC151 next to centrin-3 is not shown image 3I.

The localization of CCDC151 in close proximity of Centrin-3 is now shown on the new **Fig. 5E**.

11. It is clear from the images that TTC17 localises to more structures than just TGN46 positive structures. A colocalisation with GM130 would be interesting to show. In general the colocalisation should be quantified.

As indicated previously, for co-localization analyses shown on **Fig. 5I, 6D, 6F, 6H and 7D**, confocal images from ~30 cells per condition were acquired and quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Bitplane AG.
An important consideration for the interpretation of results presented in **Fig. 5D to 5H** is that immunofluorescence microscopy has been performed on cells permeabilized with digitonin, and then fixed with PFA. As stated in "Material and methods" section, this procedure allows the removal of soluble cytoplasmic pool of proteins, highlighting their potential association with intracellular compartments. For this reason, a residual signal is detected throughout the cytosol, but our results clearly revealed the presence of TTC17 and CCDC157 mainly on Golgi membranes, detected using an anti-TGN46 antibody. Quantification of these experiments are shown on **Fig. 5I**. However, as noticed by the reviewer, TTC17 on **Fig. 5F** seems also to be associated to additional structures. While the reviewer suggests performing co-

localization analysis with GM130, a marker of *cis* Golgi membranes, these experiments would probably not provide additional information compared to those performed using an anti-TGN46 antibody. Indeed, co-staining using anti-GM130 and anti-TGN46 antibodies has been performed (**Fig.7A**), and it clearly shows a very close proximity of these 2 markers of Golgi membranes.
TTC17 is a protein with a poorly characterized function, however a previous study suggested its involvement in actin organization (Bontems et al., PLoS One, 2014). Thus, in addition to its critical role on protein transport and secretion as shown in our study, we can not rule out the possibility that TTC17 can regulate other intracellular functions or that it is recruited on other structures, but these studies are beyond the scope of this paper.

12. Also the TfR should undergo image analysis. In the images, TfR vesicles appear smaller. A kinetic analysis of TfR uptake should be carried out to demonstrate that the endocytosis of TfR is normal, but that the recycling of the cargo is affected.

As suggested by the reviewer, we performed image analysis of TfR staining with the CellProfiler Analyst in order to assess the size of TfR-containing vesicles. Approximately 30 cells in each condition were subjected to analysis, and quantification showed high variability in these measurements without revealing significant differences between the conditions tested. For this reason, these results are not included in the revised manuscript.
As suggested, we also investigated TfR endocytosis and recycling upon TTC17 and CCDC157 knockdown. The procedure used is described in the "Material and methods" section. Briefly, HeLa cells were detached with 0.5 mM EDTA during 10 min, washed in serum-free medium and incubated at 4°C for 30 min in presence of 50 µg/ml transferrin (Tf) conjugated with Alexa Fluor 647. Cells were then incubated at 37°C and at different type points (0, 2, 5, 10, and 15 min), Tf internalization was stopped by placing cells on ice for 10 min. Cells were then fixed with 4% (w/v) PFA in PBS for 15 min at room temperature and analyzed by flow cytometry on a LSR Fortessa (BD Biosciences). For recycling assay, cells were incubated with 50 µg/ml Tf conjugated with Alexa Fluor 647 for 30 min at 37°C, washed and incubated at 37°C with 100 µg/ml unlabeled Tf for different time points (0, 5, 10, 15, and 30 min). Cells were then fixed with 4% PFA and analyzed by flow cytometry.
In line with our results showing the accumulation of TfR in the perinuclear area in the form of enlarged TfR-positive vesicles, surrounded by Golgi membranes in CCDC157-depleted cells, the uptake and the recycling of Tf, which are both tightly dependent of TfR trafficking, were inhibited upon CCDC157 knockdown. These new results are presented in the Supplemental **Fig. S4**.

13. The co-localization of TGN46 and GM130 needs to be quantified, the experiment is not interpretable otherwise.

To assess the co-localization of TGN46 and GM130, cells were incubated in presence of nocodazole resulting in Golgi stack dispersion. This procedure facilitates TGN46 and GM130 staining visualization for co-localization analysis. For the results shown on **Fig. 7D**, confocal images from ~30 cells per condition were acquired and co-localization quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Bitplane AG.

14. Other comment: when analysing 3 independent experiments, the SEM should be reported not the SD.

We have corrected this point and results of quantification are presented as mean ± SEM.

15. Further suggestions:
The author should consider doing EM to study the structure of the Golgi

As suggested, we performed Transmission Electron Microscopy in order to visualize the ultrastructure of Golgi membranes. Briefly, three days after transfection with individual specific siRNA, control HeLa cells and cells knockdown for TTC17 and CCDC157 were fixed with 2% PFA-2% glutaraldehyde solution in Sodium Cacodylate buffer, for 1 h at room temperature. Cells were carefully detached using a plastic cell scraper, collected into Eppendorf tubes and centrifuged to obtain the pellet. Cells were then post-fixed for 30 min in 1% OsO4 at room temperature, washed three times in distilled water and post-fixed for 1 h in 1% Uranyl Acetate. The pellets were dehydrated in graded steps of ethanol (50, 70, 90, 96 and 100%), two times with 100% of Propylene Oxide and embedded into Epon. Sections (60-nm thick) were cut on a Leica UC7 ultramicrotome and examined with a Fei Tecnai 12 BioTwin Spirit transmission electron microscope.
We observed that in a given cell with altered Golgi membranes, almost all individual Golgi stacks had altered ultrastructure characterized in TTC17-depleted cells by enlarged and swollen Golgi cisternae (**Fig. 4F,** blue arrowheads), and in CCDC157-depleted cells, most likely caused by accumulated and coalesced transport carriers in close proximity of Golgi membranes (**Fig. 4F,** red arrowheads)

16. The authors have identified several interacting genes. How did these genes score in the genome wide screen? The authors should do knock down of these genes and compare to the phenotypes of TCC17, CCDC151 and C10orf88. Epigenetic studies could be undertaken to reinforce that the interactors do indeed function together.

The Rab protein GDI2 and the clathrin heavy chain, which have been identified as interacting partners of C10orf88 using the BioPlex network (**Fig. 5C**), belong to the list of hit genes (p-value < 0.01 and a log2 fold change > 0.5) of the pooled genome-wide CRISPRi screen (**Table S1**). However, no interacting partners of TTC17 and CCDC151 score as a hit gene. We hypothesize that to phenocopy TTC17 and CCDC151 knockdown, it is most likely that simultaneous, but not individual knockdown of interacting partners is required.
In overall, results presented in **Fig. 5A to 5C** strongly strengthened a direct role of the newly identified factors on the secretory pathway function. This was also reinforced by their intracellular localization presented on **Fig. 5D to 5I**. Then, our results on **Fig. 6 and 7** identified CCDC157 as an important factor required for fusion events with Golgi membranes, and TTC17 as a critical component for maintaining the polarized arrangement of Golgi cisternae and post-translational modifications. The next obvious questions will be to decipher how CCDC157 and TTC17 are recruited to membranes and how their functions are coordinated with other structural factors and components of the tethering and fusion machinery, but these studies are beyond the scope of this paper.

August 5, 2019

RE: JCB Manuscript #201902028R

Dr. Julien Villeneuve
University of Cambridge
Metabolic Research Laboratories
Wellcome Trust-Medical Research Council Institute of Metabolic Science
Cambridge CB2 0QQ
United Kingdom

Dear Dr. Villeneuve:

Thank you for submitting your revised manuscript entitled "New factors for protein transport identified by a genome-wide CRISPRi screen in mammalian cells". Two of the original reviewers have assessed the revisions and both recommend publication. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

-- Please ensure that for all immunofluorescence images, magnifications also include a scale bar

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Sincerely,

Ira Mellman, Ph.D.
Editor
The Journal of Cell Biology

Rebecca Alvania, Ph.D.
Executive Editor
JCB

Reviewer #2 (Comments to the Authors (Required)):

I'm satisfied with the revision and responses to my concerns.

Reviewer #3 (Comments to the Authors (Required)):

The manuscript of Bassaganyas and et al is much improved compared to the first version and is a very agreeable read. I congratulate the authors on shedding so much clarity on their work. The description of the screening assay is now very clear, the logic of the follow up experiment are well explained and the data on TTC17 and CCD157 are compelling. I believe this publication will be a valuable resource for the community, strongly recommend the publication of the manuscript as is.

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Journal of Cell Biology
16th June 2019

Dear Editor

We thank the reviewers for their advice and assistance on our paper. We have revised the paper extensively and included new analysis and results to address the reviewer's concerns. As suggested, the revised manuscript is now formatted as a "Tool", allowing us to explain in more details key aspects of our study. We hope that our revised manuscript is suitable for publication in Journal of Cell Biology.

Best wishes,

Julien Villeneuve

In the following section we address the reviewers specific concerns.

Reviewer #1

This study uses a pooled sgRNA library and FACS-based CRISPRi approach to examine how systematic gene knockdown effects secretory trafficking in a human cell line (HeLa). As a reporter for secretory trafficking to the cell surface, the authors develop a ratiometric approach which compares expression of a fluorescent fusion protein-the single pass protein TAC-to the ability to detect surface delivery of that protein with an antibody. The authors validate a subset of these hits with an HRP secretion assay, and then focus on tow novel factors: CCDC157 and TTC17.

Major Criticisms:
1. The screen and supporting data appear generally sound, and the novel reporter and genome-wide CRISPRi represent some technical progress. However, neither of the new hits validated is followed up in significant mechanistic detail. It is not clear-in its current state-if there is major novelty or advance from this work. There have already been many screens along these lines and much is already known, as the authors note. Given these points, at the very least could the authors include a figure identifying which hits from their screen are novel secretory factors (not previously identified as such), and what fraction of previously known factors were identified in the present screen? In other words, try to put this screen in context with previous work to fill in the gaps of how close to (or far from) saturation such approaches are in detailing basic protein export.

We apologize for the lack of details provided in the original manuscript formatted as a "Brief report", on novelty and advances from our work. In the revised manuscript, formatted as a "Tool", an accurate description of results obtained as well as an extended discussion is included to address these concerns.

- First, a new **Table S2** provides the list of 62 interesting hit candidates with either unknown or poorly characterized function selected from the lower or upper quartiles of the pooled CRISPRi screen. They

represent potential new secretory factors that we tested using the arrayed secondary screen based on an HRP assay.

- Second, given the high degree of redundancy between paralog genes involved in protein trafficking and secretion, and taking into account the specificity of the transport machinery required following cargo proteins and cell type, it is not conceivable to identify the overall list of genes known to be involved along the secretory pathway, regardless of the approach used. In this context, an important added value of our study, as compared to other previously published screens, is that we developed a strategy allowing the efficient identification of genes involved in secretory pathway function and organization (See **Fig. 2B to 2D**), which can be easily adapted in different cell types, with various cargo proteins and environmental conditions. A detailed description of genes previously known to be involved in these processes, which we also identified with our approach is now included in the "Results" section. The following is mentioned: "[…] In the lower quartile, Gene Ontology (GO) term enrichment analysis of the 100 top ranked genes revealed that knockdown of genes encoding components of "Golgi vesicle transport" was the main functional category inhibiting TAC transport (**Fig. 2B and 2C**). Genes involved in "toxin transport", "antigen processing and presentation", "Golgi organization" were also highly enriched. More specifically, we identified as top hits most of the COPI subunits (COPA, COPB1, COPB2, COPG1, COPZ1 and ARCN1), as well as the COPII subunits Sec24A, Sec24B and Sec13. We also identified the SNARE Sec22B and the SNARE associated factors, SCFD1 and NBAS; several subunits of the TRAPP complex (TRAPPC3, TRAPPC8, TRAPPC11 and TRAPPC12) and the Conserved Oligomeric Golgi complex (COG1, COG2, COG3 and COG8); the small GTPase SAR1 and the Rab GTPase RAB1A, the Sec23-interacting protein Sec23IP and the exocyst complex component EXOC2, among others (**Table S1 and Fig. 2B**). Interestingly, factors belonging to small ARL and ARF GTPases, Golgins or additional SNAREs were not identified among the top hits. This is consistent with previous reports (Simpson et al., 2012; Wendler et al., 2010), highlighting the high degree of redundancy between paralog genes that could explain why the knockdown of these components did not alter robustly TAC transport."

- Third, the novelty and advances from our work are now clearly stated in the extended "Discussion" as described below:

"**Discussion**. Although a large number of gene products involved in protein transport were identified in recent decades (Novick et al., 1980; Braell et al., 1984; Bard et al., 2006; Wendler et al., 2010), the list is far from complete and several crucial issues are emerging. Indeed, in eukaryotic cells, evolution has given rise to an incredible diversity and complexity of cargo molecules, whose efficient and accurate transport along the secretory pathway is of paramount importance for organismal development, cell and tissue homeostasis, immunity, metabolic regulation, nerve transmission and healthy aging (Lee et al., 2004; Zanetti et al., 2012; Guo et al., 2014; Malhotra and Erlmann, 2015; Rothman and Orci, 1992; Söllner and Rothman, 1994). An increasing number of studies have demonstrated that cells are endowed with specific structural and functional machinery that can be tightly regulated to cope with the whole spectrum of cargo proteins (Cancino et al., 2014; Ma et al., 2018; Raote et al., 2018; Lopes-da-Silva et al., 2019). In this context, it is essential to develop versatile and high-throughput screening strategies to uncover how secretory pathways are adapted to and are regulated in response to intrinsic demands, environmental cues, and altered in diseases.

In this study, we implemented an unbiased pooled genome-wide CRISPRi screen allowing us to efficiently identify new components of the secretory pathway. Major benefits have already been associated with pooled CRISPR screens, establishing such approaches as powerful tools for systematically defining gene function in mammalian cells (Gilbert et al., 2014; Shalem et al., 2015; Canver et al., 2018). The efficiency of our strategy relies on the combination of key parameters that were optimized in our experimental approach. First, we utilized a complementary reporter system associated with cell selection based on the ratio of two fluorescent signals. This reporter system allowed monitoring surface/total TAC expression ratio variations and therefore differential FACS-based cell sorting, directly in relative to the transport of TAC protein to the cell surface. The strengths of this strategy were exemplified with Sec61A knockdown, where we could avoid following up undesirable phenotypes such as defective TAC protein

synthesis. Second, as the secretory pathway is of critical importance for cell homeostasis and viability, gene expression was systematically downregulated using the dCas9 fused to a KRAB effector domain for CRISPR interference, instead of the active Cas9 for CRISPR knockout (Kampmann, 2018; Shalem et al., 2015). The resulting transcriptional repression of target genes preserved minimal gene expression, preventing massive cell death after down regulation of key factors for protein and membrane trafficking. Third, the combination of the dual fluorescent reporter with the CRISPRi system allowed us to monitor TAC transport and perform FACS-based cell sorting at a relatively early time point (seven days after lentivirus transduction), which also would reduce excessive cell death after gene knockdown. Altogether, combining these technical parameters make our approach a noteworthy advance compared to previous RNA interference-based genome-wide screens, and resulted in a powerful platform allowing us to focus on the identification of genes involved in secretory pathway function and organization.

Our work highlighted several factors, whose knockdown inhibited or promoted the trafficking of both exogenous and endogenous cargo proteins. Various genes encoding proteins known to be involved either in calcium homeostasis, signaling pathways, or lysosomal activity, among others, (**Table S2**), strengthen the idea that there are functional links between these cellular processes and the secretory pathway. In addition, several proteins with unknown or poorly characterized function were also identified. In particular, TMEM167A, FAM46A and USP32, which promote TAC transport and HRP secretion upon knockdown, as well as C10orf88, WDR7, YPEL5, TMEM161, FAM162B, and GPR162, which inhibit TAC transport and HRP secretion upon knockdown, deserve greater attention. For example, USP32 is a membrane-bound ubiquitin protease localized in the Golgi apparatus and overexpressed in breast cancer (Akhavantabasi et al., 2010); and GPR162 an orphan GPCR. Interestingly, compelling evidence suggests that the activation of GPCRs on Golgi membranes is critical for protein and membrane trafficking but their identities remain elusive (Eichel and von Zastrow, 2018; Cancino et al., 2014; Diaz Anel et al., 2005). Altogether, our results provide a valuable resource to gain future important insights into fundamental mechanisms governing protein and membrane transport along the secretory pathway.

Our study further characterized two of the newly identified factors, TTC17 and CCDC157, as new actors of critical importance for the structure and function of Golgi membranes. Although additional studies will be required to fully decipher their roles, our results suggest that like other coiled-coil proteins (Wong and Munro, 2014; Cheung and Pfeffer, 2016), CCDC157 could be part of a tethering complex required for fusion events at the Golgi membranes. Furthermore, phenotypes observed in TTC17-depleted cells associated with TTC17 interacting partners involved in sphingomyelin metabolism (ENPP7 and NAAA, **Fig. 5A**) (Tsuboi et al., 2007; Duan et al., 2003), suggest that TTC17 could play a role in the production and distribution of particular lipids, a critical parameter for Golgi membrane organization (Campelo et al., 2017; van Galen et al., 2014). To decipher how CCDC157 and TTC17 are recruited to membranes and how their functions are coordinated with other structural factors and components of the tethering and fusion machinery will be of major interest.

In conclusion, while initial screen performed in yeast revealed the basic principles conserved across species (Novick et al., 1980), and that more recently, arrayed RNA interference screens revealed key players that function in metazoans (Bard et al., 2006; Saito et al., 2009; Von Blume et al., 2009), our pooled CRISPRi screen unveiled new components that further refine the steps along the secretory pathway. In addition, we anticipate that the adaptation of our screening platform to specific cargo proteins, different professional secretory cell types, and to particular intrinsic or environmental challenges, will open new and stimulating perspectives for a better understanding of the secretory pathway architecture in health and disease, for both conventional and unconventional secretion (Chiritoiu et al., 2019; Cruz-Garcia et al., 2018, Villeneuve et al., 2018; Zhang and Schekman, 2013)".

2. The characterization of knockdown as reported is superficial and some of it, such as scoring for Golgi fragmentation (Figure 3), appears subjective and difficult for this reviewer to accept from looking at the example micrographs shown.

We have now further quantified all experiments in order to ensure an unbiased and robust characterization of

reported phenotypes after knockdown of hit genes. In the corresponding "Material and methods" section and "Figures legends", the following is described:

- For the surface/total TAC expression ratio analyzed by flow cytometry in **Fig. 1G and 3C**, quantifications of the enrichment of cells in the lower quartile compared to the upper quartile are shown in **Fig. 1H and 3D**, respectively (mean ± SEM, n=4).
- To assess the surface MHC-I expression, in addition to results obtained by immunofluorescence microscopy (**Fig. 3G**), we have performed flow cytometry experiments and results are shown in **Fig. 3E**. Quantification of the mean of fluorescence of surface MHC-I expression for these additional experiments is presented on **Fig. 3F** (mean ± SEM, n=4).
- For unbiased analysis of Golgi membrane surface area (**Fig. 4D**), we analyzed cells (465 control cells and 388 TTC17-depleted cells) with the CellProfiler Analyst (Broad Institute) (Dao et al, 2016). For each image analyzed, area of Golgi membranes, identified as object, was extracted using the module "MeasureObjectSizeShape".
- For unbiased analysis of Golgi membrane morphology (**Fig. 4C and 4E**), we trained the classifier of the CellProfiler Analyst (Broad Institute) (Dao et al, 2016) using cells randomly chosen from the whole experiment. These were classified as having a ring shape structure, fragmented or intact Golgi. The classifier of the CellProfiler Analyst was then used to define for all cells, Golgi membrane morphology on object level. At least 200 cells were analyzed for each independent experiment (mean ± SEM, n=3).
- All co-localization experiments performed by immunofluorescence microscopy are now quantified (See **Fig. 5I, 6D, 6F, 6H and 7D**). Confocal images from ~30 cells per condition were acquired and co-localization quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Biptplane AG.

3. The authors find many RNA related genes (Figure 1H). Given their screening approach (surface vs total), loss of expression (through general mechanisms reducing mRNA) alone should not score as a hit. Could the authors explore or discuss this finding further? Is there some non-synthetic function of the RNA related hits identified?

The identification of false positives or genes with indirect effects is inherent to any screening approach. But as suggested by the reviewer, there is also the possibility that some of the RNA related genes that scored as hits have additional non-synthetic functions. We did not experimentally test this possibility but the following is now mentioned in the "Results" section: " […] Of note, genes involved in mRNA homeostasis and protein translation were also identified among hit genes. Most likely, their knockdown indirectly alters TAC transport, but a subset of them may also be part of transcriptional or translational programs required for the expression of components specifically involved in the secretory pathway. We can also not exclude the possibility that some of these genes may have additional non-synthetic functions. To test these possibilities will require further investigation". Yet, this class of genes did not represent the main functional category contributing to TAC transport, making our approach a noteworthy advance compared to previous RNA interference-based genome-wide screens (Bard et al., 2006; Simpson et al., 2012), where the list of hit genes after primary screening was to a large extent composed of genes with indirect effects.

4. The trafficking interpretation of TfR localization relative to Golgi (Figure 4) requires the assumption that TfR goes from early endosomes to the Golgi. The cited reference (Snider and Rogers 1985) did report such a route as stated, but it didn't claim this as the only route or even the major route. In fact, the kinetics of resialylation measured in that study was slow relative to receptor cycling. I think the literature overall supports transit through the Golgi as a minor pathway, accounting for the relatively slow kinetics of TfR resialylation, and transit bypassing Golgi (recycling endosomes) as major. If this is the case, I don't think the interpretation of steady state TfR localization is valid as claimed.

We agree with the reviewer that the transport of TfR through the Golgi complex after endocytosis is a minor pathway, whereas TfR recycling towards the plasma membrane is the main route. To avoid confusion, in the

"Results" section, it is now indicated: "[…] To further test a role in membrane fusion, we assessed the impact of CCDC157 knockdown on the distribution of transport carriers derived from the endocytic pathway that fuse with Golgi membranes (Johannes and Popoff, 2008). We performed immunofluorescence microscopy experiments using an antibody targeting the transferrin receptor (TfR), which, although being mainly recycled to the plasma membrane after endocytosis (Huebers and Finch, 1987), is also retrieved to Golgi membranes (Snider and Rogers, 1985; Jin and Snider, 1993; Woods et al., 1986), and an antibody targeting EEA1, a marker of early endosomes, critical for endosomal trafficking (Barysch et al., 2009). We hypothesized that while TfR-containing transport carriers do not mainly fuse with Golgi membranes (Snider and Rogers, 1985; Woods et al., 1986), alteration of these fusion events could lead over time to the accumulation of transport carriers in the vicinity of the Golgi apparatus."
As shown in **Fig. 6E to 6H and S3**, our results demonstrated that in CCDC157-depleted cells, the distribution of both TfR and EEA1 were strongly altered, most of the signal being restricted to the perinuclear area in the form of enlarged TfR- and EEA1-positive vesicles, surrounded by Golgi membranes. In addition, we also showed that in CCDC157-depleted cells, Golgi membrane reassembly after BFA washout was strongly inhibited (**Fig. 6A and 6B**), and we included additional immunofluorescence experiments demonstrating that the ER-Golgi intermediate compartment (ERGIC) was more dispersed thorough the cytoplasm upon CCDC157 knockdown compared to control cells (**Fig. 6C and 6D**). This compartment, which is a collection of tubulovesicular membrane clusters, allows delivering secretory cargo from ER-exit sites to the Golgi complex in a COPII vesicle-dependent manner via homotypic and heterotypic fusion events (Lorente-Rodríguez and Barlowe, 2011). Altogether, these results strongly support CCDC157 as being an important factor for the fusion of transport carriers with the Golgi complex.

Minor Criticisms:
5. In the materials and methods, the authors state that 10E6 cells were sorted for each quartile. Back of the envelope estimate suggests this would provide slightly less than 100-fold coverage of the pooled library. Field standard, as I understand it, is >500-fold (e.g., PMID: 28333914). I understand that sorting the >50 million cells is probably not practical using adherent cells. However, I am slightly concerned that the general reader might not be aware of this key point, or grasp the cost-benefit analysis that the authors decided upon when they chose a pooled, genome-wide, library approach with lower coverage. I think it would be useful to discuss this in the materials and methods section.

Today, in the vast majority of studies using pooled genome-wide CRISPR screening, the phenotypic selection is based on cell viability or proliferation, allowing the identification of genes essential for viability or genes that confer resistance or sensitivity to drugs, toxins and pathogen infections. For such applications, it is relatively straightforward to obtain a high coverage at > 500 cells per sgRNA during the screening selection. However for FACS-based CRISPR screens in which the phenotypic selection is based on the alteration of protein expression detected by fluorescence, a coverage of ~ 100 cells per sgRNA is generally accepted during FACS sorting. Concretely, to collect 10 millions of cells in each fraction by FACS, as performed in our study, ~ 400 cells per second must be sorted efficiently and specifically for each quartile during 8 hours. For this reason, to collect 50 millions of cells, the sorting speed must be increased, but this will inevitably reduce the accuracy of cell selection. The following references included in the "Material and methods" section, correspond to studies performed by leading laboratories using FACS-based CRISPR screens where a coverage of ~ 100 cells per sgRNA, or even less, was used: (Menzies S.A. et al., The sterol-responsive RNF145 E3 ubiquitin ligase mediates the degradation of HMG-CoA reductase together with gp78 and Hrd1, 2018, Elife), (Park R.J. et al, A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors, 2017, Nat Genet), (Parnas O. et al., A genome-wide CRISPR screen in primary immune cells to dissect regulatory networks, 2015, Cell). .

6. The flow cytometry plots are pixelated and hard to read. a minimum, the text within the plots should be re-written to improve legibility and size (such as PE-1.36 in Figure 1D(i)). We have found that exporting FlowJo figures as .svg can help with this issue.

The revised manuscript includes figures with higher resolution.

7. For figure 1G, not clear that red/blue vs black dots on volcano plot represent. Presumably, the highlighted categories from 1H. A quick line in Figure legend would help.

In the legend of **Fig. 2B**, it is now mentioned: "[…] Each gene targeted by the library of sgRNA is indicated with a black dot. Genes included in the 100 top ranked genes and belonging to functional categories of interest (as highlighted in **Fig. 2C and 2D**) are indicated with red dots for genes inhibiting TAC transport and with blue dots for genes stimulating TAC transport".

8. No scale bars for any micrographs after Figure 1.

Scale bars have been added on each micrograph.

9. In introduction, "gaining continuous complexity" is a little hard for this reviewer to parse.

In the introduction, we changed the sentence by: "Recent demonstrations indicate that cell compartments establish cross-regulatory mechanisms with numerous membrane contact sites (Wu et al., 2018), are endowed of tightly regulated dynamics (Valm et al., 2017), and stand at the crossroad of signaling pathways where inputs and outputs are integrated and coordinated (Luini and Parashuraman, 2016). Thus, protein transport and secretion processes are clearly more complex than previously thought".

10. In introduction, it appears this sentence may be missing citations, " [...] high throughput or flow cytometry systems."

Corresponding references have been added.

11. Following the pooled siRNA approach for Figure 2A, the authors' write "specific siRNA" in description of subsequent experiments. It was not clear if these experiments were still using the pooled siRNA from Dharmacon.

For experiments involving siRNA, it is now specified in the text if experiments have been performed using "specific smart pool siRNAs" or "specific individual siRNA", directed against targeted genes.

12. It may be that the word 'knockdown' would be more appropriate than 'downregulation' in this sentence, "Flow cytometry analysis revealed that downregulation of the selected genes..."

The word "downregulation" has been changed by "knockdown" as suggested.

13. As the authors state in their introduction, aspects of the machinery which mediate protein secretion have been mapped in yeast, biochemical studies, and with RNAi. It would be useful to see a figure in which the CRISPRi hits (passing a p-value and FC cutoffs) are novel.

As indicated in the comment #1, a new **Table S2** with 62 hit genes selected from the lower or upper quartiles of the pooled CRISPRi screen and tested in the arrayed secondary screen is included in the revised manuscript. It is now explained in the corresponding "Figure legends" and the "Material and methods" section that: "As we intended to validate hit genes with a secondary screen, interesting hit candidates with unknown or poorly characterized function were selected as potential new secretory factors among those with a p-value < 0.01 and a log2 fold change (lfc) < -0.5 for genes enriched in the lower quartile, and a lfc > 0.3 for genes enriched in the upper quartile. The difference of lfc cutoff applied for the selection of candidate

genes relied on the weaker phenotypes observed on TAC transport for genes enriched in the upper quartile compared to those enriched in the lower quartile".

Reviewer #2

This is a straightforward study that uses CRISPR screening to identify proteins involved in exocytosis. The strategy is clever and based on a cell line expressing a TAC-GFP fusion protein that allows them to assess PM versus intracellular expression as wells the repressor CRISPR system that allows repression of gene expression. They identify several novel proteins and focus very superficially on two of them TTC17 and CCDC157 and show that each are GOLGI proteins that have distinct functions. The analysis is interesting but not very intensive, but since I judge this mainly as a methods paper, this is probably sufficient to make their point. I have a few minor comments.

1. While there is a long list of proteins that are identified, it seems a bit surprising that known regulators of GOLGI morphology and of exocytic transport were not identified. The authors should discuss this.

As discussed above (reviewer #1 - comment #1), given the high degree of redundancy between paralog genes involved in protein trafficking and secretion, and taking into account the specificity of the transport machinery required following cargo proteins and cell type, it is not conceivable to identify the overall list of genes known to be involved along the secretory pathway, regardless the approach used. In this context, an important added value of our study, as compared to other previously published screens, is that we developed a strategy allowing the efficient identification of genes involved in secretory pathway function and organization (See **Fig. 2B to 2D**), which can be easily adapted in different cell types, with various cargo proteins and environmental conditions. A detailed description of genes previously known to be involved in these processes, which we also identified with our approach is now included in the "Results" section. It is mentioned: "[...] In the lower quartile, Gene Ontology (GO) term enrichment analysis of the 100 top ranked genes revealed that knockdown of genes encoding components of "Golgi vesicle transport" was the main functional category inhibiting TAC transport (**Fig. 2B and 2C**). Genes involved in "toxin transport", "antigen processing and presentation", "Golgi organization" were also highly enriched. More specifically, we identified as top hits most of the COPI subunits (COPA, COPB1, COPB2, COPG1, COPZ1 and ARCN1), as well as the COPII subunits Sec24A, Sec24B and Sec13. We also identified the SNARE Sec22B and the SNARE associated factors, SCFD1 and NBAS; several subunits of the TRAPP complex (TRAPPC3, TRAPPC8, TRAPPC11 and TRAPPC12) and the Conserved Oligomeric Golgi complex (COG1, COG2, COG3 and COG8); the small GTPase SAR1 and the Rab GTPase RAB1A, the Sec23-interacting protein Sec23IP and the exocyst complex component EXOC2, among others (**Table S1 and Fig. 2B**). Interestingly, factors belonging to small ARL and ARF GTPases, Golgins or additional SNAREs were not identified among the top hits. This is consistent with previous reports (Simpson et al., 2012; Wendler et al., 2010), highlighting the high degree of redundancy between paralog genes that could explain why the knockdown of these components did not alter robustly TAC transport."

2. Along with a list of proteins that appear to be critical for exocytic trafficking, there are also many proteins that appear to enhance secretion. There is no comment on this.

As shown in **Fig. 2B**, we identified many genes whose the knockdown stimulated TAC transport (genes identified from the upper quartile). In **Fig. 2D**, Gene Ontology (GO) term enrichment analysis of the 100 top ranked genes from the upper quartile, revealed that these genes encode mainly known components belonging to the following functional categories: "protein exit from the ER", "response to topologically incorrect protein", "ER to cytosol transport", "response to ER stress" and "post-translational modification". We mention in the revised manuscript that, "[…] This suggests that inhibition of the machinery required for the maintenance of ER homeostasis and quality control along the secretory pathway may favor transport and

secretion of cargo proteins that are most likely misfolded or incompletely processed." In addition, we indicate that (1) on a total of 63 genes selected for a secondary screen, 23 genes were selected from the upper quartile based on having unknown or poorly characterized function; (2) that for several of them such as FAM46A, TMEM167A, USP32 and C19orf33, we demonstrated that their knockdown promoted HRP secretion; and (3) that future analysis will help to dissect their role on secretory pathway function.

3. The description of TfR is that is is synthesized travels through the Golgi to the PM and then returns to the Golgi. This is surprising to me as my understanding is that TfR is the model protein for rapid recycling from the early endosome to the PM. The way the experiment is performed, isn't it possible that most of the protein detected is from newly synthesized protein that could be aberrantly trafficked in an ER/Golgi like compartment?

As mentioned previously (reviewer #1 - comment #3), we agree with the reviewer that the transport of TfR through the Golgi complex after endocytosis is a minor pathway, whereas TfR recycling towards the plasma membrane is the main route. To avoid confusion, in the "Results" section, it is now indicated:
"[...] To further test a role in membrane fusion, we assessed the impact of CCDC157 knockdown on the distribution of transport carriers derived from the endocytic pathway that fuse with Golgi membranes (Johannes and Popoff, 2008). We performed immunofluorescence microscopy experiments using an antibody targeting the transferrin receptor (TfR), which, although being mainly recycled to the plasma membrane after endocytosis (Huebers and Finch, 1987), is also retrieved to Golgi membranes (Snider and Rogers, 1985; Jin and Snider, 1993; Woods et al., 1986), and an antibody targeting EEA1, a marker of early endosomes, critical for endosomal trafficking (Barysch et al., 2009). We hypothesized that while TfR-containing transport carriers do not mainly fuse with Golgi membranes (Snider and Rogers, 1985; Woods et al., 1986), alteration of these fusion events could lead over time to the accumulation of transport carriers in the vicinity of the Golgi apparatus."
As shown in **Fig. 6E to 6H and S3**, results obtained demonstrated that in CCDC157-depleted cells, the distribution of both TfR and EEA1 were strongly altered, most of the signal being restricted to the perinuclear area in the form of enlarged TfR- and EEA1-positive vesicles, surrounded by Golgi membranes. In addition, we also showed that in CCDC157-depleted cells, Golgi membrane reassembly after BFA washout was strongly inhibited (**Fig. 6A and 6B**), and we included additional immunofluorescence experiments demonstrating that "the ER-Golgi intermediate compartment (ERGIC) was more dispersed thorough the cytoplasm upon CCDC157 knockdown compared to control cells (**Fig. 6C and 6D**). This compartment, which is a collection of tubulovesicular membrane clusters, allows delivering secretory cargo from ER-exit sites to the Golgi complex in a COPII vesicle-dependent manner via homotypic and heterotypic fusion events (Lorente-Rodríguez and Barlowe, 2011). Altogether, these results strongly strengthened CCDC157 as an important factor for the fusion of transport carriers with the Golgi complex".

Reviewer #3

Bassaganyas et al describe the characterisation of four genes they isolated from a genome wide CRISPRi screen on secretion in HeLa cells. They uncover several genes that either inhibit or increase the transport of their test protein TAC.

1. General comment: images are not quantified in this manuscript. This is important for the interpretation of the images and to allow others to reproduce and compare results (FAIR data). In my opinion the manuscript can only be published after quantification of all phenotypes reported.

As described previously (reviewer #1 - comment #1), we have now further quantified all experiments in order to ensure an unbiased and robust characterization of reported phenotypes after knockdown of hit genes.

- For the surface/total TAC expression ratio analyzed by flow cytometry in **Fig. 1G and 3C**, quantifications of

the enrichment of cells in the lower quartile compared to the upper quartile are shown in **Fig. 1H and 3D**, respectively (mean ± SEM, n=4).

- To assess the surface MHC-I expression, in addition of results obtained by immunofluorescence microscopy (**Fig. 3G**), we have performed flow cytometry experiments and results are shown in **Fig. 3E**. Quantification of the mean of fluorescence of surface MHC-I expression for these additional experiments is presented on **Fig. 3F** (mean ± SEM, n=4).
- For unbiased analysis of Golgi membrane surface area (**Fig. 4D**), we analyzed cells (465 control cells and 388 TTC17-depleted cells) with the CellProfiler Analyst (Broad Institute) (Dao et al, 2016). For each image analyzed, area of Golgi membranes, identified as object, was extracted using the module "MeasureObjectSizeShape".
- For unbiased analysis of Golgi membrane morphology (**Fig. 4C and 4E**), we trained the classifier of the CellProfiler Analyst (Broad Institute) (Dao et al, 2016) using cells randomly chosen from the whole experiment. These were classified as having a ring shape structure, fragmented or intact Golgi. The classifier of the CellProfiler Analyst was then used to define for all cells, Golgi membrane morphology on object level. At least 200 cells were analyzed for each independent experiment (mean ± SEM, n=3).
- All co-localization experiments performed by immunofluorescence microscopy are now quantified (See **Fig. 5I, 6D, 6F, 6H and 7D**). Confocal images from ~30 cells per condition were acquired and co-localization quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Bitplane AG.

2. The reporter system is not explained anywhere in the text. The reader is left figuring out how it works and how the authors can distinguish between total production and cell surface expression. Please add a sentence or two explaining the dual reporter system so that people from other fields might understand what was done. The demonstration of the reporter system with BFA, trypsin and the knock down of various genes is convincing.

In the "Results" section, the dual reporter system used is now described in more detail. We included the following sentence: "[...] A GFP signal allows monitoring of the total expression of the TAC protein, whereas its cell surface expression can be assessed by immunofluorescence microscopy and flow cytometry analysis using a phycoerythrin (PE)-conjugated antibody, which recognizes the extracellular domain of the TAC protein (**Fig. 1A to 1C and Fig. S1**)".

3. The experiments of figure S1 are not explained in the text and the legend is difficult to understand and has no motivation for the experiments. The authors should either remove the data or explain it more thoroughly.

Fig. S1 describes experiments characterizing optimal conditions to monitor TAC surface expression by flow cytometry using a phycoerythrin (PE)-conjugated antibody. As the dual reporter system is now described in more details in the "Results" section as mentioned above, it is now more relevant to refer to these results. The corresponding figure legend has been simplified to facilitate understanding.

4. In Figure 1E lower panel, the couloring is puzzling as the boundaries between color and gray are not perpendicular to the x-axis. If the lower or upper quartiles are selected, the lines should be straight, please explain. The authors should explicitly state that the definition of quartiles is set on the sgGal4 population and applied to other experimental conditions, if this is indeed the case. Readers are left guessing.

For these experiments shown now in **Fig. 1G**, a homogenous cell population was analyzed by flow cytometry, and as expected, the surface/total TAC expression ratio presented a Normal distribution for the control condition (sgGal4). The lower and upper quartiles were then selected on this control cell population and applied to other experimental conditions. This is now explicitly stated in the figure legends (**Fig. 1G and 3C**).

The strength of flow cytometry is that it allows a rapid analysis of large populations of cells with a typical throughput of several hundred cells per second, requiring a trade-off between the accuracy of signals detected and the flow cell speed. Given these reasons, when thresholds are selected as the lower and upper quartiles, slight deviations in comparison of parameters set up occur inevitably. Therefore, as observed on panels **Fig. 1G and 3C**, boundaries between color and gray are not exactly perpendicular to the x-axis, characterizing the Normal distribution of cell populations.

5. In figure 1F, the subplot #4 is unclear to me. What is the scale bar? Why do the read counts of the lower quartile correlate with the read counts of the upper quartile? Should they not anticorrelate?

In **Fig. 2A** (previously Fig. 1F) we have shown a schematic representation of the pooled CRISPRi screen workflow. The scatter plot in step 4 represents the sgRNA read counts derived from each cell fraction obtained after FACS sorting and Illumina DNA sequencing. The x- and y-axis indicate sgRNA read counts (log2) from the lower and upper quartile, respectively. For most of the sgRNAs, we expect that they do not affect TAC transport and no enrichment in one or the other fraction is detected. In this case, read counts of these sgRNAs correlate, defining a phenotype with a value of ~ 0. sgRNAs with a phenotype < 0 indicate an enrichment in the lower quartile compared to the upper quartile, and inversely, sgRNAs with a phenotype > 0 indicate an enrichment in the upper quartile compared to the lower quartile. The colored bar on the right of the scatter plot indicates the values of phenotype (fold change (log2)) obtained for each individual sgRNA. This explanation is now included in the Figure legend 2A.

6. Screen:
Table S1 is not included in the manuscript.

We apologize for this mistake. The **Table S1** is now associated with the revised manuscript.

7. How were 63 genes out of 200 selected for the secondary assay?

Given the efficiency of our dual fluorescent reporter and of our experimental procedure to identify genes involved in protein transport and secretion (see **Fig.1G, 2C and 2D**), we hypothesized that many genes enriched in our pooled genome-wide CRISPRi screen results might be new secretory factors. Thus, we restricted our selection of interesting hit candidates to 63 genes with unknown or poorly characterized function with a p-value < 0.01 and a log2 fold change (lfc) < -0.5 for genes enriched in the lower quartile, and a lfc > 0.3 for genes enriched in the upper quartile. The difference of lfc cutoff applied for the selection of candidate genes relied on the weaker phenotypes observed on TAC transport for genes enriched in the upper quartile compared to those enriched in the lower quartile. This is now explained in the corresponding figure legends and the "Material and methods" section.

8. Figure 2D, why is MHC expression downregulated in the siRNA experiments? There appears to be very little cytoplasmic fluorescence after silencing of the target genes. This should be quantified as this control is important. The lack of MHC expression could suggest that the genes have an effect on expression, although the FACS analysis does not show the same reduction in total signal.

In **Fig. 3G** (previously Fig. 2D), immunofluorescence experiments were performed on HeLa cells fixed with PFA without permeabilization, in order to detect exclusively the cell surface expression of MHC-I. The anti-MHC-I antibody used recognizes the extracellular domain of this transmembrane protein. In all conditions tested, although a faint signal corresponding to background can be visualized in the cytosol, a clear signal can be detected at the plasma membrane in control cells. However, in accordance with results showing that hit gene knockdown inhibited TAC transport and HRP secretion, hit gene knockdown also reduced MHC-I expression at the cell surface as demonstrated in **Fig. 3G**. To quantify these effects, we performed flow cytometry analysis on cells fixed with PFA without permeabilization. Flow cytometry profiles and

quantification of these additional experiments are shown on **Fig. 3E and 3F**, respectively, and they confirmed that hit gene knockdown reduced MHC-I expression at the cell surface.

9. Figure 3: The changes in morphology should be quantified in the images. Scoring cells containing fragmented Golgi or not is not good enough. The morphology and distribution of the ER and ERES look to be profoundly affected in the images in Figures 3B and S2B, yet the authors report no difference. Quantification of the structures would help resolve this.

As indicated above, for unbiased analysis of Golgi membrane morphology (now on **Fig. 4C and 4E**), we trained the classifier of the CellProfiler Analyst (Broad Institute) (Dao et al, 2016) using cells randomly chosen from the whole experiment. These were classified as having a ring shape structure, fragmented or intact Golgi. The classifier of the CellProfiler Analyst was then used to define for all cells, Golgi membrane morphology on an object level. At least 200 cells were analyzed for each independent experiment (mean ± SEM, n=3). Then, for unbiased analysis of Golgi membrane surface area (**Fig. 4D**), we analyzed cells (465 control cells and 388 TTC17-depleted cells) with the CellProfiler Analyst, but here, for each image analyzed, the area of Golgi membranes identified as object was extracted using the module "MeasureObjectSizeShape".
We agree with the reviewer that the distribution of the ER exit sites as shown in **Fig. S2A** is disturbed following hit gene knockdown. However, based on the overall data presented in **Fig. 4 to 7**, this phenotype is most likely the consequence of changes in Golgi membrane organization. Indeed, it is well known that ER exit sites are more concentrated in the perinuclear area in close proximity to Golgi membranes, and that Golgi stack dispersion upon genetic or chemical perturbations, concomitantly alter ER exit sites distribution. This tight structural relation between Golgi membranes and ER exit sites is demonstrated in SCFD1-depleted cells used as positive control on **Fig. 4A, 4B and S2A**. For these reasons, we think that the quantification of ER exit sites distribution would not provide additional information.
Finally, based on data presented in **Fig. S2B**, we believe that any attempt of quantification of ER organization upon hit gene knockdown would only show very slight or moderate effects and would also not provide additional information.
It is now mentioned in the "Results" section that: "[…] Along with the perturbed Golgi membrane architecture, the distributions of ER exit sites were also altered upon candidate gene knockdown (**Fig. S2A**), with no obvious changes in the distribution and morphology of the ER (**Fig. S2B**)."

10. The localisation of CCDC151 next to centrin-3 is not shown image 3I.

The localization of CCDC151 in close proximity of Centrin-3 is now shown on the new **Fig. 5E**.

11. It is clear from the images that TTC17 localises to more structures than just TGN46 positive structures. A colocalisation with GM130 would be interesting to show. In general the colocalisation should be quantified.

As indicated previously, for co-localization analyses shown on **Fig. 5I, 6D, 6F, 6H and 7D**, confocal images from ~30 cells per condition were acquired and quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Bitplane AG.
An important consideration for the interpretation of results presented in **Fig. 5D to 5H** is that immunofluorescence microscopy has been performed on cells permeabilized with digitonin, and then fixed with PFA. As stated in "Material and methods" section, this procedure allows the removal of soluble cytoplasmic pool of proteins, highlighting their potential association with intracellular compartments. For this reason, a residual signal is detected throughout the cytosol, but our results clearly revealed the presence of TTC17 and CCDC157 mainly on Golgi membranes, detected using an anti-TGN46 antibody. Quantification of these experiments are shown on **Fig. 5I**. However, as noticed by the reviewer, TTC17 on **Fig. 5F** seems also to be associated to additional structures. While the reviewer suggests performing co-

localization analysis with GM130, a marker of *cis* Golgi membranes, these experiments would probably not provide additional information compared to those performed using an anti-TGN46 antibody. Indeed, co-staining using anti-GM130 and anti-TGN46 antibodies has been performed (**Fig.7A**), and it clearly shows a very close proximity of these 2 markers of Golgi membranes.
TTC17 is a protein with a poorly characterized function, however a previous study suggested its involvement in actin organization (Bontems et al., PLoS One, 2014). Thus, in addition to its critical role on protein transport and secretion as shown in our study, we can not rule out the possibility that TTC17 can regulate other intracellular functions or that it is recruited on other structures, but these studies are beyond the scope of this paper.

12. Also the TfR should undergo image analysis. In the images, TfR vesicles appear smaller. A kinetic analysis of TfR uptake should be carried out to demonstrate that the endocytosis of TfR is normal, but that the recycling of the cargo is affected.

As suggested by the reviewer, we performed image analysis of TfR staining with the CellProfiler Analyst in order to assess the size of TfR-containing vesicles. Approximately 30 cells in each condition were subjected to analysis, and quantification showed high variability in these measurements without revealing significant differences between the conditions tested. For this reason, these results are not included in the revised manuscript.
As suggested, we also investigated TfR endocytosis and recycling upon TTC17 and CCDC157 knockdown. The procedure used is described in the "Material and methods" section. Briefly, HeLa cells were detached with 0.5 mM EDTA during 10 min, washed in serum-free medium and incubated at 4°C for 30 min in presence of 50 µg/ml transferrin (Tf) conjugated with Alexa Fluor 647. Cells were then incubated at 37°C and at different type points (0, 2, 5, 10, and 15 min), Tf internalization was stopped by placing cells on ice for 10 min. Cells were then fixed with 4% (w/v) PFA in PBS for 15 min at room temperature and analyzed by flow cytometry on a LSR Fortessa (BD Biosciences). For recycling assay, cells were incubated with 50 µg/ml Tf conjugated with Alexa Fluor 647 for 30 min at 37°C, washed and incubated at 37°C with 100 µg/ml unlabeled Tf for different time points (0, 5, 10, 15, and 30 min). Cells were then fixed with 4% PFA and analyzed by flow cytometry.
In line with our results showing the accumulation of TfR in the perinuclear area in the form of enlarged TfR-positive vesicles, surrounded by Golgi membranes in CCDC157-depleted cells, the uptake and the recycling of Tf, which are both tightly dependent of TfR trafficking, were inhibited upon CCDC157 knockdown. These new results are presented in the Supplemental **Fig. S4**.

13. The co-localization of TGN46 and GM130 needs to be quantified, the experiment is not interpretable otherwise.

To assess the co-localization of TGN46 and GM130, cells were incubated in presence of nocodazole resulting in Golgi stack dispersion. This procedure facilitates TGN46 and GM130 staining visualization for co-localization analysis. For the results shown on **Fig. 7D**, confocal images from ~30 cells per condition were acquired and co-localization quantifications were performed using Coste's method of thresholding with object Pearson's analysis using Imaris 8.2.0 by Bitplane AG.

14. Other comment: when analysing 3 independent experiments, the SEM should be reported not the SD.

We have corrected this point and results of quantification are presented as mean ± SEM.

15. Further suggestions:
The author should consider doing EM to study the structure of the Golgi

As suggested, we performed Transmission Electron Microscopy in order to visualize the ultrastructure of Golgi membranes. Briefly, three days after transfection with individual specific siRNA, control HeLa cells and cells knockdown for TTC17 and CCDC157 were fixed with 2% PFA-2% glutaraldehyde solution in Sodium Cacodylate buffer, for 1 h at room temperature. Cells were carefully detached using a plastic cell scraper, collected into Eppendorf tubes and centrifuged to obtain the pellet. Cells were then post-fixed for 30 min in 1% OsO4 at room temperature, washed three times in distilled water and post-fixed for 1 h in 1% Uranyl Acetate. The pellets were dehydrated in graded steps of ethanol (50, 70, 90, 96 and 100%), two times with 100% of Propylene Oxide and embedded into Epon. Sections (60-nm thick) were cut on a Leica UC7 ultramicrotome and examined with a Fei Tecnai 12 BioTwin Spirit transmission electron microscope.
We observed that in a given cell with altered Golgi membranes, almost all individual Golgi stacks had altered ultrastructure characterized in TTC17-depleted cells by enlarged and swollen Golgi cisternae (**Fig. 4F,** blue arrowheads), and in CCDC157-depleted cells, most likely caused by accumulated and coalesced transport carriers in close proximity of Golgi membranes (**Fig. 4F,** red arrowheads)

16. The authors have identified several interacting genes. How did these genes score in the genome wide screen? The authors should do knock down of these genes and compare to the phenotypes of TCC17, CCDC151 and C10orf88. Epigenetic studies could be undertaken to reinforce that the interactors do indeed function together.

The Rab protein GDI2 and the clathrin heavy chain, which have been identified as interacting partners of C10orf88 using the BioPlex network (**Fig. 5C**), belong to the list of hit genes (p-value < 0.01 and a log2 fold change > 0.5) of the pooled genome-wide CRISPRi screen (**Table S1**). However, no interacting partners of TTC17 and CCDC151 score as a hit gene. We hypothesize that to phenocopy TTC17 and CCDC151 knockdown, it is most likely that simultaneous, but not individual knockdown of interacting partners is required.
In overall, results presented in **Fig. 5A to 5C** strongly strengthened a direct role of the newly identified factors on the secretory pathway function. This was also reinforced by their intracellular localization presented on **Fig. 5D to 5I**. Then, our results on **Fig. 6 and 7** identified CCDC157 as an important factor required for fusion events with Golgi membranes, and TTC17 as a critical component for maintaining the polarized arrangement of Golgi cisternae and post-translational modifications. The next obvious questions will be to decipher how CCDC157 and TTC17 are recruited to membranes and how their functions are coordinated with other structural factors and components of the tethering and fusion machinery, but these studies are beyond the scope of this paper.