INTRODUCTION
Although eukaryotic cells share membrane compartmentalization and many aspects of the secretory pathway, they also harbor differences. The organization of the Golgi apparatus is one example. In the yeast Saccharomyces cerevisiae, the Golgi is constituted of distant single cisternae (Franzusoff et al., 1991); in flies, the Golgi is constituted of stacks dispersed throughout the cytoplasm (Kondylis and Rabouille, 2003); and, finally, in mammals, the Golgi stacks are concentrated at the perinuclear area, where they form a ribbon structure that results from the lateral fusion of many individual Golgi stacks (Ladinsky et al., 1999). Although the Golgi ribbon has a function during the cell cycle (Rabouille and Kondylis, 2007), it is unclear why a ribbon is needed during secretion in interphase cells. Why do mammals harbor such a complex Golgi architecture, when the individual and disconnected Golgi cisternae of yeast seem to be as efficient in secreting proteins? This question is especially vexing, because the transport of secretory proteins is generally not affected when the ribbon structure in mammalian cells is disrupted to produce numerous individual Golgi stacks (Rogalski et al., 1984; Van De Moortele et al., 1993; Cole et al., 1996; Trucco et al., 2004).

Many mechanisms for intra-Golgi transport have been proposed (Glick and Luini 2011), the two most extreme being the maturation and the vesicular models. The main limitation of the vesicular model is that specialized cargoes (such as certain collagens) are too large to be accommodated by typical transport vesicles such as coatomer proteins I (COP1) vesicles (~70-nm internal diameter). One possibility, which we explore here, is that the ribbon structure serves the special purpose of facilitating the anterograde transport of such large cargoes.

We have shown that forward flow of large aggregated cargoes through the Golgi requires their concentration within the enlarged rims of Golgi cisternae and that they remain in the close vicinity of the cisternae during anterograde transport (Volchuk et al., 2000; Lavieu et al., 2013; Pellett et al., 2013); this process differs fundamentally from the transport of small cargoes, which seems to mainly utilize diffusible carriers (Pellett et al., 2013). We speculated that aggregate transport may instead occur by a proposed process of “rim progression,” in which the large cargo–laden rim of one cisterna may be itself transferred to another cisterna in a process of membrane fission–fusion. A process of rab conversion occurring vectorially across the Golgi stack (envisioned in an analogous model termed “cisternal progenitor” [Pfeffer, 2010]) could provide a ready mechanism to drive rim progression. In these models (Pfeffer, 2010; Lavieu et al., 2013; Mironov et al., 2013), contact between adjacent stacks within the ribbon should be required for efficient transport of large cargoes (but not small cargoes, which are accommodated within COPI vesicles or potentially in tubules of similar diameter).
Thus rim progression and similar models make the striking prediction that disruption of the ribbon should selectively prevent anterograde transport of large but not small cargoes. In this study, we tested this prediction in a variety of different and complementary ways.

RESULTS
Nocodazole treatment inhibits the secretion of artificial aggregates
To test whether Golgi ribbon fragmentation affects intra-Golgi transport, we used nocodazole, a drug that prevents microtubule polymerization and that is well known to break down the Golgi ribbon of many cells into dispersed ministacks (Thyberg and Moskalewski, 1985; Cole et al., 1996).

First, we combined nocodazole treatment with a drug-controlled aggregation system that allows for positioning soluble aggregates at different stages of the secretory pathway (Volchuk et al., 2000; Lavieu et al., 2013). The chimeric cargo is constituted of a signal sequence fused to a fluorescent protein, four repeats of the self-aggregation domain (FM4), a furin cleavage site, and the sequence encoding the human growth hormone (Figure 1B). In the absence of the disaggregating drug, the chimeric protein is aggregated and stays in the endoplasmic reticulum (ER). In the presence of the drug, at 16°C for 2 h, the now-disaggregated protein reaches the cis face of the Golgi, where it can be reaggregated by subsequent drug removal. Shifting the temperature back to 37°C in the absence or presence of the disaggregating drug allows for comparison of intra-Golgi transport and further secretion of large aggregated cargo versus small disaggregated cargo, respectively. Importantly, such a chase needs to be performed in the presence of cycloheximide (CHX), an inhibitor of protein synthesis.

We preincubated the cells for 2 h with nocodazole to disrupt the Golgi ribbon (Figure 1A) before manipulating the aggregation/positioning of the chimeric protein. Note that nocodazole as well as CHX were present during the entire course of the experiment, except if mentioned otherwise. The proteins were reaggregated or not within the cis-Golgi (using 16°C incubation), and then transport was resumed by shifting the temperature back to 37°C and we assessed the rate of secretion for each kind of cargo (Figure 1C). In the absence of nocodazole, aggregated and disaggregated cargoes showed similar rates of secretion, consistent with previous work. When the ribbon was broken by nocodazole, the secretion of aggregates that were prepositioned in the cis-Golgi was strongly inhibited (70% reduction), whereas the secretion of disaggregated cargo showed only a modest inhibition (15% reduction; Figure 1D).

We then assessed the intracellular localization of the nonsecreted fraction of the reaggregated protein using confocal microscopy. Working with nocodazole-induced ministacks allows for discriminating cis- from trans-Golgi cisternae, even at the light microscopy level (Deijgaard et al., 2007). Within the very same cells, the localization of the green fluorescent protein (GFP)-tagged disaggregated or aggregated cargo was compared with a cis-Golgi marker (Gpp130 labeled with a red dye) and a trans-Golgi marker (p230 labeled with a far-red dye). As expected, cis- and trans-Golgi markers were easily distinguishable, as judged by the lack of overlay and the low
whereas the reaggregated cargo remained associated with the cis-Golgi cisternae (Figure 2, A and B, condition III). We observed the same cis-Golgi retention of the aggregates when the chase was performed at 37°C (Supplemental Figure S1).

These observations were qualitatively confirmed by electron microscopy (EM), which showed that aggregates prepositioned at the cis face of the nocodazole-induced ministack remained at the same position, even when the temperature was shifted to 20°C, which normally allows for cis→trans movement (Figure 2C, left panel). On the contrary, in the control condition, when reaggregation was triggered only at the end of the 20°C chase (in order to visualize the cargo by transmission EM), the cargo was now localized within the trans-Golgi (Figure 2C, right panel), made identifiable by its swollen aspect, which is typical at 20°C (Griffiths et al., 1989). This indicated again that disaggregated cargo passed through the ministacks, whereas the re-aggregated cargo remained mostly within the cis-Golgi.

**Nocodazole treatment inhibits the secretion of endogenous collagen I**

The cargo used above was artificially aggregated and may not perfectly reflect the behavior of physiologically large cargos. Collagen I, an abundant component of the extracellular matrix, is an example of a physiological large cargo (Ricard-Blum, 2011). We repeated our experiments using Saos-2 cells that secrete endogenous collagen I (Saito et al., 2009; Lavieu et al., 2013). We took advantage of the ascorbate-dependent folding of collagen to control the wave of collagen secretion (Harwood et al., 1976; Mironov et al., 2001). Cells were cultured overnight in ascorbate-depleted media to block collagen within the ER and then, during the last 2 h, cells were pretreated with nocodazole before ascorbate was added back to allow for collagen folding and subsequent exit from the ER. We then assessed the secretion of endogenous collagen I (large cargo) and endogenous MMP2 (small cargo) simultaneously within the same samples. This allows for the direct comparison of the secretion rates of the two endogenous cargos using a bulk biochemical secretion assay that leaves no room for biased interpretation. The rate of secretion of collagen I was strongly diminished (70% reduction) in nocodazole before ascorbate was added to starve the endo-Golgi (I). The disaggregating drug was removed (II) or not (II) for 30 min before the temperature was shifted to 20°C for 30 min. Cells were fixed and prepared for immunofluorescence against the cis-Golgi marker gpp130 (labeled with a red dye) and against the trans-Golgi marker p230 (labeled with a far-red dye). (B) Graph represents the Pearson’s coefficient for each marker combination for each condition. For each condition, we analyzed between 70 and 150 ministacks. *, p values < 0.01. (C) Electron micrographs show the retention of aggregates within the cis face of nocodazole-induced ministacks. The temperature was shifted to 20°C for 1 h, and cells were prepared for EM (left panel). As a positive control for cis→trans transport at 20°C, cells subjected to the same nocodazole treatment were incubated for 1 h with the disaggregating drug at 20°C, and reaggregation was triggered by drug removal for 30 min at 20°C to allow for visualization of the aggregates by conventional EM (right panel).
A previous study reported that the classical cargo vsv-G and procollagen are both efficiently transported through nocodazole-induced stacks (Trucco et al., 2004). Although our results agree with the results for the classical cargo, they diverge with regard to collagen transport. We do not have a full and rational explanation for this discrepancy, but we noted at least two major differences between the respective studies. First, the authors used a different cell type, and, more importantly, they added in their protocol a 40/15/40°C temperature shift cycle to control the cargo release wave, which implies that their intra-Golgi transport assay was performed at 40°C, while our experiments were conducted at either 20 or 37°C.

**Grasp55/65 small interfering RNA (siRNA)-mediated ribbon disruption also inhibits rim progression**

One interpretation of the results reported above would be to consider that the phenotype is mainly due to the alteration of the microtubule network and is only an indirect consequence of the ribbon breakdown. To test this hypothesis, we decided to knock down grasp55/65, which is known to disrupt the ribbon structure of the Golgi without affecting the microtubule organization (Pathanweedu et al., 2006; Feinstein and Linstedt, 2008; Lee et al., 2014). As judged by immunoblotting and immunofluorescence analysis (Figure 4, A–C), at least 75% of the grasp55/65 proteins were efficiently silenced within cells treated with specific siRNA. As previously reported (Feinstein and Linstedt, 2008), we confirmed that these cells harbored a mildly scattered Golgi (Figure 4A and graph), without showing any alteration of the microtubules (Figure S3).

First, we used the disaggregation/reaggregation assay and showed that grasp55/65 knockdown (KD) inhibited the secretion of cis-Golgi reaggregated cargo by a factor of two (Figure 4B, lanes 2 and 5), whereas the secretion of the disaggregated cargo was not decreased at all (Figure 4B, lanes 1 and 4) and even seemed to be slightly increased. Note that, in our hands, this increase was not statistically significant, although previous studies reported a robust 40% increase of the secretion of classical cargo under similar conditions (grasp55/65 KD; Xiang et al., 2013).

We then tested whether the grasp55/65 KD had a similar effect on the secretion of large and small endogenous cargoes. Collagen I secretion was inhibited by 60% when grasp55/65 were silenced, whereas MMP-2 secretion was not diminished (Figure 4C). These results recapitulated the observations made under nocodazole treatment and suggest that the transport inhibition of large cargoes is directly due to the disruption of the ribbon structure. It is important to note that the grasp55/65 KD-mediated scattering of the Golgi is less pronounced than the nocodazole-mediated ribbon disruption. This may explain why the inhibition on large cargo transport mediated by grasp55/65 siRNA (~50%) is less severe than the inhibition triggered by nocodazole (~70%).

**Fly cells harbor dispersed Golgi stacks and poorly secrete reaggregated cargo**

Drosophila S2 cells naturally harbor Golgi stacks that are dispersed through the cytoplasm (Kondylis and Rabouille, 2003). As for microtubule-induced ministacks, cis- and trans-Golgi cisternae can be resolved at the light level (Figure 5A and graph). We first generated a plasmid compatible with the expression of the drug-controlled GFP aggregate within S2 cells. S2 cells normally grow at 25°C, and this prevented the direct application of the 16°C reaggregation temperature shift block that we established with HeLa cells, which normally grow at 37°C. We empirically tested several lower temperatures to determine which one would be most appropriate to slow down trafficking of the disaggregated human growth hormone (hGH) cargo, such
that it would be positioned within the cis-Golgi before its reaggregation was triggered. We found that, at 10°C, the disaggregated cargo reached the cis-Golgi after 5 min and then progressively reached the trans-Golgi within the next 20 min (Figure 5B and graph). Importantly, after 40 min at 10°C, the vast majority of the disaggregated cargo remained at the trans-Golgi (Figure 5B), and only a very small portion could be detected in the media (<5%, Figure 5C). We concluded that the 10°C incubation slowed down the trafficking within S2 cells and triggered retention of the cargo within the trans-Golgi, thereby mimicking the well-known 20°C temperature block often used in mammalian cells. Now knowing precisely the kinetics of the sequential ER→cis-Golgi→trans-Golgi transport at 10°C, we decided to incubate the S2 cells at 10°C for 5 min in the presence of the disaggregating drug to position the cargo within the cis-Golgi before triggering (or not) its reaggregation for 10 min on ice, which did not alter the microtubule network (Figure S4). The cells were then either incubated for 20–40 min at 10°C before being processed for confocal microscopy or were incubated at 20°C to analyze and compare the rate of secretion of each reaggregated and disaggregated cargo. As judged by confocal microscopy, the reaggregated cargo remained associated longer within the cis-Golgi (Figure 5D and graph; up to 40 min instead of 5–10 min for the disaggregated cargo). A portion of the aggregates reached the trans-Golgi, but again was more than two times slower than the disaggregated cargo. Analysis of the bulk secretion when transport was resumed at 20°C revealed that the secretion of the aggregates was inhibited by a factor of two when compared with the disaggregated cargo (Figure 5E and graph). Note that almost 100% of the disaggregated hGH was released from the S2 cells, whereas only 50% was released from the HeLa cells. We attribute this difference to the transfection efficiency and the protein overexpression level, which is considerably higher in HeLa cells than in S2 cells, resulting in a large portion of the ER aggregates remaining insensitive to the disaggregating drug in HeLa cells. This, however, has no impact on our interpretation of the results.

DISCUSSION

Our previous results (Volchuk et al., 2000; Lavieu et al., 2013; Pellett et al., 2013) and the current results suggest that cargoes follow distinct secretory tracks within the Golgi according to their size. To explain these results (Figure 6), we propose that large
cargoes freshly arrived at the cis-Golgi are first concentrated at the enlarged rim of the cis-Golgi cisternae, where they are physically sequestered from other classical small cargoes. This separation, which remains to be further demonstrated, could simply result from the fact that large aggregates are too big to be accommodated within the narrow confines of the flattened central portions of the stacked cisternae. Fission of the enlarged rims may occasionally occur and would result in a more robust sequestration of the large cargo from the rest of the cisternae (Volchuk et al., 2000). This may be the purpose of the so-called mega-vesicles, which have been reported to contain 20% of the soluble aggregates (Volchuk et al., 2000), but in theory, the enlarged rims may remain physically connected to the cisternae in many cases. Making the assumption that the Golgi ribbon is going through continuous fission–fusion cycles, we propose that single stacks emanating from the ribbon and containing large cargoes within their cis-Golgi cisterna will fuse laterally with one another to form a new ribbon. When the fusion is homotypic (the cis cisterna of one stack fusing with the cis cisterna of an adjacent stack), no net anterograde movement occurs, though such an event may not be entirely unproductive because it could allow additional time for efficient posttranslational modification. However, when the lateral fusion is heterotypic (the cis cisterna of a stack containing the cargo fusing with the medial cisterna of the adjacent stack), this would allow for forward movement of the cargo into the next compartment and so on across the stack.

The Golgi ribbon (facilitated by microtubule-based motility of individual stacks) would ultimately be formed and maintained by a very dynamic process of fission–fusion.

FIGURE 5: S2 cells poorly secrete aggregates, which are retained within the cis-Golgi. (A) Confocal micrograph showing separated cis- and trans-Golgi within single stacks through the cytoplasm of S2 cells. S2 cells were fixed and prepared for immunofluorescence against Gm130 (green-labeled cis-Golgi marker) and G245 (red labeled trans-Golgi marker). Graph shows Pearson’s coefficient value for each imaged single Golgi stack. Red line represents the average value over 50 analyzed stacks. (B) Transport kinetics of disaggregated cargo at 10°C. The S2 cells expressing GFP-FM4-hGH were incubated at 10°C for various time points in the presence of the disaggregating drug, before being fixed and prepared for immunofluorescence against Gm130 (cis-Golgi) or G245 (trans-Golgi) labeled with a red dye, the green signal emanating from the GFP-tagged cargo. Confocal micrographs show representative Golgi stacks at each time point. Graph shows the Pearson’s coefficient over time for each combination. Data represent the mean ± SD of three independent experiments. For each time point of each experiment, at least 30 stacks were analyzed. *, p values < 0.01. (C) Immunoblot showing the secretion block at 10°C. The S2 cells expressing GFP-FM4-hGH were incubated at 10 or 20°C for 0 or 40 min in the presence of the disaggregating drug. Media and cell contents were analyzed by immunoblot and densitometry. Graphs show the secretion over time for each condition. Data represent the mean ± SD of two independent experiments. (D) Confocal micrographs illustrate the inhibition of intra-Golgi transport of aggregates. As in B, cells were incubated at 10°C for 5 min in the presence of the disaggregating drug to position the cargo within the cis-Golgi. Then, reaggregation was triggered by drug removal (15 min on ice) before shifting the temperature back to 10°C for 20 or 40 min. Cells were then fixed and prepared for immunofluorescence as in B. Confocal micrographs show the transport kinetics of GFP aggregates within Golgi stacks labeled with cis or trans markers. Graph shows the Pearson’s coefficient over time for each combination. Data represent the mean ± SD of two independent experiments. For each time point of each experiment, at least 30 stacks were analyzed. *, p values < 0.01. (E) Immunoblot shows the inhibition of aggregate secretion. Cells were treated as in C, except that after the 15-min reaggregation at 4°C, the temperature was shifted to 20°C with or without the drug to allow for secretion. Media and cell fractions were analyzed by immunoblot and densitometry. Graphs show the secretion over time for each condition. Data represent the mean ± SD of two independent experiments. The dashed line indicates the percent of secretion at 10°C for disaggregated cargo, as illustrated on the gel in B.
that would simultaneously result in the meandering, if not unidirectional transport, of the large cargoes from the cis to the trans face. This transfer process would require collisions between the individual stacks and therefore would be much reduced when the stacks are separated from one another; precisely as we report here, whether the separation is artificial (disrupting microtubule motility [nocodazole] or Golgi adhesion [Grasp KD1]) or occurs naturally (fly cells).

Natural, oversized cargoes, such as collagen or chylomicrons, have first to be released from the ER (Fromme and Schekman, 2005). Using a cargo that can be artificially reaggregated within the Golgi allowed us to decouple ER exit from intra-Golgi trafficking, thereby assessing exclusively how oversized cargoes are transported across the Golgi. Because we have shown that natural cargoes such as collagen behave similarly, this suggests that the mechanism described here is physiologically relevant.

During the revision of our manuscript, Cutler and colleagues reported that the size of the Golgi determines copackaging of the von Willebrand factors (vWF), a giant cargo secreted by endothelial cells (Ferraro et al., 2014). We considered whether the size of our soluble aggregates may be affected within nocodazole-induced ministacks. However, this was ruled out, because our EM data showed that the average size of soluble aggregates formed within nocodazole-induced Golgi stacks was 209 nm ± 90 nm (n = 38; Figure 2 and Supplemental Figure S5A) and very similar to the size reported previously within the full ribbon (Volchuk et al., 2000).

We also considered that the presence of the soluble aggregates within the ministacks might alter the proper flow of endogenous cargo. However, this was ruled out, because mmp-2 secretion was unaltered by the presence of the aggregates within nocodazole-induced ministacks (Figure S6).

Another possibility is that a second process, such as cisternal fusion, allows for forward transport of the aggregates.

At first glance, the different Golgi morphologies encountered through evolution seem to correlate with the presence of large cargo. For instance, yeasts do not secrete collagen or, as far as we are aware, any similarly large polymeric cargo (note that chitin is secreted in a dispersed form and is assembled outside the cell; Cabib et al., 2008), and they harbor single Golgi cisternae. Many insect cells harbor dispersed single stacks and secrete homologues of type IV mammalian collagens (network-forming collagens) (Yasothornsrikul et al., 1997). Unlike type I, III, V, and XI collagens (fibril-forming collagens; Thyberg and Moskalewski, 1985; Ferraro et al., 2014), type IV collagens harbor interruptions within the triple-helical domain repeats that are thought to introduce flexibility and instability in the triple helix (Bella et al., 2006; Li et al., 2007; Hwang et al., 2010). However, it remains to be accurately determined to what extent such an increased flexibility would make collagen IV a better candidate than collagen I for loading into putative small transport carriers during intra-Golgi transport. Finally, mammalian cells secrete numerous fibril-forming collagen species that extensively aggregate within the cell, and it is these cells that display an extensive Golgi ribbon. “Professional” collagen/matrix-secreting cells, such as fibroblasts and osteoblasts, often contain a single elongated Golgi ribbon (Cho and Garant, 1985). These correlations broadly match the expectations of rim progression.

However, other observations do not match such predictions and incite us to temper our statement. Some algae have numerous separate Golgi stacks and yet efficiently secrete the macromolecule scale (Brown et al., 1970). Other plants harboring individual Golgi stacks process aggregated cell wall precursors (Mollenhauer and Morre, 1991). Specific tissues of insect cells secrete dumpy collagen (Wilkin et al., 2000), a protein predicted to be 1 μm in size. These observations suggest that the ribbon structure is not absolutely required for the forward transport of oversized cargoes and that other mechanisms, such as cisternal maturation, may back up the lack of ribbon organization or complement the ribbon-dependent rim progression in mammals.

Interestingly, in our study, although the decrease of large cargo transport when ribbons were disrupted was substantial (~70%), this inhibition was never complete.

One possibility is that ribbon disruption is not complete. Paired Golgi stacks have been described within S2 cells and nocodazole-treated mammalian cells and could support such a possibility (Kondylis et al., 2007). However, treatment with latrunculin B, which depolymerizes F-actin and triggers Golgi unpairing (Kondylis et al., 2007), did not perturb the secretion of either small or large cargoes within S2 cells or nocodazole-treated HeLa cells (Figure S7). It remains unclear whether the paired Golgi are physiologically connected (Kondylis et al., 2007), which would be required to favor rim progression; therefore it may be that Golgi pairing is not involved in protein transport and is exclusively involved in cell cycle regulation, as originally proposed (Kondylis et al., 2007).

Another possibility is that a second process, such as cisternal progression, accounts for the residual transport of aggregates.

Considering all these aspects, we state that alteration of the ribbon structure promotes at least a delay in the transport of large cargoes, and we conclude that the ribbon structure facilitates the anterograde transport of oversized cargoes.

**MATERIAL AND METHODS**

**Cell culture and transfection**

HeLa cells were maintained at 37°C in 5% CO2 in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Life Technologies). Saos-2 cells were maintained with the same media supplemented with ascorbate (25 μg/ml). The HT1080 human fibrosarcoma stable cell line expressing ssGFP-FM4-FCs-hGH was previously generated in our lab by Allen Volchuk (Volchuk et al., 2000). HT1080 cells were grown similar to HeLa cells, except with the addition of 0.5 mg/ml geneticin and...
50 U/ml penicillin-streptomycin (Life Technologies) to their medium. S2 cells were maintained at 25°C in Schneider’s Drosophila medium (Life Technologies) supplemented with 10% FBS (Life Technologies). HeLa cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or RNAiMax (Invitrogen) as recommended by the manufacturer. S2 cells were transfected using Effectene transfection reagent (Qiagen, Venlo, Netherlands) as recommended by the manufacturer. Saos-2 cells were transfected using electroporation (Nepa21 type II model from Nepa Gene, Chiba, Japan).

**Plasmids and siRNA**

pC4-sGFPFM4hGH (mammalian cell expression plasmid) was published previously (Lavieu et al., 2013). For expression within S2 cells, ssGFPFM4hGH was subcloned into the pENTR/SD/DTOPO vector (Invitrogen) and then into the pAW destination backbone vector from the Drosophila Gateway vector system (https://dgrc.cgb.indiana.edu). grasp55/65 siRNA sequences were reported previously (Lee et al., 2014).

**Secretion assay, SDS–PAGE, and immunoblot analysis**

These experiments were performed as described previously (Lavieu et al., 2013) with slight modifications. Cells were grown in six-well plates. For HeLa cells and Saos-2 cells, secretion experiments were performed in Hanks’ balanced salt solution (HBSS; Life Technologies) supplemented with 0.1% FBS (Life Technologies) and 100 μg/ml CHX (Sigma-Aldrich, St. Louis, MO). When required, we used nocodazole (Sigma-Aldrich) at 1 μg/ml. For S2 cells, HBSS was replaced with Schneider’s Drosophila medium. The disaggregating drug (D/D solubilizer from Clontech, Mountain View, CA) was used at 1 μM. Incubations were performed at 10, 16, 20, 32, and 37°C, using temperature-controlled incubators. Chase media were collected and precipitated overnight at 4°C with 10% trichloroacetic acid (TCA). After centrifugation, TCA pellets were washed with acetone before being resuspended in loading buffer and analyzed by immunoblotting. Cells were washed, detached, and collected by centrifugation. Pellets were resuspended in loading buffer. Extracted proteins were first separated in SDS–polyacrylamide gels and then transferred onto nitrocellulose membranes for immunoblotting. After being blocked with fat-free milk, the membranes were incubated with appropriate primary antibodies. We used anti-GFP (Roche, Basel, Switzerland), anti-collagen I (SP1.D8 from the Developmental Studies Hybridoma Bank, Iowa City, IA), anti-Grasp55 (Proteintech, Iowa City, IA), anti-Grasp65 (Santa Cruz Biotechnology, Dallas, TX), anti-GAPDH (Sigma-Aldrich), anti-actin (Cell Signaling Technology, Danvers, MA), and anti-MMP2 (Cell Signaling). Primary antibodies were detected by chemiluminescence using horseradish peroxidase–conjugated secondary antibodies. Fluorographs were quantitatively scanned using ImageJ software.

**Confocal and STED imaging**

Confocal images were obtained using a Zeiss LSM510 confocal microscope. Two-color STED images were acquired using a Leica TCS STED microscope. The dyes Star520P (Nizamov et al., 2012) and Star635P (Wurm et al., 2012) (both from Abberior [Göttingen, Germany]) were excited with 532-nm and 640-nm pulsed diode lasers, respectively. For depletion, a tunable, mode-locked Ti:Sapphire laser was used (760 nm for Star520XP and 770 nm for Star635P). Imaging was performed with a 100×/1.4 NA oil-immersion objective lens. Fluorescence was split by a dichroic mirror (650-nm long-pass), band-pass filtered (FF01-685/40 for 640-nm excitation or FF01-582/75 for 532-nm excitation), and detected by avalanche photo-diodes (APD1 for Star520XP and APD2 for Star635P). Images were then smoothed with a 0.7 pixel full-width half-maximum Gaussian filter using ImageJ software.

Images were analyzed using Zeiss LSM510 software or using ImageJ (colocalization finder). Images were analyzed using Zeiss LSM510 software or using ImageJ (colocalization finder plug-in).

**Statistical analysis**

Analyses were performed with StatPlus software. A two-tailed unpaired t test type was used to determine p values. N (number of individual experiments) and n (number of objects [ministacks]) are noted in the figure legends.

**Electron microscopy**

For conventional EM, cells were fixed with 2% glutaraldehyde, buffered with 0.1 M sodium phosphate (pH 7.4), detached from their substrate, postfixed with osmium tetroxide, stained with uranyl acetate, dehydrated in ethanol, and embedded in Epon.

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