Exomer complex regulates protein traffic at the TGN through differential interactions with cargos and clathrin adaptor complexes

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Abstract
Protein sorting at the trans-Golgi network (TGN) usually requires the assistance of cargo adaptors. However, it remains to be examined how the same complex can mediate both the export and retention of different proteins or how sorting complexes interact among themselves. In Saccharomyces cerevisiae, the exomer complex is involved in the polarized transport of some proteins from the TGN to the plasma membrane (PM). Intriguingly, exomer and its cargos also show a sort of functional relationship with TGN clathrin adaptors that is still unsolved. Here, using a wide range of techniques, including time-lapse and BIFC microscopy, we describe new molecular implications of the exomer complex in protein sorting and address its different layers of functional interaction with clathrin adaptor complexes. Exomer mutants show impaired amino acid uptake because it facilitates not only the polarized delivery of amino acid permeases to the PM but also participates in their endosomal traffic. We propose a model for exomer where it modulates the recruitment of TGN clathrin adaptors directly or indirectly through the Arf1 function. Moreover, we describe an in vivo competitive relationship between the exomer and AP-1 complexes for the model cargo Chs3. These results highlight a broad role for exomer in...
INTRODUCTION

The trans-Golgi network (TGN) is a major intracellular cargo sorting station, where newly synthesized proteins and endocytosed proteins need to be accurately identified and sorted to distinct subcellular destinations. Cells utilize sophisticated cargo sorting machinery to meticulously package the cargo molecules into the right transport carriers. During this process, cargo adaptors often play pivotal roles in both cargo recognition and in coat assembly, while coat assembly ultimately leads to membrane deformation and fission. One critical coat at the TGN is the clathrin coat, which generates clathrin-coated vesicles (CCV). Although it is clear that in most eukaryotes the clathrin adaptor complex-1 (AP-1) plays a critical role in TGN sorting, growing evidence suggests that it may play a role in both export and retention, moreover, AP-1 shows genetic interaction with several other sorting complexes, suggesting that communication between complexes may help maintain the protein sorting function. CCV assembly has been extensively analyzed in the yeast Saccharomyces cerevisiae. In this yeast, several clathrin adaptors are sequentially recruited to TGN membranes through a coordinated mechanism that depends on PtdIns P and the Arf1 GTPase. The adaptor complex GGA is first recruited, followed by AP-1. Deletion of Gga2 alters the dynamics of the recruitment of AP-1. In contrast, AP-1 has minor mechanistic effects in the assembly of the other clathrin adaptor complexes. Chlathrin and its adaptor complexes have a general role in the regulation of the traffic of multiple proteins to the pre-vacuolar compartment (PVC) facilitating their recycling back to the TGN. Accordingly, the involvement of these complexes in the late endosomal traffic of multiple amino acid transporters has been described. Recycling from the PVC has been well characterized for TGN resident proteins like Kex2, Vps10, and Tlg1. However, a role for clathrin in the formation of a distinct subset of secretory vesicles has also been reported, but the nature of these vesicles and their cargos has remained elusive.

The function of clathrin and its adaptors in the anterograde traffic from the TGN to PM is poorly understood, yet another sorting complex, exomer, has a more established role in this TGN to PM traffic. The exomer complex is a cargo adaptor required for the delivery of three cargoes to the PM, the major chitin synthase, Chs3, Fus1, and Pin2, all three integral transmembrane proteins. Exomer consists of a tetramer formed by a dimer of the scaffold protein Chs5 and two accessory proteins that are encoded by four different genes: the paralogous BCH1/BUD7 and CHS6/BCH2 gene pairs. Together these four proteins are called the ChAPs (Chs5-Arf1 binding proteins). The ChAPs are thought to bind directly to cargos, Arf1, and membranes thereby acting as the cargo recognition face of the complex. The current view is that any two of the four ChAPs can be incorporated into the exomer complexes, providing different functionalities. For example, only an exomer containing the Chs6 ChAP is able to mediate the traffic of Chs3 to the PM, because Chs3 interacts physically with the exomer through the Chs6 ChAPs. In contrast, the Bch1 Bud7 paralogous proteins appear to interact directly with the TGN membrane to favor the membrane curvature required for vesicle formation.

Interestingly, all known exomer cargos are also subject to AP-1-mediated traffic. This was first reported for Chs3, where the deletion of the AP-1 complex restores Chs3 PM localization in cells lacking exomer. This restoration is thought to indicate a role for clathrin and AP-1 in the retention of Chs3 at the TGN, which allows the exomer to deliver Chs3 to the PM in a cell cycle or stress-regulated manner. The mechanistic relationship between exomer and AP-1 complexes in this retention mechanism is unclear. In addition, exomer is well conserved in other fungi, and it has been recently reported that Schizosaccharomyces pombe exomer interacts functionally with clathrin adaptors as a means to maintain the integrity of diverse cellular compartments. Taken together, these results, reported in several organisms, highlight the potential multiple levels of interaction among exomer and other TGN complexes in order to facilitate protein traffic.

In this work, we explored additional roles for exomer in protein traffic and its unsolved relationship with TGN clathrin adaptors using multiple approaches. We show that exomer not only facilitates the anterograde traffic of several integral PM proteins from the TGN, but also plays a general role in protein traffic by modulating the assembly of TGN clathrin adaptors thus regulating proper traffic from the TGN to the PVC. Finally, we conclude that exomer maintains different associations with cargos and clathrin adaptors, which differ along the fungal lineage.
2 | MATERIALS AND METHODS

2.1 | Yeast strains construction

The yeasts strains used throughout this work were made in the W303, BY4741 or X2180 genetic backgrounds as indicated in Table 1. Cells were transformed using the lithium acetate/polyethylene glycol procedure. Gene deletions were made using a PCR-mediated gene replacement technique, using different deletion cassettes based on the natMX4, kanMX4, or hphNT1 resistance genes. For the insertion of the GAL1 promoter in front of ORFs, the cassette was amplified from pFA6a-kanMX4::pGAL1 (Table 2). Proteins were tagged chromosomally at their C-terminus with 3xHA, GFP, mCherry, and Venus CT or NT fragments, employing integrative cassettes amplified from pFA6a-3xHA::hphMx6/pFA6a-GFP::hphMx6/pFA6a-GFP::natMx4/pFN21/pFA6a-VN::HIS3Mx6/pFA6a-VC::kanMX6. The Delitto Perfetto technique was performed to generate the internal gene modifications within the genome. In brief, this approach allows for in vivo mutagenesis using two rounds of homologous recombination. The first step involves the insertion of a cassette containing two markers at or near the locus to be altered and the second involves complete removal of the cassette and transfer of the expected genetic modification to the chosen DNA locus as previously described.

2.1.1 | Construction of TAT252-53-3xHA

To obtain a fully functional tagged version of Tat2, we generated a chromosomally internally tagged version of Tat2 in a region suitable for causing less reduced interference in Tat2 function, regulation, and transport (between amino acids 52-53) using the Delitto Perfetto technique. In contrast to the GFP versions, this HA-tagged protein fully complemented the tat2Δ-associated phenotypes, and importantly, had no effect on the chs5A requirement for external tryptophan. For microscopic localization, we used a GFP C-terminus tagged version of the protein. This protein is functionally based on the complementation of the tat2Δ mutant, but showed a reduced rate of endocytosis.

The C. albicans mutants were generated as previously described.

2.2 | Media and growth assays

Yeast cells were grown at 28°C in YEPD (1% Bacto yeast extract, 2% peptone, 2% glucose), in SD medium (2% glucose, 0.7% Difco yeast nitrogen base without amino acids) or SD-N (2% glucose, 0.16% Difco yeast nitrogen base without ammonium and amino acids) supplemented with the pertinent amino acids and 2% agar in the case of solid media. Calcofluor white (CW) sensitivity was always tested on YEPD or SD medium buffered with 50 mmol/L potassium phthalate at pH 6.2 as described.

2.2.1 | C. albicans media

LEE (2% agar, 0.5% (NH4)2SO4, 0.25% K2HPO4, 0.02% MgSO4·7H2O, 0.5% NaCl, 0.05% proline, 1.25% glucose), LEE NAGA (LEE w/o glucose +1.25% N-acetylglucosamine), LEE SERUM (LEE +4% fetal bovine serum) and M199 (M199 [Gibco BRL], 2% agar, 80 mg/L of uridine).

2.2.2 | Drop tests

To assess the growth phenotypes, cells of each tested strain from early logarithmic cultures were resuspended in water and adjusted to an OD600 of 1.0. Tenfold serial dilutions were prepared and drops were spotted onto the appropriate agar plates containing media supplemented as indicated. Plates were incubated at 28°C for 2-5 days.

2.2.3 | Quantification of half maximal inhibitory concentration (IC50)

Sensitivity to myriocin and sertraline was analyzed in liquid YEPD media by growing strains in a 96-well plate with different drug concentrations and measuring the OD600 using a Spectra Max 340PC plate reader as described.

2.3 | Fluorescence microscopy

Yeast cells expressing GFP/mCherry/Venus-tagged proteins were grown to early logarithmic phase in SD medium supplemented with 0.2% adenine. Living cells were visualized directly by fluorescence microscopy. The bimolecular fluorescence complementation (BIFC) technique was used to analyze proximity among different proteins in vivo. For CW staining, 50 μg/mL of CW was directly added to the fresh cells growing in YEPD and the cultures were incubated at 28°C for 1 hour before images were taken.

For non-quantitative purposes, images were routinely obtained using a Nikon 90i Epifluorescence microscope (x100 objective; NA: 1.45) equipped with a Hamamatsu ORCA ER digital camera, specific Chroma filters (49000 ET-DAPI, 49002 ET-GFP, 49003 ET-YFP, 49005 ET-DsRed) and controlled by MetaMorph software. Images for quantitative purposes, such as co-localization, particle description or stream time-lapse of
**TABLE 1**  Yeast strains used

| Strain   | Genotype                                                                 | Origin/Reference |
|----------|---------------------------------------------------------------------------|------------------|
| CRM67    | W303, mat a (leu2-3,112 trp1-1 can1-100 ara3-1 ade2-1 his3-11,15)           | Lab. collection  |
| CRM2268  | W303, mat a, chs5Δ::natMx4                                                 | Lab. Collection  |
| CRM3066  | W303, mat a, bch1Δ::kanMx4 bud7Δ::natMx4                                   | 36               |
| CRM3081  | W303, mat a, chs6Δ::kanMx4 bch2Δ::natMx4                                   | 36               |
| CRM3851  | W303, mat a, tat2Δ::hphNT1                                                  | This study       |
| CRM3853  | W303, mat a, chs5Δ::natMx4 tat2Δ::hphNT1                                   | This study       |
| CRM3909  | W303, mat a, PGAL1-TAT2::KanMx4                                            | This study       |
| CRM3917  | W303, mat a, chs5Δ::natMx4 PGAL1-TAT2::KanMx4                               | This study       |
| CRM2868  | W303, mat a, PGAL1-GFP-SSY1::KanMx4                                        | This study       |
| CRM2871  | W303, mat a, chs5Δ::natMx4 PGAL1-GFP-SSY1::KanMx4                           | This study       |
| CRM3811  | W303, mat a, STP1-3xHA::hphNT1                                              | This study       |
| CRM3825  | W303, mat a, STP1-3xHA::hphNT1 chs5Δ::natMx4                                | This study       |
| CRM3813  | W303, mat a, GLN3-3xHA::hphNT1                                              | This study       |
| CRM3827  | W303, mat a, GLN3-3XHA::hphNT1 chs5Δ::natMx4                                | This study       |
| CRM3023  | W303, mat a, GTR1-GFP::hphNT1                                               | This study       |
| CRM3032  | W303, mat a, chs5Δ::natMx4 GTR1-GFP::hphNT1                                 | This study       |
| CRM3017  | W303, mat a, TCO89-GFP::hphNT1                                              | This study       |
| CRM3020  | W303, mat a, chs5Δ::natMx4 TCO89-GFP::hphNT1                                | This study       |
| CRM2972  | W303, mat a, GAP1-GFP::hphNT1                                               | This study       |
| CRM2979  | W303, mat a, chs5Δ::natMx4 GAP1-GFP::hphNT1                                 | This study       |
| CRM2894  | W303, mat a, TAT2-GFP::hphNT1                                               | This study       |
| CRM2903  | W303, mat a, chs5Δ::natMx4 TAT2-GFP::hphNT1                                 | This study       |
| CRM3531  | W303, mat a, MUP1-GFP::KanMx4                                               | This study       |
| CRM3540  | W303, mat a, MUP1-GFP::KanMx4 chs5Δ::natMx4                                 | This study       |
| CRM3882  | W303, mat a, TAT252-53-3xHA (internal by Delitto Perfetto)                  | This study       |
| CRM3890  | W303, mat a, TAT252-53-3xHA chs5Δ::kanMx4                                   | This study       |
| CRM3862  | W303, mat a, bul1Δ::kanMx4                                                  | This study       |
| CRM3903  | W303, mat a, bul2Δ::hphNT1                                                  | This study       |
| CRM3880  | W303, mat a, bul1Δ::kanMx4 bul2Δ::hphNT1                                    | This study       |
| CRM3864  | W303, mat a, chs5Δ::kanMx4 bul1Δ::kanMx4                                    | This study       |
| CRM3915  | W303, mat a, bul2Δ::hphNT1 chs5Δ::natMx4                                   | This study       |
| CRM3888  | W303, mat a, bul1Δ::kanMx4 bul2Δ::hphNT1 chs5Δ::natMx4                     | This study       |
| CRM1700  | W303, mat a, rcy1Δ::kanMx4                                                  | Lab. Collection  |
| CRM2160  | W303, mat a, rcy1Δ::kanMx4 chs5Δ::natMx4                                   | Lab. Collection  |
| CRM3155  | W303, mat a, aps1Δ::kanMx4                                                  | This study       |
| CRM3157  | W303, mat a, chs5Δ::natMx4 aps1Δ::kanMx4                                    | This study       |
| CRM3520  | W303, mat a, gga1Δ::natMx4                                                  | Lab. Collection  |
| CRM3523  | W303, mat a, gga2Δ::hphNT1                                                  | Lab. Collection  |
| CRM3621  | W303, mat a, gga2Δ::hphNT1 gga1Δ::natMx4                                   | Lab. Collection  |
| CRM3905  | W303, mat a, gga2Δ::hphNT1 gga1Δ::natMx4 chs5Δ::kanMx4                     | This study       |
| CRM3949  | W303, mat a, gga1Δ::natMx4 chs5Δ::kanMx4                                    | This study       |
| CRM3950  | W303, mat a, gga2Δ::hphNT1 chs5Δ::kanMx4                                    | This study       |
| CRM3526  | W303, mat a, rcy1Δ::kanMx4 gga1Δ::natMx4                                   | Lab. Collection  |

(Continues)
## Table 1 (Continued)

| Strain   | Genotype                                                                 | Origin/Reference       |
|----------|---------------------------------------------------------------------------|------------------------|
| CRM3602  | W303, mat a, rcy1Δ:: kanMx4 gga2Δ:: hphNT1                                 | Lab. Collection        |
| CRM4025  | W303, mat a, rcy1Δ:: kanMx4 aps1Δ:: hphNT1                                 | This study             |
| CRM3432  | W303, mat a, APL4-VC::kanMx4 CHS5-VN::HIS3                                | This study             |
| CRM3477  | W303, mat a, CHS5-VC::kanMx4                                             | This study             |
| CRM3977  | W303, mat a, CHS5-VN::kanMx4                                             | This study             |
| CRM4003  | W303, mat a, CHS5-VN::kanMx4 MUP1-VC::HIS3                                | This study             |
| CRM4070  | W303, mat a, CHS5-VN::kanMx4 GGA2-VC::HIS3                                | This study             |
| CRM2879  | W303, mat a, SEC7-mRuby2::kanMx4                                         | This study             |
| CRM2882  | W303, mat a, chs5Δ::natMx4 SEC7-mRuby2::kanMx4                           | This study             |
| CRM4088  | W303, mat a, arf1Δ::kanMx4                                               | This study             |
| CRM4090  | W303, mat a, chs5Δ::natMx4 arf1Δ::kanMx4                                 | This study             |
| CRM1278  | W303, mat a, chs3Δ:: URA3 chs5Δ:: natMx4                                 | Lab. Collection       |
| CRM1590  | W303, mat a, chs3Δ:: natMx4                                              | Lab. Collection       |
| CRM3089  | W303, mat a, bch1Δ::kanMx4 bud7Δ:: natMx4 chs3Δ:: hphNT1                 | This study             |
| CRM3091  | W303, mat a, chs6Δ::kanMx4 bch2Δ:: natMx4 chs3Δ:: hphNT1                 | This study             |
| CRM4098  | W303, mat a, chs3Δ:: URA3 chs6Δ::kanMx4                                  | This study             |
| CRM3534  | W303, mat a, CHS5-VN::kanMx4 gga2Δ:: hphNT1                               | This study             |
| CRM3511  | W303, mat a, CHS5-VN::kanMx4 chs6Δ:: natMx4 bch2Δ:: hphNT1               | This study             |
| CRM3668  | W303, mat a, CHS5-VN::kanMx4 bch1Δ:: hphNT1 bud7Δ:: natMx4               | This study             |
| CRM3641  | W303, mat a, CHS5-VN::kanMx4 chs7Δ:: hphNT1                               | This study             |
| CRM3674  | W303, mat a, CHS5-VN::kanMx4 chs3Δ:: URA3                                  | This study             |
| CRM3511  | W303, mat a, CHS5-VN::kanMx4 chs6Δ:: natMx4                               | This study             |
| CRM3676  | W303, mat a, CHS5-VN::kanMx4 chs6Δ:: natMx4 chs3Δ:: URA3                 | This study             |
| CRM3432  | W303, mat a, APL4-VC::kanMx4                                             | This study             |
| CRM3453  | W303, mat a, APL4-VC::kanMx4 gga2Δ:: hphNT1                               | This study             |
| CRM3436  | W303, mat a, chs3Δ::URA3 APL4-VC::kanMx4                                 | This study             |
| CRM2248  | W303, mat a, CHS5-mCherry::natMx4 APL4-GB:: hphNT1                        | Lab. Collection       |
| CRM2533  | W303, mat a, CHS5-mCherry::natMx4 GGA1-GB:: hphNT1                        | Lab. Collection       |
| CRM808   | BY4741, mat a (his3Δ1, leu2Δ0, met15Δ0, ura3Δ0)                            | EUROSCARF             |
| CRM1435  | BY4741, mat a, chs3Δ::natMx4                                             | Lab. Collection       |
| CRM2453  | BY4741, mat a, chs3Δ::natMx4 chs5Δ::hphNT1                                | Lab. Collection       |
| CRM3922  | BY4741, mat a, chs5Δ::natMx4                                             | Lab. Collection       |
| CRM3924  | BY4741, mat a, art1Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3926  | BY4741, mat a, art2Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3928  | BY4741, mat a, art3Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3930  | BY4741, mat a, art4Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3932  | BY4741, mat a, art5Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3934  | BY4741, mat a, art6Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3935  | BY4741, mat a, art7Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3937  | BY4741, mat a, art8Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3939  | BY4741, mat a, art9Δ::kanMx4 chs5Δ::natMx4                                | This study             |
| CRM3941  | BY4741, mat a, art10Δ::kanMx4 chs5Δ::natMx4                               | This study             |
| CRM4019  | BY4741, mat a, GGA2-GFP::hphNT1 APL4-mCherry:: natMx4                    | This study             |
| CRM2761  | X2180-1A, mat a (SUC2 mal mel gal2 CUP1)                                   | Francisco del Rey     |
TGN-tagged proteins were acquired in a Spinning Disk confocal microscope (Olympus IX81 with Roper technology) with an Evolve EMCCD camera, 100X/1.40 Plan Apo lens, 488 nm/561 nm lasers, 525/45 - 609/54 Semrock emission filters and controlled by MetaMorph 7.7 software.

### 2.4 Protein extracts and immunoblotting

The trichloroacetic acid (TCA) protocol was used for protein processing for the Western blot analyses. Extracts were made using equal numbers of cells from logarithmic growing cultures. Cells were centrifuged, resuspended in 20% TCA, and frozen at −80°C for at least 3 hours. The samples were then thawed on ice and centrifuged cells were disrupted in 1.5 mL tubes with 100 μL of 20% TCA and glass beads (0.45 mm, SIGMA), during 3 pulses of 30 seconds with an intensity of 5.5 in a Fast prep (FP120, BIO101). Extracts were transferred to new tubes and 5% TCA was added to dilute TCA concentration to 10%. Precipitated proteins were collected by centrifugation at 900 g for 10 minutes and the supernatant was completely discarded. Pelleted proteins were resuspended in 50 μL of 2x Sample Buffer (100 mmol/L Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 25 mmol/L DTT, and traces of bromophenol blue) by vortexing, followed by the addition of 50 μL of 2 mol/L Tris-HCl pH 7.5. Samples were maintained on ice throughout this process. Finally, the extracts were heated to 37°C for 30 minutes (for multipass transmembrane proteins) or 95°C for 5 minutes (for other proteins) and centrifuged for 5 minutes at 15000 g. The supernatant was collected and 15 μL were used for Western blot analysis.
TABLE 2  Plasmids used

| Plasmid        | Genotype          | Origin/Reference |
|----------------|-------------------|-----------------|
| CRM160         | pRS313 (HIS3)     | Lab collection  |
| CRM161         | pRS314 (TRP1)     | Lab collection  |
| CRM264         | pRS315 (LEU2)     | Lab collection  |
| CRM265         | pRS316 (URA3)     | Lab collection  |
| CRM166         | pRS426 (URA3)     | Lab collection  |
| CRM2546        | pAG25 (natMX4)    | 32              |
| CRM1188        | pUG6 (kanMX4)     | 32              |
| CRM2546        | pUG72 (URA3)      | 32              |
| CRM1451        | pFA6a-hphNT1      | 32              |
| CRM1807        | pFA6a-3xHA::hphNT1| 34              |
| CRM1995        | pFA6a-GFP::natMX4 | 34              |
| CRM1811        | pFA6a-GFP::natMX4 | 34              |
| CRM2653        | pFN21 (mCherry::natMX4) | 34         |
| CRM2037        | pFA6a-kanMX4-pGAL1| 33              |
| CRM2827        | pFA6a-kanMX4-pGAL1-GFP | 33          |
| CRM2360        | pGSHU (CORE Delitto Perfetto) | 35          |
| CRM3469        | pFA6a-VenusCterminal::HIS3 | 38        |
| CRM3470        | pFA6a-VenusNterminal::HIS3 | 38        |
| CRM3471        | pFA6a-VenusCterminal::kanMX4 | 38       |
| CRM3472        | pFA6a-VenusNterminal::kanMX4 | 38       |
| CRM2328        | pFA6a-link-yomRuby2::CaURA3 | 39       |
| CRM1131        | pRS315::CHS3-GFP  | 40              |
| CRM3456        | pRS315::CHS3-YN::HIS3 | This study        |
| CRM2084        | pRS315::CHS3L245-GFP | 41        |
| CRM1868        | pRS315::GFP-SNC1  | Anne Spang      |
| CRM3236        | pFA-CalHIS1       | 42              |
| CRM3238        | pFA-CalARG4       | 42              |
| CRM3240        | pFA-CalURA3       | 42              |
| CRM2583        | pFA-GFP-SAT1      | 43              |

Extracts were separated on 7.5% SDS-PAGE and transferred to PVDF membranes. The membranes were then blocked with TBST (Tris-buffered saline with 0.1% Tween 20) supplemented with 3% non-fat dry milk for 1 hour and incubated with the corresponding antibodies in TBST with 3% milk for 2 hours at room temperature (RT) or overnight (O/N) at 4°C: anti-GFP JL-8 monoclonal antibody (Living colors, Clontech), anti-HA 12CA5 (Roche), anti-tubulin (T5162 Sigma). The blots were developed using anti-Rsp6-P (Cell Signaling Tech: Phospho-Ser/Thr Akt Substrate Antibody #9611s) and 5% BSA replaced the non-fat dry milk in all steps. After three washes with TBST, the membranes were incubated for 50 minutes together with the secondary antibodies in TBST with 3% milk: polyclonal anti-mouse or anti-Rabbit conjugated with horseradish peroxidase. After three washes with TBST, the blots were developed using the ECL kit (Advansta).

2.5  Subcellular fractionation by centrifugation in a sucrose gradient

For subcellular fractionations, 50 mL of culture with an OD₆₀₀ of 0.8-1 were collected and NaN₃ and NaF were added up to a final concentration of 20 mmol/L. The cultures were centrifuged at 4°C at 3500 g for 4 minutes, resuspended with ice-cold water in 1.5 mL tubes, centrifuged at 3500 g for 2 minutes, and resuspended in 1 mL of Azide Buffer (10 mmol/L DTT, 20 mmol/L NaN₃, 20 mmol/L NaF, 100 mmol/L Tris-HCl pH 9.4). After a 10-minute incubation step at RT, the samples were centrifuged at 3500 g for 2 minutes and resuspended in 600 μL of Spheroplast Buffer (1 mol/L sorbitol, 20 mmol/L NaN₃, 20 mmol/L NaF, 10 mmol/L Tris-HCl pH 7.5 in YEPD medium). Afterward, 60 μL of zymolyase (100T, unfiltered, 4.76 mg/mL) were added and incubated at 30°C during 30-40 minutes under gentle mixing until spheroplasts were produced based on microscopic analysis. The spheroplast samples were then washed twice with spheroplast buffer and collected by 500 g centrifugation for 4 minutes at 4°C. The washed spheroplasts were incubated with 300 μL of lysis buffer (10% sucrose, protease inhibitors, 1 mmol/L PMSF, 1 mmol/L EDTA, 20 mmol/L Tris-HCl pH 7.5) and incubated 10 minutes at RT with gradual pipetting (6-8 times). Lysis was microscopically assessed. Then, cell debris was removed by centrifugation at 500 g for 4 minutes at 4°C, the supernatants were collected and 250 μL of the supernatants were layered on the top of a mini-step sucrose gradient (EDTA 5, 50 mmol/L Tris-HCl pH 9.4) made as follows: 300 μL 55%, 750 μL 45%, 500 μL 20% sodium citrate, pH 4.5, and 20 mmol/L (NH₄)₂SO₄, and 2% glucose. The OD₆₀₀ was measured to refer values to cell number. 2.6  Tryptophan uptake assay

Tryptophan was measured adapting protocols previously described. Specifically, cells were grown to early log phase in 50 mL of SD medium (without Trp) at 30°C until an OD₆₀₀ of 0.4-0.8. Then, the cells were washed twice with wash buffer (10 mmol/L sodium citrate, pH 4.5, and 20 mmol/L (NH₄)₂SO₄) and resuspended in 3.6 mL of incubation medium (10 mmol/L sodium citrate, pH 4.5, 20 mmol/L (NH₄)₂SO₄, and 2% glucose). The OD₆₀₀ was measured to refer values to cell number.
The assay was initiated by the addition of 400 μL of radiolabeled tryptophan solution (390 μL of the incubation medium and 10 μL of L-[5-3H]-tryptophan at 31 Ci/mmol GE healthcare, UK). Two aliquots (500 μL) were collected at each time point and chilled by the addition of 1 mL of the ice-cold incubation medium. Cells were collected by filtration through a nitrocellulose filter (0.45 μm pore size, 25 mm diameter [Millipore HAWP]) and washed three times with chilled water. Moist filters were transferred to Filter Count solution (Perkin Elmer). Radioactivity was measured using a Tri-Carb® 4910 TR liquid scintillation counter (Perkin Elmer).

2.7 Digital quantification, statistics, and figure design

Microscopy and Western blot image processing and quantification were performed using ImageJ-FIJI software (1.48k version, NIH). For the quantification of dot co-localization, pre-filtering with a custom built ImageJ Macro (Macro1, see Table 3) was used followed by the analysis of the co-localization using the JACoP ImageJ plugin (co-localization based on centers of mass-particles coincidence, particle size 4-∞ pixels). For the particle descriptors (intensity and area), ROIs were selected using a custom built Macro (Macro2, see Table 3), applying the same intensity threshold per experiment and loaded to ROI Manager with Analyze Particles (0.07-∞ μm², exclude on edges). In the case of Chs3-GFP TGN-EE structures, due to the difficulty of the segmentation, dots were selected manually for maximum intensity and maximum diameter quantification. A more detailed description of the macros used is presented in the supplementary materials.

For the quick time-lapse experiments, continuous images were acquired through the streaming mode on a Spinning Disk microscope in three z-planes separated by 0.2 μm to avoid loss of the highly dynamic TGN-structures in z-axis and to partially reduce photo-bleaching. Z-maximum intensity projections were analyzed manually or with the TrackMate ImageJ plugin. For the Sec7-mR2 structures, tracking was performed using the TrackMate ImageJ plug-in (LoG detector, Diameter 0.5 μm, Threshold 80, Median filter, Sub-pixel loc.; LAP Tracker; Frame to Frame 0.5 μm, No Gap, No Split, No Merge, Duration ≥8s) and the Extract track stack option (half of the vesicles extracted from each channel). The average recruitment duration (temporal region with intensity ≥25% of the maximum intensity per channel), as well as the temporal distance between maximum intensity peaks referring to Chs5-mCh for 30 trajectories, were manually calculated.

To obtain an unbiased measurement of the cellular polarization of PM proteins, the daughter/mother plasma membrane signal coefficient (polarization coefficient) of single cells was determined as described.26

Image measurements were statistically analyzed using the T test for unpaired data in GraphPad Prism 6 software (GraphPad Software, Inc, La Jolla, USA). Significantly different values (P < .05, P < .01, P < .001, P < .0001) are indicated (*, **, ***, ****).

The presented images were prepared using Adobe Photoshop CS5 and Adobe Illustrator CS5 (San José, CA, USA) software. All images shown in each series were acquired under identical conditions and processed in parallel to preserve the relative intensities of fluorescence for comparative purposes. If not indicated, the scale bar represents 5 μm.

3 RESULTS

3.1 Exomer mutants show ammonium sensitivity due to the reduced uptake of tryptophan

Exomer has been described to function as a cargo adaptor complex based on its role in the transport of Chs3, the catalytic subunit of the major chitin synthase in budding yeast.17,21,24 However, our recent work, based on the evolutionary characterization of exomer function across the fungi kingdom, suggests that exomer may have additional functions.29 More importantly, S. cerevisiae exomer mutants showed multiple phenotypes that cannot be explained by known cargoes of exomer such as sensitivity to ammonium.21,29

In an effort to better understand the functionality of exomer, we first confirmed the ammonium sensitivity of the exomer mutant chs5Δ by showing that this mutant grew poorly in YEPD supplemented with 0.2 mol/L ammonium (Figure 1A). This sensitivity was also observed in SD media (Figure S1A). Interestingly, the absence of the two paralogous pairs of ChAPs produced different growth phenotypes (Figure 1A). The bch1Δ bud7Δ double mutant was as sensitive to ammonium as chs5Δ; however, chs6Δ bch2Δ double mutant was not sensitive to ammonium. This result is notable since Chs6/Bch2 are known to function as cargo adaptors, whereas Bch1/Bud7 are thought to function in membrane association without cargo selectivity.22-24 These results could,
therefore, reflect a role for exomer that is independent of its function as cargo adaptor.

A first indication for the source of the ammonium sensitivity of the exomer mutants came from the observation that the \textit{chs5} \textit{\Delta} mutant was not sensitive to ammonium in the prototrophic X2180 background (Figure S1A). Although ammonium toxicity in yeast is poorly understood, one mechanism for ammonium detoxification involves the active excretion of amino acids across the PM.\textsuperscript{49} We hypothesized that as cells excrete amino acids as a response to ammonium toxicity, they deplete internal pools of amino acids and the prototrophic strain is able to compensate by synthesizing higher levels of the essential amino acids. We further pinpointed the key auxotrophic requirement through the observation that the \textit{chs5} \textit{\Delta} mutant was not sensitive to ammonium in the BY strain, which differs from W303 in that it is not auxotrophic for tryptophan. In order to confirm this, we transformed the \textit{chs5} \textit{\Delta} mutant in the W303 background with different plasmids that restore the ability to synthesize amino acids for each auxotrophy. The restoration of the ability to synthesize tryptophan by a plasmid encoding \textit{TRP1}, strongly reduced the ammonium sensitivity of the \textit{chs5} \textit{\Delta} mutant, while the plasmid containing \textit{HIS3} and \textit{URA3} genes showed no effect on ammonium sensitivity (Figure S1B). In contrast, restoration of the ability to synthesize leucine by a plasmid encoding \textit{LEU2} slightly reduced ammonium sensitivity. Moreover, the addition of tryptophan to the medium also abolished ammonium sensitivity in all strains. These results could indicate that the \textit{chs5} \textit{\Delta} mutant has a reduced uptake of tryptophan, and, therefore, this mutant may require higher amounts of external tryptophan for growth. Consistent with this model, the wild-type auxotroph strain in SD media could form colonies with as little as 0.001 mg/mL of tryptophan, while the \textit{chs5} \textit{\Delta} mutant required eight times more tryptophan in the medium to obtain substantial colony growth (Figure 1B). In order to confirm this, we measured tryptophan uptake directly (Figure 1C). Consistent with the increased requirement for extracellular tryptophan, tryptophan uptake was severely reduced in the absence of exomer compared to wild-type yeast. However, uptake was not as low as cells lacking the tryptophan permease Tat2.\textsuperscript{50} Together these results show that in exomer mutants, the deficient uptake of tryptophan restricts the growth of tryptophan auxotrophs in a low concentration of tryptophan or in the presence of ammonium.

### TABLE 3 ImageJ Macros used

**Macro1: Pre-filtering for dot co-localization**

```
showMessage("Open Channel Red"); //Select Channel Red open();
red =getTitle();
showMessage("Open Channel GFP"); //Select Channel GFP open();
gfp =getTitle(); //Then, we are going to filter both channels selectWindow(gfp);
run("Median...", "radius=1 stack"); //Filtering
run("Unsharp Mask...", "radius=2 mask=0.5 stack"); //Filtering
selectWindow(red);
run("Median...", "radius=1 stack"); //Filtering
run("Unsharp Mask...", "radius=2 mask=0.5 stack"); //Filtering
run("JACoP "); //Open JACoP plugin
```

**Macro2: Analyze dots**

```
showMessage("Open the Image"); //Select image open();
run("Duplicate...", ""); //We want to do the segmentation in a copy of the original, therefore we duplicate
run("Median...", "radius=1"); //Filtering
run("Unsharp Mask...", "radius=2 mask=0.50"); //Filtering
run("Threshold..."); // to open the threshold window if not opened yet
waitForUser("Set the threshold and press OK, or cancel to exit macro"); // pauses the execution and lets you access ImageJ manually
run("Analyze Particles..."); //to take the ROIs
waitForUser("Finally, use these ROIs in the original window"); //A note to correctly continue after the macro
```

**Macro3: Counting colonies**

```
showMessage("Open the Image"); //Select image with colonies open();
rename("Initial");
run("Duplicate...", "");
run("Threshold..."); // to open the threshold window if not opened yet
waitForUser("set the threshold and press OK, or cancel to exit macro"); // pauses the execution and lets you access ImageJ manually as long as you don't press OK, which resumes the macro execution
run("Convert to Mask"); // to binarize, if you use this command, don't press ‘Apply’ in the threshold window
run("Watershed"); //to separate close colonies
rename("Mask");
waitForUser("Calibration: Draw a line along the Petri dish and introduce the known size in Analyze/SetScale, then press OK");
run("Threshold...");
```

(Continues)
**FIGURE 1** Ammonium sensitivity of the exomer mutants is due to defects in amino acid uptake. A. Overnight (O/N) cultures of the indicated strains in the W303 genetic background were diluted and plated onto YEPD media supplemented with 0.2 mol/L NH₄Cl. Note the similar level of sensitivity of the *chs5Δ* and *bch1Δ/bud7Δ* mutants. B, O/N cultures of the indicated strains in the W303 background were diluted and spread on synthetic defined media (SD) supplemented with the indicated concentrations of tryptophan. Note that low concentrations (%) of tryptophan (Trp) allowed the wild-type strain to completely grow, but was unable to efficiently support the growth of the *chs5Δ* mutant. The numbers indicate the average diameter ± standard deviation of the colonies growing on the different media quantified using ImageJ (Macro3). Also, refer to supplemental Figure S1 for additional data on ammonium sensitivity. C, L-[5-3H]-tryptophan uptake in SD media by the indicated X2180-derived prototrophic strains. Numeric values indicate the incorporation rate (dpm/A600/min) calculated as the slope of the linear regression made using 10 to 40 min time points. Note the absence of Trp incorporation in the *tat2Δ* mutants used as the control. D, Sensitivity of the indicated X2180-derived strains to toxic amino acids analogs. Growth was analyzed in YEPD supplemented with the indicated concentrations of the following analogs: Sulfometuron methyl; L-canavanine; L-azetidin-2-carboxylate (AzC); 3-aminotriazole (3-AT) and L-histidine as indicated. E, Western blot of the total protein from the cellular extracts of strains carrying *STP1*-3xHA integrated at the chromosomal locus. Cultures starved for 1 h in YNB-N-aa media were transferred to rich YEPD media and samples were taken at 0 and 30 min. Note the similar processing of the Spt1 transcription factor in the wild-type and *chs5Δ* strains. F, Induction of Rsp6 phosphorylation after adding glutamine. X2180 strains grown on SD complete media were transferred to media lacking a nitrogen source for 1 h; glutamine (500 μg/mL) was then added to the media. Note that the kinetics of the phosphorylation induced in the wild type and *chs5Δ* are similar. Phosphorylation was determined by Western blot using a phospho-(Ser/Thr) Akt substrate antibody (#9611s, Cell Signaling Tech). Also, refer to supplemental Figure S2 for the rationale behind these experiments.
3.2 | Deficient Trp uptake of exomer mutants is directly linked to Tat2 permease but independent of nitrogen source regulation

In yeast, tryptophan is primarily transported by one of the two permeases, the general amino acid permease, Gap1, and the high-affinity specific Trp-permease, Tat2.\textsuperscript{51} In media containing high ammonium levels, such as SD, Gap1 is not expressed; therefore, we hypothesized that the \( \text{chs5} \Delta \) defect could be associated with defects in Tat2 localization or function. Consistent with this hypothesis, overexpression of \( \text{TAT2} \) suppressed the ammonium sensitivity of \( \text{chs5} \Delta \) (Figure S1C). These results strongly suggest that ammonium and tryptophan phenotypes observed in \( \text{chs5} \Delta \) are caused by the defective function of Tat2 permease. Notably, the ammonium sensitivity of the double mutant \( \text{tat2} \Delta \text{chs5} \Delta \) was not fully suppressed by external tryptophan (Figure S1C.D), suggesting that the absence of exomer may affect additional amino acid transporters. This conclusion is consistent with the partial alleviation of ammonium sensitivity after \( \text{LEU2} \) introduction (Figure S1B). As an additional test of the effect of exomer on amino acid transporters, we analyzed the sensitivity of exomer mutants to toxic analogs of different amino acids.\textsuperscript{52-56} Sensitivity to these analogs can indicate changes in the plasma membrane levels or activity of the relevant amino acid transporter. In the X2180 background, the exomer mutant \( \text{chs5} \Delta \) was moderately more sensitive to the arginine analog Canavanine, but significantly more resistant to the proline analog AzC, to the \( \text{HIS3} \) inhibitor 3-AT, and to toxic concentrations of histidine (Figure 1D). These results are consistent with a potential defect in the uptake of several amino acids, owing to a defect in the localization or function of several amino acid permeases (AAPs).

Alternatively, the observed phenotypes could simply reflect a defect in the regulation of nitrogen metabolism in the absence of exomer. To test this possibility, we first investigated whether the \( \text{chs5} \Delta \) mutant showed altered signaling through the \( \text{Ssy1p-Ptr3p-Ssy5} \) (SPS) sensor of extracellular amino acids.\textsuperscript{51} (Figure S2B). We first compared the phenotypes of the cells lacking SPS to those of cells lacking exomer. We found that the phenotype of \( \text{ssy1} \Delta \), the core SPS sensor, was not identical to that of \( \text{chs5} \Delta \). Unlike \( \text{chs5} \Delta \), \( \text{ssy1} \Delta \) was resistant to canavanine and sensitive to 3-AT, although similar to \( \text{chs5} \Delta \) it was resistant to AzC (Figure S2A). Moreover, \( \text{chs5} \Delta \text{ssy1} \Delta \) double mutant exhibited phenotypes of sensitivity to canavanine and resistance to AzC, 3-AT, and to toxic concentrations of histidine as that of \( \text{chs5} \Delta \). We then monitored whether loss of exomer disrupted SPS function. We found that the localization of Syyl-GFP in the \( \text{chs5} \Delta \) strain was indistinguishable from the wild type (Figure S2C). More importantly, loss of exomer did not affect the proteolytic processing of the SPS effector Stp1 in response to amino acids, a key step in the SPS signaling pathway\textsuperscript{51} (Figure 1E). Taken together, these observations indicate that the SPS signaling pathway is fully functional in the absence of exomer.

As an additional test for whether exomer controls amino acids signaling, we investigated the TORC1 pathway, which regulates many AAPs.\textsuperscript{51} TORC1 signaling occurs in preferred nitrogen sources like glutamine and during this signaling, two kinase regulatory subunits, Gtr1 and Tco89, are recruited to the vacuolar membrane, from where they trigger the phosphorylation of the small ribosomal subunit, Rsp6, and the Nitrogen Catabolite Repression (NCR) signaling pathway transcription factor, Gln3.\textsuperscript{57,58} (Figure S2D). We found that Gtr1 and Tco89 localized normally at the vacuolar membrane in the \( \text{chs5} \Delta \) mutant (Figure S2E), the phosphorylation timing of Rps6 occurred normally in this mutant upon growth in different nitrogen sources (Figure 1F) and the levels of Gln3 phosphorylation were indistinguishable from control under different nutritional conditions (Figure S2F).

All of these results strongly indicate that nitrogen signaling occurs normally in the absence of exomer. We, therefore, hypothesized that the observed phenotypes might be associated directly with a defective transport of one or several AAPs.

3.3 | Exomer is required for proper intracellular traffic of the Tat2 and Mup1 permeases

In order to test this hypothesis, we first monitored the localization of the amino acid permeases Tat2 and Mup1. These proteins localized at the PM in induction media under steady-state conditions (Figure 2A) in either the wild-type or \( \text{chs5} \Delta \) mutant strains. However, the \( \text{chs5} \Delta \) mutant, Tat2 was conspicuously absent at the vacuole and some of the protein was localized in intracellular spots. Similarly, Mup1 was also localized in intracellular spots in the absence of exomer. Terminally tagged versions of Tat2 show impaired endocytosis (see Materials and Methods section and references therein). Therefore, to confirm whether Tat2 localization changes in \( \text{chs5} \Delta \), we performed subcellular fractionations using an internally HA-tagged version of Tat2 (Figure 2B). In the wild-type strain, Tat2-3xHA localized primarily in the lightest fractions of the gradient together with Pma1, a marker of the plasma membrane fraction. However, the \( \text{chs5} \Delta \) mutant Tat2-3xHA showed a bimodal distribution, with part of the protein co-migrating with Pma1 and the other significant part co-migrating with the TGN/endosomal marker Pep12 in the heavier fractions. These observations suggest that \( \text{chs5} \Delta \) causes partial intracellular accumulation of Tat2 and Mup1 at the TGN/endosomal compartment. This event would lead to a reduction in the levels of the permeases at the PM, leading to the tryptophan and amino acid analog responses described above.

The steady-state localization of AAPs reflects the balance of anterograde transport of newly synthesized proteins,
endocytosis, and recycling. Therefore, the mislocalization of Tat2 or Mup1 could reflect a defect in any of these steps. To test whether exomer contributes to anterograde traffic of Tat2 and Mup1, we used a regulated expression system based on the GAL1 promoter. Growth on galactose induces expression thus allowing us to examine anterograde transport based on...
the arrival of each permease at the PM. One hour after induc-
tion, both proteins were readily apparent at the plasma mem-
brane in both wild-type and chs5Δ cells, suggesting that the
overall rate of anterograde traffic is not dramatically reduced
in chs5Δ cells (Figure 2C). However, both Tat2 and Mup1
were less polarized in the chs5Δ mutant. In wild-type cells,
both Tat2 and Mup1 were highly polarized in the growing
bud, whereas in the chs5Δ mutant a significant amount of
each transporter was observed spread along the PM of the
mother cell. A quantitative analysis of Tat2 and Mup1 dis-
tribution indicated that the PM signal in the daughter cells is
significantly reduced in the absence of the exomer for both
proteins (Figure 2D). Although the functional significance of
the defect in polarized distribution is unclear, these results
indicate that exomer contributes to the polarized delivery of
these proteins to the PM, similarly to what has been described
for Ena1.36

Following on, we examined the effect of exomer on the be-
havior of AAPs after endocytosis. Tat2 is endocytosed after
the depletion of tryptophan from the media and trafficked to
the vacuole for degradation.59 In a wild-type strain, the Tat2-GFP
signal was increased in the vacuole after tryptophan depletion
and the total amount of the protein was significantly reduced
(Figure 2E). However, in the chs5Δ mutant, fluorescence in the
vacuole was reduced compared to the wild type, and intracel-
lar spots became more numerous and intense (Figure 2E). In
addition, the total amount of Tat2 was significantly higher in
the chs5Δ mutant than in the wild type after tryptophan deple-
tion. Mup1 is also rapidly endocytosed, trafficked to the vacu-
ole, and degraded in presence of an excess of methionine in
wild-type cells (Figure 2F).60 Similar to Tat2, in cells lacking
exomer Mup1 traffic to the vacuole was reduced 20 minutes
after adding methionine, with fewer cells showing vacuolar
fluorescence and more cells showing substantial plasma
membrane signal in the chs5Δ mutant compared to the wild
type. This defect was associated with an increase in the num-
ber of cells with bright intracellular spots. However, vacuolar
localization was apparent in chs5Δ after 45 minutes. Analysis
of the total levels of Mup1 by Western blot after adding me-
thionine indicated that protein degradation was significantly
delayed in the absence of exomer (Figure 2F). These results are
consistent with a function for exomer in the proper traffic of
AAPs and may explain the phenotypes associated with chs5Δ
in terms of the cells being sensitive to different levels of tryp-
tophan and toxic amino acids.

3.4 Exomer-dependent recycling of Tat2
differs from that of Chs3

Based on the proposed role of exomer in the recycling of
Chs3,27 we tested whether exomer controlled the recycling of
Tat2. In yeast, the recycling of Tat2 and Mup1 depends
on the f-box protein Rcy1.61 To determine whether exomer
contributes to this step in recycling, we first compared the
phenotypes of rcy1Δ and chs5Δ mutants. We found that
rcy1Δ was sensitive to ammonium, and, as previously
reported, required an external supply of tryptophan for
growth61 (Figure 3A,B). These results are consistent with
a model that proposes that both exomer and Rcy1 may
participate in the recycling of AAPs. Interestingly, the
double rcy1Δ chs5Δ mutant required a greater concentra-
tion of tryptophan for growth than the single-gene dele-
tion mutants, which could suggest Rcy1 and exomer act at
different steps in recycling or that the recycling pathway
is only partially functional in the absence of either factor
(Figure 3B).

To distinguish between these possibilities, we explored
the genetic interactions between chs5Δ and rcy1Δ and addi-
tional regulators of Tat2. We first examined clathrin adap-
tors Gga1 and Gga2, which alter cell surface levels of Tat2
in some mutant backgrounds by controlling sorting from
the TGN to the vacuole.44 We found that chs5Δ and rcy1Δ
showed different effects when combined with the deletion
of gga2Δ, or the gga1Δ gga2Δ double mutant (Figure 3C). The ammonium sensitivity of chs5Δ was suppressed by gga2Δ and more so by the gga1Δ gga2Δ double mutation. In contrast, the rcy1Δ phenotype was not suppressed by gga2Δ. Interestingly, the gga1Δ mutant was sensitive to ammonium on its own, a sensitivity that was additive with the chs5Δ mutant, but not with the rcy1Δ mutant. Together these results suggest that exomer and Rcy1 may affect different steps of AAPs trafficking.

The suppression of the ammonium sensitivity of chs5Δ by gga2Δ is reminiscent of the previously reported suppression of the calcofluor resistance of chs5Δ by gga2Δ.27,62 We, therefore, sought to determine whether the exomer-mediated traffic of Tat2 was similar to the exomer-mediated traffic of Chs3. In addition to Gga1 and Gga2, the clathrin adaptor protein complex AP-1 alters the traffic of Chs3 in exomer mutant cells.27 We first asked whether the deletion of the small subunit of AP-1 (aps1Δ) suppressed the ammonium sensitivity of chs5Δ. Unlike calcofluor resistance, aps1Δ did not suppress the ammonium sensitivity of chs5Δ (Figure 3D). Similarly, aps1Δ did not suppress the sensitivity of chs5Δ to low tryptophan, while gga2Δ did it efficiently (Figure S3), suggesting that the traffic of Chs3 and AAPs are strikingly different.

To further explore the differential requirements for Chs3 and AAP traffic, we explored the role of the arrestin family of ubiquitin ligase adaptors because the recycling of Tat2 is controlled by its ubiquitination mediated by the Bul1 arrestin-like protein.63 Accordingly, bul1Δ suppressed the ammonium sensitivity of the chs5Δ mutant (Figure 3A). In contrast, after analyzing calcofluor sensitivity and staining, the deletion of BUL1 did not restore Chs3 PM transport in
the chs5Δ mutant (Figure 3A,E). Because Bul1 is one of a number of arrestin-like adaptors, we also tested the partially redundant Bul2 ligase, the bul1Δ bul2Δ double mutant, and individual deletions of ten additional arrestin ligases. None of the arrestin mutants suppressed the calcofluor resistance of chs5Δ, highlighting the difference between AAPs and Chs3 (See Figure S4). In summary, while both Tat2 and Chs3 proteins can be re-routed by Gga2 proteins in the absence of exomer, the two proteins likely diverge at one or more steps in their traffic, suggesting that the mechanistic requirement for exomer differs for the two proteins.

3.5 | Exomer is involved in traffic to late endosomes by modulating the proper assembly of the clathrin adaptor complexes

Exomer and clathrin adaptors mediate the traffic of common cargoes including Chs3 and AAPs; however, their functions appear to be largely antagonistic to one another. Mechanistically, this antagonism could be explained by direct physical competition between exomer and clathrin adaptors for cargo, or something more complex. In order to understand the antagonistic roles of exomer and clathrin adaptors, we explored their proximity to one another using bi-molecular fluorescence complementation (BIFC).38 Exomer comes into close proximity to both AP-1 and Gga2, based on the appearance of fluorescent puncta in strains containing Chs5-VN and either Apl4-VC or Gga2-VC. Our BIFC results also confirmed the previously reported physical interaction of exomer with its cargo Chs3 (Figure 4A), but also revealed close proximity of exomer with Mup1 (Figure 4A), suggesting that exomer may play a direct role in Mup1 traffic.

Because BIFC can trap transient proximity between proteins and does not report on the dynamic changes in protein localization, we next addressed the dynamic TGN localization of exomer and clathrin adaptors using two-channel spinning disk-confocal microscopy (Figure 4B, Figure S5). Previous work has established ordered recruitment of clathrin adaptors, with Gga2 reaching peak fluorescence several seconds before AP-1.6,64 We found that exomer is recruited shortly after GGA, and significantly before (21.9 seconds) AP-1 (Figure 4B), a temporal distribution that overlaps with both clathrin adaptor complexes. This distribution is also consistent with the co-localization observed between exomer and GGA and AP-1 complexes (Figure S5E).

Next, we explored whether the absence of exomer affected the localization of clathrin adaptors. In the chs5Δ mutant, Gga2 collapsed in significantly brighter and larger puncta compared to wild-type cells (Figure 4C,D) and similar results were observed for the AP-1 (Apl4) complex. Moreover, co-localization between Gga2 and Apl4 was significantly increased in the chs5Δ mutant compared with the control (Figure 4C,E).

In order to confirm the physiological relevance of these defects, we determined the sensitivity of the chs5Δ mutant to sertraline and myriocin. These drugs affect membrane fluidity and lipid biosynthesis, and selectively inhibit the growth of yeast with impaired AP-1 functions.65,66 We found that chs5Δ mutant was significantly more sensitive to both drugs, showing a half maximal inhibitory concentration (IC50) of 0.087 ± 0.005 and 6.60 ± 0.33 mmol/L to myricin and sertraline, respectively. IC50s significantly (P < .01) lower than those observed for the wild-type strain (0.178 ± 0.011 and 8.16 ± 0.19 mmol/L). This increased sensitivity to both drugs is consistent with altered AP-1 function in the chs5Δ mutant (Figure 4F).

3.6 | Re-exploring the functional link between Arf1 GTPase and the exomer complex

Exomer was described as an Arf1 GTPase-dependent protein complex13,15 and was later co-crystallized with Arf1.14 Interestingly, TGN clathrin adaptors also bind Arf1 GTPase.6,20 Therefore, the antagonistic functions of these complexes may be through direct competition for active Arf1.

We next sought to monitor the effects of exomer on Arf1 localization. Unfortunately, GFP tagging of Arf1 disrupts its function67 and alters TGN/EE morphology (Figure S6A), similarly to what has been described for the null mutant.68 We, therefore, analyzed as an alternative the localization of Sec7, a guanine nucleotide exchange factor (GEF) that stimulates Arf1 activity at the TGN69 promoting clathrin adaptor localization.6 We found that similar to clathrin adaptors, the intensity, and area of Sec7 puncta were greater in the chs5Δ mutant (Figure 5A). Moreover, time-lapse analysis showed that in the exomer mutant chs5Δ the Sec7 dots moved significantly slower and showed reduced track displacement, although the lifespan of the structures was only slightly increased (Figure 5B, Figure S6B,C). The functional significance of this altered movement is unclear but, altogether, our results suggest that exomer could influence the behavior of clathrin adaptors through Arf1/Sec7. In view of this implication of exomer in Arf1/Sec7 dynamics, we sought to revisit the role of Arf1/Sec7 in exomer functionality.

We first addressed the effect of the arf1Δ mutation. Surprisingly this mutant showed increased levels of chitin based on calcofluor staining (Figure 5C, upper panels), which were in clear agreement with increased levels of Chs3 at the bud neck (Figure 5C, lower panels, see arrows and amplified insets), despite the partial accumulation of part of this protein in aberrant TGN/EE structures (Figure 5C, lower panels, see arrowheads). The absence of Arf1 reversed the effect of the chs5Δ mutation on calcofluor staining and Chs3 localization as previously described,27 likely through rerouting Chs3 to the PM in a less polarized fashion (Figure 5C).
Similar results were obtained when we depleted Arf1 by growing the pGAL1-ARF1 strain in glucose. (Figure 5D,E). Moreover, Arf1 depletion in glucose also relieved the calcofluor and tryptophan phenotypes associated with the chs5Δ mutant (Figure 5D,E and S6D). Although the effects of Sec7 were difficult to assess due to its absence being lethal, the transient depletion of Sec7 in the pGAL-SEC7 strains after growth in glucose slightly increased chitin synthesis in the
wild-type strain and restored chitin synthesis in the chs5Δ mutant (Figure 5D,E).

Interestingly, overexpression of either Arf1 or Sec7 in the wild-type strain after growth in galactose caused hypersensitivity to calcofluor (Figure 5D), which in the case of Sec7 could be linked to an increased deposition of chitin toward the bud (Figure 5E). However, the overexpression of either protein did not restore chitin synthesis in chs5Δ. In contrast, overexpression of Sec7, but not of Arf1, alleviated the tryptophan and ammonium phenotypes of chs5Δ mutant (Figure 5D and Figure S6D), suggesting that Sec7 has a different effect on the traffic of amino acid permeases.

Finally, given the ability of exomer to influence the localization of clathrin adaptors, we tested the effect of GGA overexpression on exomer function using the GAL1 promoter. We found that the overexpression of Gga2 in a wild-type strain significantly reduced the recruitment of exomer and AP-1 complexes at the TGN (Figure S7A-D). However, this overexpression did not produce a significant physiological effect on chitin synthesis or ammonium sensitivity, probably because Gga2 exerts a pleiotropic effect on both complexes (Figure S7E,F). Remarkably, in the absence of the exomer complex, overexpression of Gga2 partially recovered chitin synthesis and diminished sensitivity to ammonium (Figure S7E,F). While the chitin phenotype could be explained by the alteration of the AP-1 complex, which in turn promotes the aperture of the alternative route for the chitin synthase to the PM, the ammonium phenotype is probably more complex and likely associated with general alterations of the TGN.

Altogether our results support the existence of a complex network of functional interactions between Arf1, exomer, and clathrin adaptors. Moreover, although it is known that Arf1 activity favors the polarized delivery of Chs3 by exomer, this activity, in contrast to previous reports, was found not to be essential for exomer function since the polarization of Chs3 occurred normally in the absence of Arf1 (Figure S8). However, exomer was still present at the TGN/EE membranes (Figure S6E). In contrast, the ablation of Arf1/Sec7 function reroutes Chs3 and amino acid permeases to the PM, independently of exomer, through alternative routes likely to be associated with the effects of this ablation in the recruitment of clathrin adaptors. Interestingly, overexpression of Sec7 only had a significant effect on the traffic of amino acid permeases in the absence of exomer, reinforcing our previous findings (see above) that suggest that different links exit between exomer and Chs3 and amino acid permeases.

3.7 | The intracellular traffic of the exomer bona fide cargoes is dependent on their competitive interactions between exomer and AP-1 complexes

Previous studies suggest that exomer assembled by different ChAP subunits may have dramatically different functions. The Chs6/Bch2 pair is proposed to directly mediate association with selected cargoes like Chs3, whereas the Bch1/Bud7 pair contributes to exomer association with membranes and membrane remodeling together with Arf1. Given the effects of exomer on clathrin adaptors, we revisited the roles of the different exomer subunits in the traffic of Chs3.

In the absence of a functional exomer (chs5Δ), or when both members of a group of ChAP paralogs are deleted (chs6Δ bch2Δ or bch1Δ bud7Δ), yeast cells become resistant to calcofluor owing to the intracellular retention of Chs3 (review in 70). However, we found that in the complete absence of functional exomer the subcellular localization of Chs3 differed compared to the loss of the cargo binding paralogs Chs6/Bch2. In the absence of a functional exomer (chs5Δ), Chs3 was found in significantly brighter puncta compared to the wild type (Figure 6A), similar to what was observed for Gga2/Apl4 in this mutant (Figure 4C). A similar phenotype was also seen in the bch1Δ bud7Δ double mutant (Figure S8B). However, in the chs6Δ bch2Δ mutant, Chs3 puncta were similar to that of the wild type. This suggests that exomer complexes associated with different ChAP paralogs may have different effects on Chs3 traffic. We hypothesized that exomer containing the Chs6 and/or Bch2 paralogs may strictly act as a cargo receptor whereas the exomer containing Bch1 and/or Bud7 paralogs may have more general effects on traffic, as described above for AAPs.

To test this hypothesis, we explored the effect of the exomer mutants on the localization of Chs33,24A mutant protein which cannot bind the AP-1 complex. We found that chs5Δ cells expressing Chs33,24A were sensitive to...
calcofluor white, whereas \textit{chs6}\textDelta cells expressing Chs3\textsuperscript{L24A} were moderately resistant to the same calcofluor concentration (Figure 6B). This suggests that in cells lacking Chs5, Chs3\textsuperscript{L24A} reaches the cell surface, while in cells lacking Chs6 Chs3\textsuperscript{L24A} does not reach the plasma membrane. As an independent confirmation, we monitored the localization of Chs3\textsuperscript{L24A}-GFP in \textit{chs5}\textDelta, \textit{chs6}\textDelta \textit{bch2}\textDelta, and \textit{bch1}\textDelta \textit{bud7}\textDelta cells. We found Chs3\textsuperscript{L24A}-GFP on the cell surface in \textit{chs5}\textDelta
and bch1Δ bud7Δ cells but not in chs6Δ bch2Δ cells, indicating that the two ChAP paralogous pairs play different roles in Chs3 traffic (Figure S8B).

We sought to explore this hypothesis further by examining the localization of Chs5-Chs3 BIFC complexes in different ChAP mutant backgrounds. We hypothesized that if complexes containing Chs6/Bch2 were exclusively required for cargo loading in exocytic vesicles then the formation of the Chs5-Chs3 BIFC complexes could be able to bypass this requirement. Surprisingly, we found that Chs5-Chs3 BIFC complexes localized along the cell surface in both chs6Δ bch2Δ or bch1Δ bud7Δ cells (Figure 6C, Figure S8A). However, these BIFC complexes could not reach the PM in the absence of Chs7 (chs7Δ strain), a specific chaperon implicated in a Chs3-exocytic step prior to exomer complex function.34 This suggests that, under this condition, complexes containing only Bch1/Bud7 are competent for exocytosis, and that exocytosis may not be the only role of exomer containing Chs6 and Bch2. Consistent with these findings, we found that the concomitant expression of Chs5-VC and Chs3-VN was able to restore calcofluor white sensitivity to chs6Δ (Figure 6D). Interestingly, the Chs5-Chs3 BIFC complexes were conspicuously absent from the neck region in both double mutants, chs6Δ bch2Δ and bch1Δ bud7Δ, consistent with a lower polarization of the protein similar to the localization observed for the Chs3L34A protein, which is unable to bind the AP-1 complex (Figure S8A).

One explanation for these phenotypes is that the artificially stable Chs3-Chs5 dimer induced by BIFC tags prevents AP-1 from retaining Chs3 in the TGN and therefore the protein can reach the PM without Chs6 or Bch1/Bud7 following an alternative route.77 To test if the converse could be true, we tested the effect of the formation of Apl4-Chs3 BIFC complexes on global Chs3 localization. We found Apl4-Chs3 BIFC complexes were only detected as intracellular dots, consistent with the intracellular localization of the AP-1 complex (Figure 6E, upper panel). The formation of these complexes was highly specific because they were clearly altered in the absence of Gga2 (Figure 6E), a finding that clearly agrees with the proposed role for GGAs in the recruitment of AP-1 to the TGN.6 More important, cells expressing these BIFC complexes were moderately resistant to calcofluor, indicating that the artificial stable interaction of Chs3 with AP-1 reduced the exit of Chs3 to the plasma membrane (Figure 6E, lower panel).

Altogether our results suggest that the exomer and AP-1 complexes can compete for some cargoes in S. cerevisiae, such as Chs3, but not others such as AAPs. These findings may be highly relevant and influence our understanding of how protein sorting at the TGN (see discussion) may have evolved.

### 3.8 Exploring the physiological relationship between the exomer and AP-1 complexes in Candida albicans

These multiple lines of evidence support the idea of a TGN niche in where exomer and AP-1 complexes maintain competitive and regulated relationships between themselves and with respect to their cargoes. Thus, we sought to analyze whether these relationships have been conserved along evolution. Considering the evolutionary distribution of the exomer complex,71 we decided to analyze this relationship in Candida albicans where both complexes exist. Given the effect of exomer on polarized exocytosis in Saccharomyces cerevisiae, we examined filamentous growth which strongly depends on polarized exocytosis. We examined the filamentous growth of cells lacking exomer and AP-1 in liquid (Figure 7A,B) and on different solid media (Figure 7C). Surprisingly, loss of exomer only weakly affected filamentous growth whereas the AP-1 complex was indispensable for hyphal formation on solid media and its absence strongly reduced the rate of hyphal growth in liquid media. However,
exomer deletion only partially altered the morphology of filaments growing on solid and in liquid media, and slightly reduces hyphal growth in liquid media. Interestingly, the double mutant showed additive phenotypes, with stronger alterations in colony morphology and a lower hyphal extension rate in liquid media. These results highlight the significant differences between the role of the exomer and AP-1 complexes in yeast or hyphal cells, and suggest that the AP-1 complex has an important role in maintaining polarity during mycelial growth.

4 | DISCUSSION

4.1 | Exomer facilitates the polarized delivery of several proteins to the PM

The TGN is a major platform for the intracellular sorting of proteins where anterograde and recycling pathways converge.\(^3,72\) Surprisingly, even in yeast, the mechanisms for protein sorting to the PM remain unclear.\(^7\) Some years ago, the discovery of exomer as an adaptor complex at the TGN,
required for the delivery of Chs3 to the PM, opened a pathway to study these mechanisms. However, the number of proteins that depend on exomer for their transport is limited, this observation was striking given the evolutionary maintenance of this sophisticated machinery. Moreover, the phenotypes of mutants lacking exomer, as well as the range of its genetic interaction, suggested additional roles in protein trafficking.
The characterization of the sensitivity of exomer mutants to ammonium (21 and this work) expands the previously reported role of exomer in protein traffic regulation. Here, we have shown that the ammonium sensitivity of exomer mutants is linked to the absence of the unique scaffold Chs5 or the pair of ChAPs Bch1/Bud7, but not to the absence of the other pair of ChAPs Chs6/Bch2, which has been described as cargo adaptor for the Chs3 and Pin2 proteins. This suggests additional functions for exomer containing Bch1/Bud7 that are independent of the cargo binding ChAPs.

Our work unequivocally links the ammonium hypersensitivity of the exomer mutant chs5Δ to a deficient uptake of tryptophan. Still, our results also suggest that the absence of exomer affects the uptake of other amino acids, based on the partial alleviation of chs5Δ ammonium sensitivity by the LEU2 gene, or the altered sensitivity to several amino acid analogs. These defects in amino acid uptake are not caused by deficient signaling through the major signaling pathways involved in nitrogen assimilation. Rather, the ammonium sensitivity is directly linked to defective traffic of the tryptophan permease Tat2 in the chs5Δ mutant. This traffic defect is shared with the Mup1 permease and the sodium ATPase Ena1. Therefore, our results highlight the involvement of exomer in the polarized traffic of these three proteins and likely other transporters. This specific function in polarized transport explains why these proteins have not been previously linked to exomer function, since their transport to the plasma membrane is not blocked in the absence of exomer and the defects in polarization, much more discrete, were only detectable after the use of regulatable promoters. Therefore, our work enlarges the spectrum of proteins that rely on exomer for polarized transport. Moreover, the results presented here to link this transport to the Bch1/Bud7 pair of ChAPs. These subunits lack the cargo binding activity of the Chs6/Bch2, suggesting that exomer may also contribute to the transport of proteins through its action in coat assembly in addition to its direct role as cargo adaptor. Since Bch1/Bud7 are less divergent from the ChAP representative found in the root of the fungal evolutionary tree, this may suggest that this coat function is the ancestral role of the complex.

4.2 Exomer contributes to the late endosomal traffic of several proteins through its functional relationship with clathrin adaptor complexes

Our results show that chs5Δ disrupts not only the anterograde transport of Tat2 and Mup1 to the PM but also their traffic to the vacuole. This defect is consistent with the overall alterations of the TGN dynamics associated with the modified recruitment of clathrin adaptor complexes at TGN membranes in the absence of exomer. Notably, similar cooperation between exomer and clathrin adaptors was previously reported in S. pombe. The deletion of the GGA complex suppressed both the ammonium sensitivity and the tryptophan requirement of the chs5Δ mutant. This most likely occurred by reducing Tat2 traffic to the vacuole, thereby restoring its delivery to the plasma membrane. Surprisingly, the absence of the AP-1 complex had only a marginal effect on chs5Δ phenotypes linked to tryptophan transport, suggesting that AP-1 is not linked to Tat2 traffic. Interestingly, exomer does not seem to affect the early endosomal recycling of Tat2 that is mediated by Rey1 because the effects of the absence of Rey1 and Chs5 are additive. In addition, the deletions of GGA1 or GGA2 have opposite effects on the ammonium sensitivity of the rcy1Δ or chs5Δ mutants. Altogether, these results indicate that the absence of exomer affects the late endosomal traffic of Tat2 without affecting its Rey1-dependent early endosomal recycling.

Exomer and clathrin adaptor complexes localize and mediate sorting decisions at the TGN, but also rely on the
Moreover, this model is also compatible with the action of exomer as an inhibitor of Arf-GAP (GTPase activating protein) activity, as previously proposed. Accordingly, overexpression of Arf1 or Sec7 did not suppress the chitin synthesis defect associated with the chs5Δ mutant. In contrast, depletion of Arf1 or Sec7 effectively suppressed the chs5Δ-associated phenotypes, most likely by disrupting clathrin-mediated TGN-endosome traffic. Interestingly, overexpression of Sec7 alleviated partially the ammonium sensitivity and the tryptophan requirement of the chs5Δ mutant, indicating that Chs3 and Tat2 may follow different itineraries from the TGN.

One unexpected finding of this work is the observation that the polarized delivery of Chs3 is unaffected by the absence of Arf1. This is surprising because polarized delivery of Chs3 depends on the presence of a functional exomer, which should be disrupted when Arf1 is depleted. This result is indicative of the assembly of a functional exomer complex despite the strong morphological alteration of the TGN in the arf1Δ mutant. This somehow contradicts previous indirect observations, suggesting that Arf1 is required for exomer assembly. Whether this observation is explained by the function of Arf2 or other GTPase still remains to be established.

Altogether our results, together with those previously reported, show that in addition to the direct competition between the AP-1 complex and exomer in the anterograde transport of proteins exomer also contributes to the late endosomal traffic of multiple proteins by facilitating the proper functioning of the clathrin adaptor complexes. This function appears to be independent of the cargo binding activity of exomer, and may vastly increase the number of proteins known to depend on exomer for localization.

4.3 Exomer and AP-1 complexes compete for a subset of cargoes

An interesting observation is the differential effect of the AP-1 complex on the traffic of exomer-dependent proteins. Our phenotypic analysis showed that the deletion of the AP-1 complex has little effect on Tat2 traffic in the chs5Δ mutants, which is similar to prior reports about Ena. In contrast, the absence of AP-1 efficiently reroutes Chs3, as well as other bona fide cargoes, Fus1 and Pin2, to the PM in the absence of exomer. This can be simply explained by the physical interaction between these cargoes and the AP-1 complex as it has been shown for Chs3. Our in vivo results favor a mechanistic model in which exomer and AP-1 compete for cargoes, since the artificially increased interaction between Chs3 and Chs5 through the BIFC system allows Chs3 traffic to PM avoiding the TGN-retention of Chs3 mediated by AP-1 in chs6Δ and bch1Δ bud7Δ mutants. Moreover, tighter binding of Chs3 to AP-1 using the BIFC system also reduced Chs3 traffic to the PM (Figure 6).

In addition to the direct competition between exomer and AP-1 for cargoes, we propose that competition for Arf1 also contributes to their complex interaction. Exomer and CCV are assembled at the TGN in close proximity (64 and this work), and both utilize Arf1 (Figure 7D). Moreover, exomer is strictly required for the PM delivery of a restricted number of cargoes that are recycled to the TGN through their physical interaction with the AP-1 complex (Figure 7E). However, exomer also facilitates the polarized delivery of other proteins that recycle independently of AP-1. Additionally, exomer contributes to the proper assembly of the clathrin adaptor complexes that facilitate late endosomal traffic of multiple proteins. Functional disruption of clathrin adaptors complexes reroutes proteins into alternative non-polarized ways to the PM. Our results also discriminate between the functions of the GGA and AP-1 complexes at the TGN in S. cerevisiae, a subject still under debate. The role of AP-1 in S. cerevisiae appears limited, affecting only a restricted number of cargoes that are recycled by this complex through their direct binding. The GGA complex appears to perform more general functions in the organization of the TGN. This occurs independently of the specific recognition of the cargoes thus affecting the late endosomal traffic of several proteins. This view is similar to what has been proposed for animal cells, in which AP-1 would have a distinct role in protein recycling that is not shared with GGAs.

The many functional connections between exomer and AP-1 found in yeasts help to raise the interesting question of whether these are evolutionarily conserved relationships. Our work with C. albicans (Figure 7A-C and 29) indicates that while exomer contributes to the polarization of Chs3 and only displays a secondary role in filamentous growth, AP-1 is pivotal for this hyperpolarized process (Figure 7F). More broadly, the absence of AP-1 is lethal in filamentous fungi. However, all exomer mutants characterized are fully viable (M. Riquelme, personal communication), suggesting that the functional interconnection between exomer and AP-1 may be limited to yeast cells within the fungal lineage. The AP-1 complex is conserved in eukaryotes, but exomer...
appears to be fungal specific, evidence that is consistent with the major role of AP-1 in polarized traffic in animal cells. Therefore, our results reflect the general idea that polarity determinants, and thus the mechanism of sorting at the TGN, are highly diverse, coexisting mechanisms based on cargo adaptor complexes together with others based on protein partitioning between micro-domains, with exomer somehow exerting both functions in yeast depending on the cargo to be sorted.

ACKNOWLEDGMENTS

We thank A. Spang, J. Ariño and S. Moreno for strains and reagents and for the many useful discussions that took place throughout this work and to E. Keck for the editorial correction of the manuscript. We especially acknowledge the initial work carried out by M. Berroa on C. albicans. CAP was supported by a University of Salamanca (USAL) predoctoral fellowship, an EMBO short-term fellowship, and a USA Grant for Excellence; NS was supported by an FPU fellowship from the Spanish Ministry of Education. Work at the CR laboratory was supported by grants BFU2017-84508-P from the CICYT/FEDER program (Ministerio de Economía, Industria y Competitividad, Gobierno de España) and SA116G19 from Consejería de Educación, Junta de Castilla and León. JMM laboratory was supported by grants RTC-2017-6468-2-AR and BIO2016-77776-P (Ministerio de Economía, Industria y Competitividad, Gobierno de España) and work by MCD was supported by the National Institutes of Health (USA) grant R01 GM092741 and by funds from the Michigan Protein Folding Disease Initiative. CR also thanks the financial support awarded to the Institute of Biological and Functional Genomics (IBFG) (CLU-2017-03) provided by the Junta de Castilla y León through the program "Escalera de Excelencia," co-financed by the PO FEDER of Castilla y León 14-20.

CONFLICT OF INTEREST

The authors state explicitly there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS

CR and CAP designed the research and CAP performed most of the experiments. NS, RV, JMM, and MCD did experimental work and/or provided essential reagents. CR and CAP wrote the paper and generated the figures. MCD contributed to the final text editing. All authors have read and approved the final manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.