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A POSITIVE SIGNAL PREVENTS SECRETORY MEMBRANE CARGO FROM RECYCLING BETWEEN THE GOLGI AND THE ER

Matteo Fossati, Sara Francesca Colombo and Nica Borgese

Corresponding author: Nica Borgese, Consiglio Nazionale delle Ricerche

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Editor: Andrea Leibfried

1st Editorial Decision 21 March 2014

Thank you for submitting your manuscript entitled 'A POSITIVE SIGNAL PREVENTS SECRETORY MEMBRANE CARGO FROM RECYCLING BETWEEN THE GOLGI AND THE ER'. I have now received reports from all referees, which are enclosed below.

As you will see, the referees find your study interesting. They propose some amendments and additional experiments to better support your data and claims and to improve the clarity of the paper, all of which are clearly outlined in the reports. Referee #3 also makes good suggestions on how to broaden your conclusions and proposes to add more mechanistic insight into how escape from the retrograde flux is mediated. We would appreciate if you try to address these points.
Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees. Please do not hesitate to contact me in case of further questions.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

In this manuscript, the authors have addressed an interesting issue, yet poorly investigated, e.g. how newly synthesized secretory cargos can escape the retrograde membrane flow between Golgi and the endoplasmic reticulum (ER). By comparing the behavior of several transport markers, they provide evidence that positive signal(s) is (are) required to prevent retrograde trafficking and to favor efficient intra-Golgi and post-Golgi transport.
The data, mainly based on live cell imaging approaches, are generally convincing. The weak point is that the nature of this (these) signal(s) remain(s) elusive, although several potential candidates have been tested. Nevertheless, this paper sheds new light on the transport mechanisms operating within the early secretory pathway.

Main comments:

1. Most of the described experiments are performed after a 20{degree sign}C block. If I understand the rationale for using this condition, it is nevertheless likely that such a block not only impairs post-Golgi transport, but also affects intra-Golgi transport and possibly pre-Golgi transport. In Fig. 2D, the authors verified that FP22 recycling to the ER occurs both at 20{degree sign}C and 37{degree sign}C. However, this experiment is not quantified. The arrival of FP-22 at the plasma membrane at 37{degree sign}C (Fig. E1) should be quantified as well (as percent of intracellular signal over time). It is also important to document that VSV-G recycling does not occur at 37{degree sign}C as shown at 20{degree sign}C.
2. The authors interpret the fast recovery phase following FRAP as a diffusion of FP-22 from the bulk ER to the juxtanuclear ER. I am not sure that I understand what this means exactly. What happens if FRAP is performed in a region that does not include the juxtanuclear ER? Is the diffusion process ATP-dependent?
3. Fig. 3A-B: The second slope of fluorescence decay curve is indeed slower in the case of VSV-G than for FP-22. There is still however a decay (k value should be given). Given that VSV-G fluorescence signal looks pretty stable in the Golgi (see for instance Fig. 4D), how do the authors interpret this result?
4. In Fig. 3C, the authors tested whether a transport wave of VSV-G through the Golgi complex could inhibit the retrograde machinery. This is indeed an important control. However, this experiment should be quantified by FRAP analysis.
5. The experiments shown in Fig. 5 are not very conclusive. In particular, a significant fraction of GalNac-mCherry co-localizes with TGN38 when VSV-G is present (Fig. 5B), which is not the case in the cell expressing mEGFP-22 (Fig. 5A).
6. Intra-Golgi transport of VSV-G can be blocked by Src kinase inhibitors (see Pulverenti et al, Nat Cell Biol 2008, 10:912). It would be informative and interesting to test whether VSV-G recycling can be favored under this condition.

Minor comments:

7. Which Rab6 isoform was used in these experiments?

8. There are several typo and/or English mistakes throughout the manuscript: malfolded, permessive,..
9. Introduction: arf1 and rab6 should not be written in italics.

Referee #2:

This manuscript from the Borgese lab provides new insight into the question of how secretory cargoes reach their destination at the cell surface. Focusing on the ER-Golgi interface, Fossati and colleagues ask whether forward-moving cargo escape from retrograde flux, surprisingly finding that cargo-specific differences that seem to reflect the presence or absence of distinct signals that so far correspond to ER exit signals. Overall, the experiments seem rigorous, although I am not an expert in the technical details of the FRAP methodology, which provide the bulk of the evidence. In general, I was persuaded that the proteins observed were indeed subject to distinct recycling rates. Although one could argue about the overall interpretations and models by which these observations might impact more physiologically normal situations, the findings as presented certainly add to our understanding of competing pathways that must be navigated by secretory cargoes.

I have some minor questions that might be addressed to clarify the manuscript and make the interpretations easier for a general audience to appreciate:

1. Is the FP-22 protein that is visible at 70h post-injection all membrane-associated? If some pool of the protein were still cytosolic, this could confound all of the subsequent interpretations - ie. is the rate-limiting step here delivery to the insertion machinery?
2. In Fig. 1C, there seems to still be some recovery in the second phase even during ATP depletion; could this be explained by a non-membrane associated pool?
3. In Fig. 2C, it would be helpful to also show the quantification of the ER recovery under ATP depletion conditions.
4. In Fig. 2D, the Golgi loss seems greater than at 20C, which would be consistent with PM delivery (or other internal destinations); quantifying this effect would be nice and might give a good measure of support for the authors' postulation that Golgi loss at 20C is largely retrograde.
5. In Fig. 3A, the logic eludes me. The authors state that one reason VSVG might not show ER accumulation is that it is so rapidly exported. But in this experiment, unbleached (originally Golgi) molecules could simply travel back to the ER and be rapidly packaged as stated. In order to distinguish whether this is happening, continual bleaching is required so that any Golgi molecules that make it back to the ER are removed from the analysis. Although the H89 experiments address this in a slightly different manner, the logic (and meaningfulness) of this particular experiment is unclear.
6. If FP-22 is excluded from the TGN (Fig. 5A), under conditions where intra-Golgi traffic is permitted, how do the authors propose that ultimate PM delivery occurs? The model for TM-mediated sorting would suggest that it should accumulate in the TGN where the bilayer thickness becomes more PM-like. So why doesn't it? Is there are concern that at 20C, TM thickness is affected and thus TM-

mediated sorting in the absence of other signals is affected? This is experimentally beyond the scope of this paper, but some discussion might be warranted.
7. In the EndoH experiments (Fig. E3), I'm concerned that the global fraction of VSVG-AxA in the Golgi is so small as to make it impossible to detect even if it were EndoH resistant. If there were vastly more protein loaded into the experiment, would a difference be seen?
8. In Fig. 8A, the control cells seem to lack appreciable Golgi-localized VSVG-AxA (unlike in previous images), which makes this experiment very difficult to evaluate/interpret, despite a quantification that shows seeming significance.

Referee #3:

The study by Borgese and colleagues uses quantitative imaging methods with fluorescent protein tagged cargo proteins to demonstrate that transmembrane cargos moving through the secretory pathway undergo constitutive recycling between the ER and Golgi prior to their delivery to the plasma membrane. Previous work in this area has shown this occurs for Golgi enzymes but not secretory cargo molecules themselves. The new findings are significant because they provide a potential explanation for the vastly different rates of protein transport through the Golgi and are relevant for understanding quality control mechanisms within the secretory pathway. The authors identify a potentially significant mechanism controlling partitioning of outward directed membrane proteins into retrograde carriers: which is the lack of positive signals involved in anterograde transport, such as diacidic motifs. The authors further show that the retrograde pathway is Rab6 dependent. Overall, the paper is potentially suitable for publication in EMBO J. if the authors can address the following issues.

1. The figure panels should be more clearly labeled regarding what constructs are being used (Figs 1D; 2B, C; 3A, etc).

2. The authors should broaden the list of TM secretory cargo they test regarding the role of diacidic motifs. What happens if you add this motif to a protein that doesn't have it? This is needed to broaden the conclusions of their study.

3. Is the recycling rate of cargo correlated with the processing efficiency of the cargo?

4. Any effort to delve deeper into the molecular mechanism by which the presence of di-acidic motifs limits movement into the retrograde pathway at the level of the Golgi would strengthen the paper.

1st Revision - authors' response 17 June 2014

Referee #1
The referee appreciated the value and interest of our study, although he/she notes that its weak point is the failure to identify the mechanism by which positive signals may favour

cargo progression through the Golgi. While we basically agree with this criticism, I would like to point out that if the positive signal exerts its effects through multiple, low affinity interactions, as considered in the Discussion section, it may turn out to be difficult to identify the functionally relevant interactions. Nevertheless, we believe that our study has heuristic value for future investigations on trafficking at the ER-Golgi interface. In the revised version, we have added a silver-stained gel in Fig. E4, to illustrate the pulldown approach that we applied in our attempt to identify proteins interacting with the VSVG export signal within the Golgi complex.

Main comments of Referee #1

1. *The referee is concerned that the 20°C temperature block used in most of our experiments, by affecting trafficking steps other than exit from the TGN, may not be reporting on the situation at physiological temperatures. He/she asks that we provide the following additional data:*

(1.i) Quantification of the iFRAP experiment on FP-22, carried out at 37°C (Fig. 2D)

Reply: The quantification is now provided (revised Fig. 2, panel E)

(1.ii) "The arrival of FP-22 at the plasma membrane at 37°C (Fig. E1) should be quantified as well (as percent of intracellular signal over time)"

Reply: The exact quantification of surface fluorescence for FP-22 is not possible, because, being a tail-anchored protein, there is no extracellular epitope that could be used for quantification on non-permeabilised cells (as we have instead done for the VSVG constructs in Fig. 9). We do, however, now provide quantitative data on the time course of the decrease of FP-22's Golgi fluorescence; visual inspection of the images shows the decrease in Golgi fluorescence to be accompanied by arrival of the construct at the plasma membrane (revised Fig. E1, panels D,E and new video 1).

(1.iii) "It is also important to document that VSV -G recycling does not occur at 37°C as shown at 20°C"

Reply: It is not possible to directly assess recycling of VSVG at 32°C, because at this temperature, its transport from the Golgi is extremely rapid, so that a bleach of the ER under these conditions would not be informative. I would like, however, to stress that we believe that the experiment of Fig. 9 effectively addresses the concern of the reviewer. In this experiment, we compared the effect of dominant negative Rab6 on the transport of wt and mutant (export signal-deleted) VSVG at physiological temperature. Rab6 exerts it effects at multiple steps along the secretory pathway: it is involved in the retrograde Golgi-to-ER pathway (Girod A. et al. Nature Cell Biol., 1999) as well as in post-Golgi trafficking (Storrie B., et al. Traffic, 2012). In our study, we identified the Rab6 pathway as the one involved in retrograde trafficking of outbound cargo (Fig. 8). We found, very interestingly, that dominant negative Rab6 had opposite effects on the transport to the surface of the two VSVG forms: it inhibited transport of the wt protein, but it enhanced transport of the mutant. We believe that this result strongly suggests that recycling of the wt VSVG is a minor phenomenon, so that DN Rab6's inhibitory effect on retrograde transport has little effect on this cargo's transport through the secretory pathway, allowing the inhibition of steps downstream to the Golgi to be revealed. In contrast, the stimulatory effect of DN Rab6 on export signal deleted VSVG suggests that recycling of this protein between the Golgi and the ER has an important effect in delaying its transport, so that inhibition of this pathway overrides the negative effect exerted by DN Rab6 on post-Golgi steps. We think that this experiment provides strong evidence supporting the idea that the

export signal-bearing VSVG is not engaged, or is engaged to a minor extent, in the Golgi-to-ER recycling pathway.

2. The referee is not convinced of the meaning of diffusion of FP-22 into the "juxtanuclear" ER, and would like more information on the characteristics of the recovery in the ER in a region removed from the Golgi region. ("What happens if FRAP is performed in a region that does not include the juxtanuclear ER? Is the diffusion process ATP-dependent?")

Reply: When bleaching the Golgi, the ER in the Golgi region is necessarily also bleached, thus recovery from the ER into that region is expected to include both diffusion into the ER of the bleached ROI and vesicular transport into the Golgi complex. In Fig. 1B,C, we do show FRAP data of an area of ER that does not include the juxtranuclear region. In the revised version, we also present data on the diffusion into the ER outside the Golgi region in the absence of ATP, as requested by the reviewer. These new data, which are presented in Fig. 1C and E, show that diffusion into the bleached ER is energy-independent.

3. The referee notes that there is some decrease in Golgi fluorescence in iFRAP experiments of VSVG, and would like us to provide an estimate of the first-order decay constant. In addition, he/she notes that there may be some discrepancy between the curve of Fig. 3B (which shows some diminution in Golgi fluorescence) and the images of Figs. 3A and D (previous 4D).

Reply: Our calculations of decay constants have all been done starting from cells at steady state, and assuming that the cells would return to the same Golgi to ER fluorescence ratio as before the bleach. This value is the plateau inserted in the equation used to fit the data (see Expanded Methods). In the case of the experiment with VSVG, we carried out the bleach *before* steady state was reached, because at the 20°C steady state there is no significant VSVG staining of the ER. For this reason, we cannot apply the equation used for estimating the decay constants of the other investigated proteins. We agree that there is some decrease in VSVG Golgi fluorescence after ER bleaching, and indeed, we have not claimed that VSVG is *completely* prevented from engaging the recycling pathway, and have been careful in the text to underline that it is much less efficiently recruited into the pathway than its signal-deleted counterpart, not that it is completely excluded (see for instance the first sentence of the third paragraph of the Discussion, p.17)

Concerning the apparent discrepancy between the graph of Fig. 3B and the images of Fig. 3A and D, the graph displays the average decay, and the small difference in fluorescence values (after the 10 min time point) would not be, in our mind, easily discernible in single images.

4. The referee rightly asks for quantitative analysis of retrograde flow of FP-22 when co-expressed with VSVG.

Reply: This has been done. The results are presented in revised Fig. 4 and Table I. The analysis show that there is no significant difference in the rate of retrograde transport of FP-22 when expressed together with VSVG in comparison to the situation in which it is expressed alone.

5. The experiments shown in Fig. 5 are not very conclusive. In particular, a significant fraction of GalNac-mCherry co-localizes with TGN38 when VSV-G is

present (Fig. 5B), which is not the case in the cell expressing mEGFP-22 (Fig. 5A).

Reply: We believe that the experiment of Fig. 5 is important, as the finding that VSVG has a different distribution from FP-22 in the Golgi lends additional support to the conclusion that the VSVG and FP-22 behave differently within this compartment. The important observation in the Figure is that VSVG colocalises with TGN38 (as reported in the literature) while FP-22 does not. GalNacT2 is reported to have a wide intra-Golgi distribution (Roettger et al., JCS, 1998). We do not know why it is segregated from the TGN in the FP-22-expressing cells and not in the ones expressing VSVG, however, as stated above, to us the important point is that FP-22 is distributed differently from VSVG. The same conclusion was reached on the signal-deleted form of VSVG, based on lack of acquisition of EndoH resistance (Fig. E3).

6. The reviewer suggests that we investigate whether Src kinase inhibitors (shown by Pulverenti et al. to block exit from the Golgi) might favour VSVG recycling back to the ER.

Reply: This is an extremely interesting idea, and we would like to carry out these experiments. However, we believe that it would take quite some time to characterise the effect of these inhibitors in our system. Our study opens many avenues for future research, and the effect of Src kinase inhibitors surely is one direction that we hope to pursue in the future.

Minor:

7. Which Rab6 isoform was used in our experiments?

Reply: The isoform used was Rab6A. This is now specified under Expanded Methods.

8. There are several typo and/or English mistakes throughout the manuscript

Reply: We have carefully checked the manuscript for typo and English mistakes.

9. Introduction: arf1 and rab6 should not be written in italics.

Reply: This error has been corrected.

Referee #2
The referee has some minor issues that he/she would like to see clarified, which we address in the revised text and below.

1. The referee is worried that at 60-70 min post-injection some FP-22 (which is a post-translationally inserted protein) could still be soluble. As I understand it he/she fears that such a situation could confound the interpretation of our data; e.g., upon Golgi bleaching, recovery in the Golgi could be due to fluorescent protein inserting from the cytosol and not from the ER? Or, similarly, after ER bleaching, recovery in the ER could be from the cytosol and not from the Golgi?

Reply: All the data from ours and other labs indicate that tail-anchored proteins do not insert directly into the Golgi, but reach the Golgi after prior insertion into the ER, as do other membrane proteins (e.g., Kutay U et al., EMBO J., 1995, Pedrazzini E et al., PNAS, 1996; Linstedt A.D. et al., PNAS, 1995; Bulbarelli A. et al., JCS, 2002). Therefore, the energy-dependent recovery of the Golgi that we see after bleaching can only be attributed to vesicular transport from the ER. Also, work from our and other labs indicates that the insertion of tail-anchored proteins into the ER is very rapid, as soluble pools have not been detected (with the exception, of course, of proteins whose insertion is regulated, such as the insertion of Bax into the outer mitochondria membrane). For instance, in the study by Yabal M et al., (JBC 2002), we observed that after a 5 min pulse of ^{35}S methionine, all the synthesised cytochrome b5 was inserted into the S. cerevisiae ER; a soluble pool in yeast could be detected after extremely short pulses (< 2 min). In mammalian cells, Kutay et al. (Embo J., 1995) found all of another tail-anchored protein (synaptobrevin) inserted into the ER after a 10 min pulse. Although we cannot do biochemistry on microinjected cells, the high resolution images in Ronchi P. et al (JCB 2008) acquired in CV1 cells at 60-75 min post-microinjection, show no evidence for the presence of a soluble pool of the same constructs that we have used in the present study. Finally, I note that in our study bleaching was generally carried out 30 after addition of cycloheximide, so that any "stray" protein would have had the time to insert or be degraded.

2. In Fig. 1C, there seems to still be some recovery in the second phase even during ATP depletion; could this be explained by a non-membrane associated pool?

Reply: This concern is related to the one of point 1 (see above). We believe that the residual Golgi recovery observed under conditions of ATP depletion is due to the less than 100% efficacy of the treatment (NaN_3 and 2-deoxyglucose). The effect of energy depletion is in any case large, as the estimated decay constant for FP-22 retrograde trafficking is reduced over 10 fold (Table I). We have changed the wording in the text to state that under energy depletion conditions, recovery in the Golgi was "strongly reduced" (p. 8 of revised manuscript).

3. "In Fig. 2C, it would be helpful to also show the quantification of the ER recovery under ATP depletion conditions."

Reply: This has been done (see revised Fig. 2).

4. The referee notes that FP-22 fluorescence loss from the Golgi at 37°C is more rapid than at 20°C and wonders whether this higher rate is due to transport of FP-22 to other compartments (plasma membrane for instance) other than back to the ER. He/she suggests that we provide quantitative analysis of this iFRAP experiment.

Reply: We now provide the quantitative analysis of this experiment in panel E of revised Fig. 2 (as requested also by referee #1). It should, however, be noted that, as pointed out by the referee, the interpretation of this experiment is not univocal, as it is possible that transport to locations other than the ER is contributing to some extent to the loss of Golgi fluorescence observed here. However, the stability of Golgi fluorescence in a neighbouring non-bleached cell (see Fig. 2D) does suggest that most of the observed fluorescence loss is due to retrograde transport. In the revised test (p. 9), we now call the reader's attention to the behaviour of the neighbouring cell Further strong evidence in favour of the occurrence of recycling

at physiological temperature is given in the experiment of Fig. 9, where we show that dominant negative Rab6 stimulates transport of signal-deleted VSVG to the cell surface, while inhibiting the transport of the wt protein. This experiment analysed the end point of the secretory pathway (arrival at the cell surface) and was carried out at physiological temperature. It indicates that recycling indeed delays transit through the secretory pathway; the wt protein, which appears to engage much less in recycling, served as control and its transport was affected by dominant negative Rab6 in a manner *opposite* to the signal-deleted mutant.

5. The referee criticizes the experiment of Fig. 3A, in which, with the aim of establishing whether VSVG enters or not the recycling pathway, we carried out iFRAP on VSVG-expressing cells. He/she points out that if the much more efficient accumulation in the Golgi of VSVG cargo (compared to FP-22) at 20°C were due exclusively to its more efficient anterograde transport from the ER to the Golgi (and not to absence of retrograde transport), this anterograde transport would mask the ongoing retrograde traffic, i.e., fluorescent protein traveling back to the Golgi would be immediately recaptured into forward-directed COPII transport vesicles. To distinguish what is happening he/she points out that "continual bleaching is required so that any Golgi molecules that make it back to the ER are removed from the analysis."

Reply: This point is well taken, however, the repeated bleaching experiment suggested by the referee is not feasible, as phototoxicity would damage the cells and inhibit vesicular transport. It should be noted, however, that at the initial time points after bleaching the ER, any recycled fluorescent cargo would be diluted by the non fluorescent bleached cargo in the ER; therefore, its contribution to maintaining Golgi fluorescence would be expected to be low. In any case, because the interpretation of this experiment was not univocal, we used H89 to inhibit anterograde transport and to measure Golgi emptying in the absence of bleaching. This experiment confirmed the stability of VSVG in the Golgi. Further evidence in favour of the different behaviour of VSVG and FP22 stems also from the different distribution of the two proteins within the Golgi at 20°C (Fig. 5) and from the opposite effect of dominant negative Rab6 on signal bearing and signal-deleted VSVG (Fig. 9); the latter result indicates that Golgi-to-ER retrograde transport has little effect on VSVG transport through the secretory pathway in comparison with the situation for signal-deleted VSVG.

In the revised version of the manuscript, we have better explained the rationale for carrying out the experiment of Fig. 3 (please see p. 9 of the revised manuscript). Furthermore, we have combined the iFRAP data and the H89 experiments in one figure (revised Fig. 3), so that the logical relationship between the two approaches is clearer. Finally, as any ongoing anterograde transport might affect the estimated decay constants for *all* our cargoes, we now refer to the calculated decay constants as *apparent* k.

6. The referee would like us to discuss in more depth the mechanism of exit of TMD-dependent cargoes from the TGN; specifically he/she asks why our cargoes do not accumulate in the TGN at 20°C, where membrane thickness is expected to better match the plasma membrane and is concerned that at 20°C membrane thickness may be altered, leading to altered TM-mediated sorting.

Reply: For the cargoes that we are investigating, the TM length affects exit from the ER whereas the effect on export from the Golgi is not clear (Bulbarelli A et al., JCS 2002), as is true instead for the type II Golgi enzymes investigated by other groups (e.g., Munro S., EMBO J., 1995). It seems that the short TMD may determine exclusion from ER exit sites, but whether this depends on membrane thickness or other factors is not clear at present (see Discussion in Ronchi P et al., JCB 2008). We hypothesize that the longer TMs (of tail-anchored proteins as well as of EGFR and of signal-deleted VSVG) can partition into any bilayer and this is what determines both their recruitment into the Golgi-ER recycling pathway as well as their final transport to the PM. The fact that at steady state at 20°C we see most of our recycling cargoes in early Golgi compartments does not mean that a fraction of cargo may not also travel to the TGN. At physiological temperature this fraction would be exported to the plasma membrane, and once out of the Golgi this cargo would no longer be able to return to the early secretory pathway. We discuss this Brownian ratchet type of mechanism on p. 20 of the revised manuscript. As far as the concern that TM-dependent sorting may be altered at 20°C, I again stress that the experiment of Fig. 9 was carried out at physiological temperature and confirms our observations at 20°C.

7. The referee is concerned that the absence of acquisition of EndoH resistance of signal-deleted VSVG reported in Fig. E3 could be due to the small amount of protein in the Golgi, and asks whether a difference would be seen if vastly more protein were loaded into the experiment.

Reply: We did not intend to claim that VSVGAxA remains 100% EndoH-sensitive, but that most of it does. We now have rephrased the description of Fig. E3 to state that our results indicate that "the majority of this cargo has limited access the medial cisternae of the Golgi" (p. 12). Keeping in mind that we estimate a Golgi emptying decay constant of 1.2%/min (Table 1), in 4 h a considerable amount of the ER pool of VSVGAxa is expected to have visited the Golgi, yet is not EndoH resistant. Finally, we think that an important point of Fig. 3E is the acquisition of EndoH resistance by wt VSVG, in confirmation of the immunofluorescence analysis of Fig. 5.

8. The referee notes that in the control image of Fig. 8A accumulation of signal-deleted VSVG is very poor (unlike in other images), making the interpretation of the data doubtful, despite seeming significance.

Reply: We agree that the concentration in the Golgi of the VSVGAxA cargo is very poor under these conditions. However, the images are not really comparable to the previous ones, because both the cells (HeLa vs NRK) and the temperature (32 vs 20 degrees) are different. As explained in the text, these conditions were chosen so as to be able to follow in parallel the internalisation and trafficking of Shiga Toxin. We do not understand at present why cells transfected with Arf1 Q71L show a decrease in VSVGAxA concentration in the Golgi compared to the controls, however, the important conclusion of this analysis is that the Rab6 T27N transfected cells show instead an increase, suggesting that recycling may be slowed. This is then shown directly in Fig. 8E. We now explain better in the text that the expected result in the case of a block in retrograde transport of cargo was an increase in Golgi staining and that we do not understand why dominant negative Arf1 caused a decrease (p. 15 of revised manuscript).

Referee #3
The referee appreciates the significance of our work, but raises the following concerns:

1. The figure panels should be more clearly labelled.

Reply: This has been done (revised Figs. 1D, 2B, 3A, E2)

2. The referee asks us to broaden the list of analysed secretory cargoes, specifically to test the effect of adding the VSVG export signal to a protein that doesn't have it.

Reply: we have now transplanted the YTDIE export signal of VSVG to our FP-22 model tail-anchored cargo, and find that indeed the presence of this signal reduces the rate of Golgi-to-ER retrograde transport. We think that this new result, presented in Fig. 6F,G of the revised manuscript, broadens the impact of our results, and thank the reviewer for this suggestions.

3. "Is the recycling rate of cargo correlated with the processing efficiency of the cargo?"

Reply: We are not sure whether with this question the referee intends (i) processing of cargo within the Golgi (e.g., acquisition of EndoH resistance for glycoproteins) and/or (ii) transport to the plasma membrane. Concerning the first alternative, we do show that the majority of VSVGAxA fails to acquire EndoH resistance even after 4 h of incubation at 20°C. Concerning the second alternative, the experiment of Fig. 9 shows that the reduced transport of VSVGAxA to the plasma membrane in comparison to wt VSVG is in part reverted by dominant negative Rab6. This experiment strongly suggests that recycling of cargo delays transport to the plasma membrane. It would, of course, be interesting to make quantitative comparisons on the rate of transport to the cell surface between different cargoes that recycle at different rates within the early secretory pathway; however, since other factors acting within the secretory pathway (e.g., rate of recruitment into post-Golgi carriers) are expected to influence the final rate of transport to the cell surface, such experiments might be difficult to interpret.

4. Any effort to delve deeper into the molecular mechanism by which the presence of di-acidic motifs limits movement into the retrograde pathway at the level of the Golgi would strengthen the paper.

Reply: As noted in our reply to the general comment of Referee #1, if partitioning of VSVG away from the recycling pathway involves multiple low-affinity interactions, it may be difficult to identify the functionally relevant ones. We discuss this possibility on p. 18 of the revised manuscript. We also have added a silver-stained gel to Fig. E4 to illustrate the pulldown approach, with which we attempted to identify interacting partners of VSVG within the Golgi. We feel that the manuscript, with the additional experiment on the role of the transplanted YTDIE sequence, does provide some mechanistic insights, as it (i) identifies the pathway involved in recycling (Rab6) and (ii) shows that the YTDIE sequence is both necessary and sufficient to prevent recycling of the cargo proteins investigated. We hope that in the future other "recycling prevention" signals will be identified and that the mechanisms involved will be unravelled by us or by other groups.

2nd Editorial Decision 25 June 2014

I have now received comments from the referees of your manuscript that are all satisfied with the amount of revisions and thus support publication of your article. I am thus pleased to accept your manuscript for publication here.

Thank you for contributing to the EMBO Journal!

Referee #1:

The authors have met my previous comments/criticisms in a satisfactory way.

Referee #2:

This revised manuscript from the Borgese lab, and the accompanying rebuttal, adequately resolve my previous concerns. This is a solid study that brings a new perspective to the efficiency of forward traffic through the secretory pathway and will be of broad interest to the field.

Referee #3:

The authors have addressed all of my concerns.

