COMMENTARY

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Linking membrane trafficking and intestinal homeostasis

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A major challenge for the human body is to maintain symbiotic relationships with bacterial communities that colonize their intestines. Although several molecules important for intestinal homeostasis have been discovered, the vast array still needs to be identified. We approached this task using a forward genetic approach, which revealed several molecules essential for intestinal homeostasis. One recently identified molecule is Ypt1p-interacting protein 1 domain family, member 6 (Yipf6). Mice with a null mutation in Yipf6 are hypersensitive to dextran sulfate sodium (DSS) induced colitis and develop spontaneous intestinal inflammation. Members of the Yip1 family are believed to be involved in ER to Golgi membrane transport.

In this review we summarize recent advances in the understanding of genes involved in intestinal homeostasis with a specific focus on the Yip family members. We speculate on how deficiency or dysfunction of Yip molecules may dysregulate intestinal homeostasis leading to pathogenic states.

Introduction

The mammalian intestine harbors trillions of microorganisms and has to cope constantly with a broad microbial diversity, a vast surface area and frequent challenges from pathogens ingested in food and water. Together, the intestinal immune system and the mucosal epithelium provide an extremely efficient barrier to microbial invasion. The intestinal epithelium is a critical regulator of intestinal homeostasis, both triggering immune responses to pathogenic challenges and maintaining tolerance to commensal microbiota.[2]

An important strategy to minimize bacterial invasion into deeper host tissue is to control bacterial interactions with the apical surface of the intestinal epithelium. In addition to the production of mucus and IgA, antimicrobial molecules are one important mechanism to limit bacteria-epithelial cell contact. These epithelial derived proteins typically mediate enzymatic attack of the bacterial cell wall or disrupt the inner bacterial membrane to directly kill bacteria. Antimicrobial peptides are structurally diverse and several families are known to exist, including the defensins, cathelicidins and C-type lectins.[3,4] Each may be regulated by distinct mechanisms. For example, the lectin RegIIIγ is induced by bacterial signals through the activation of Toll-like receptors (TLRs),[5,6] and a subset of α-defensins and defensin-related cryptidins is regulated by the nucleotide-binding oligomerization domain-containing (NOD) protein family.[7] Other α-defensins are expressed constitutively and do not require bacterial signals.[8]

A Forward Genetics Approach to Study Intestinal Homeostasis

Despite advances in understanding the genetic determinants of intestinal homeostasis, the full spectrum of genes necessary to monitor and respond to inflammatory agents in the intestine remains to be elucidated. Therefore, to identify genes important for intestinal homeostasis we performed a forward genetics approach using germline mutagenesis to analyze this phenomenon in mice.

Random germline mutagenesis, phenotypic screening, and positional cloning comprise the modern embodiment of the classical genetic approach. We used the germline mutagen N-ethyl-N-nitrosourea (ENU), administered to male mice to induce point mutations in spermatogonial stem cells. ENU predominantly affects A/T base pairs, while at the protein level, ENU results mainly in missense mutations (64%) and to a minor extent in splicing errors (26%) and nonsense mutations (10%).[9] Breeding to the third generation (G3) transmitted a subset of mutations from the mutagenized male to progeny in homozygous state; G3 mice were subjected to phenotypic screening, as described below.

The strength of the forward genetic approach is its unbiased nature, which allows for unexpected discoveries of "key" genes with non- redundant functions in defined biological processes. Furthermore, the random germline approach permits an estimation of the total number of essential proteins required for a particular process, in the present example intestinal homeostasis. By generating carefully defined phenovariants through a random process, we hoped to identify many of the genes important for the integrity of the intestinal epithelial barrier and tolerance to enteric flora.

Feeding mice for several days with DSS polymers in the drinking water induces an acute intestinal inflammation. It is believed that DSS is directly toxic to gut epithelial cells of the basal crypts and therefore affects the integrity of the mucosal barrier.[10] Low doses of DSS are well tolerated by wild type mice, which are able to repair epithelial damage and contain bacterial infiltration. In contrast, a mouse with a mutation that compromises repair or immune mechanisms might display elevated susceptibility to intestinal inflammation induced by a similar low dose of DSS. DSS therefore offers a model of acute intestinal injury useful for study of the epithelial barrier, its regeneration and innate immune responses.

The *Klein-Zschocher* Mutation

To identify genes important for intestinal homeostasis we screened approximately 6,000 mice with subthreshold doses of DSS (1% w/v in drinking water). Mice were weighed daily and extreme outliners were retrieved for further studies. In total, 16 transmissible mutations were identified, causing extreme weight loss in response to DSS. Of those, five mutations have been identified to date, four of which affect the genes encoding TLR9, Aqp3 and Muc2 (two alleles). The function of these proteins is important to intestinal homeostasis in several ways:[11] TLRs sense the incursion of microbes into epithelial cells, leading to the induction of amphiregulin and epiregulin, members of the EGF ligand family, and promoting epithelial repair.[12] Epithelial integrity is also dependent on signaling via the EGF receptor pathway,[12] water transport via aquaporin 3, and an intact unfolded protein response dependent on Mbtps1, also known as the site-1 protease.[13] Mutations that alter the structure of Mucin-2 lead to excessive accumulation of aberrantly folded proteins, that the ER stress machinery is incapable of resolving, and result in enhanced susceptibility to colitis.[14]

The fifth mutation, designated *Klein-Zschocher (Klz)*, was mapped (using C3H/HeN) to chromosome X with a LOD score of 17.4(1). A critical region between the markers DXMit114 and DXMit169 (95.34–97.952 MB) was identified after an analysis of 79 meioses. Using whole genome SOLiD sequencing a single coding/splicing mutation within the critical region, a T to A transversion at position 3002 of the genomic DNA sequence of *Yipf6*, encoding the 236 amino acid protein Yipf6, was identified. *Yipf6* contains seven exons, with the *Klz* mutation affecting a thymine base within intron 3, five nucleotides from the next exon (exon 4). cDNA sequencing revealed that the mutation impairs the splice acceptor site of intron 3 and results in skipping of exon 4. Splicing of exon 3 to exon 5 creates a frameshift and a premature stop codon at position 89 (the first abnormal codon after exon 3).[1] The effects of the *Klz* mutation on intestinal homeostasis were recessive.

Homozygous *Klein-Zschocher* mice showed severe weight loss after administration of 1% DSS in the drinking water. The colons of *Klz* mice displayed ulceration, dramatic leukocyte infiltrations and a complete loss of epithelial architecture after seven days of DSS treatment.[1]

Naïve *Klein-Zschocher* mice showed increased intestinal permeability as assessed by FITC dextran absorption assay. Furthermore, naïve *Klz* mice also showed reduced Paneth cell numbers that exhibited defective formation and secretion of large secretory granules. A similar phenotype was observed in goblet cells of *Klz* mice. We hypothesized that defective formation and secretion of secretory granules in Paneth and goblet cells may be responsible for the impaired intestinal homeostasis in these mutant mice. Importantly, *Klein-Zschocher* mutant mice also developed spontaneous ileitis and colitis after 16 mo of age.[1]

The Ypt1p-Interacting Protein 1 (Yip1) Family of Proteins

The founding member of the Yip1 family, the *Saccharomyces cerevisiae* protein Yip1p, was identified in a yeast two-hybrid screen for interactors of Ypt1p and Ypt31p, the yeast homologs of mammalian Rab1 and Rab11, respectively.[15] Yip1 family proteins have been most extensively studied in yeast, where they are known to regulate Rab protein-mediated ER to Golgi membrane transport, as discussed below. The yeast Yip1 family consists of Yip1p,[15] Yip4p, and Yip5p.[16] In mammals, there are seven recognized members designated Yipf1–7 of which Yipf5 and Yipf7 (also called Yip1A and Yip1B, respectively) reportedly represent the closest homologs of yeast Yip1p.[17] Families of Yip1p homologs are found in all eukaryotes so far examined (**Fig. 1**).[16-19]

Yip1 family proteins have several features in common. First, Yip1 family members are transmembrane proteins defined by the presence of the Yip1 domain, a ~200 amino acid integral membrane domain that contains four transmembrane α helices and the motifs DLYGP and GY.[15-17] Thus, Yip1 proteins share a common domain topology in which the N-terminus is oriented in the cytosol, and the C-terminus contains multiple hydrophobic segments that span or are inserted into the membrane. Second, Yip1 proteins possess an ability to interact with Rab proteins in a manner dependent on Rab

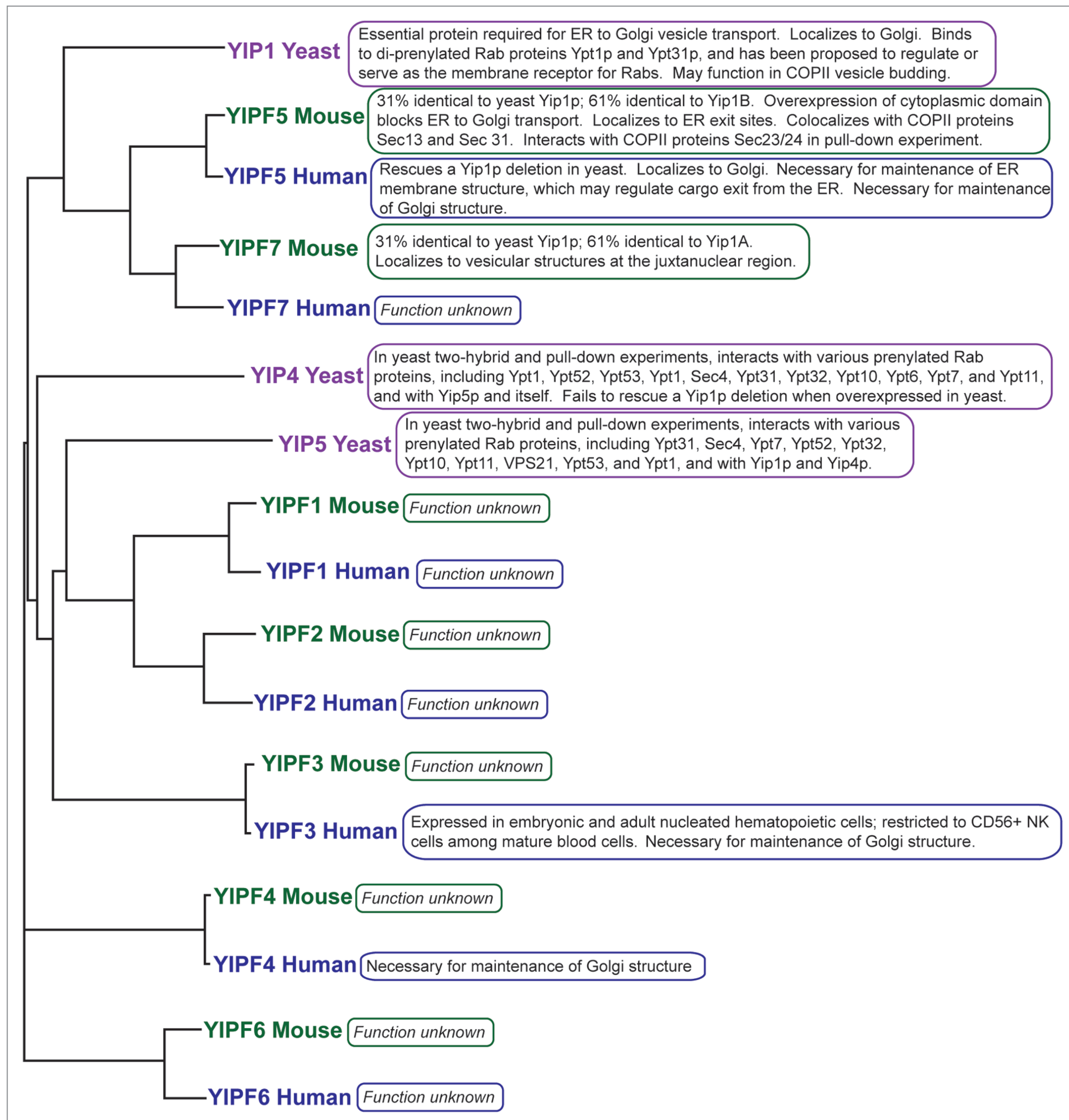

Figure 1. Inferred evolutionary relationships between known Yip1 family members based on sequence homology calculated from multiple sequence alignment using ClustalW. Protein functions, where known, are described.[18]

C-terminal dual prenylation.[20,21] Third, Yip1 proteins physically associate with other Yip1 family members,[15,16,22,23] suggesting that they carry out their functions within a larger protein complex.

Mouse Yipf6 is 21% identical and 31% similar in sequence to Yip1p. Like Yip1p, Yipf6 is predicted to consist of a soluble, cytoplasmic N-terminal domain (roughly 40% of the length of the protein) and a C-terminal domain containing five transmembrane α helices connected by short loops. Colocalization with Golgi markers[1] suggests that Yipf6 resides within Golgi and possibly ER membranes, with its C terminus oriented luminally.

The Role of Yip1p in Trafficking

Numerous studies support the idea that yeast Yip1 family proteins regulate Rab protein-mediated ER to Golgi membrane transport. This inter-organellar journey forms part of the transport pathway that serves to translocate secretory proteins to the extracellular space, and plasma membrane, endosomal and lysosomal proteins to their respective membrane residences. The transit of protein cargo between ER and Golgi is mediated by transport vesicles following a process that is reiterated for every inter-organellar transport event until the protein cargo reaches its

destination.[24] Each step of the transport process is regulated by a distinct set of proteins, including cytosolic coat proteins that mediate the generation of transport vesicles, and conserved membrane fusion factors such as SNARE proteins that are important for the site specific fusion of vesicles with target membranes.[24] GTPases of the Rab family are required for tethering transport vesicles to the target membrane before SNARE-mediated fusion.

The precise function of Yip1p in ER to Golgi membrane transport remains under study, with existing data supporting a role for Yip1p in tethering of ER-derived vesicles at the Golgi membrane prior to fusion, as well as in formation or budding of COPII vesicles from the ER. Yip1p interacts with the yeast homologs of Rab1 and Rab11 (Ypt1p and Ypt31p, respectively), both of which are localized primarily to Golgi membranes and function in the yeast exocytic pathway. Ypt1p functions as a tethering factor localized at the target (Golgi) membrane for COPII-coated vesicles arriving from the ER[24,25] (**Fig. 2**). Furthermore, studies have shown a role of Ypt1p in cis-to medial-Golgi transport.[26] Ypt1p also interacts with large heteromeric tethering complexes such as the Uso1 complex (yeast homolog of mammalian p115) and the GEF-containing TRAPP1 complex. Interaction of Ypt1p with Uso1 seems to be required for vesicle tethering and for assembly of the SNARE complex at the target membrane,[27-29] whereas TRAPP1 acts as a guanine nucleotide exchange factor for Ypt1p, activating Ypt1p preceding membrane fusion.[30-33] In contrast to Ypt1p, Ypt31p is required for the exit of transport vesicles from the trans-Golgi.[26,34] These data suggested that Yip1p, by virtue of its interaction with both Ypt1p and Ypt31p, may function in the tethering of vesicles to the destination membrane prior to vesicle fusion, and perhaps also in the exit of vesicles from the Golgi.

In support of a role for Yip1p in tethering vesicles to Golgi membranes, Yip1p has been shown to interact with several different Golgi-specific Rab proteins[20,35] in a manner dependent on C-terminal dual prenylation of the Rab, a modification that is necessary for Rab membrane association.[20,21] In addition, Yip1p forms a complex with Yif1p and Yos1p, proteins that when mutated block transport between the ER and Golgi, resulting in an accumulation of ER membranes, vesicles, and tubulovesicles.[36,37] Yip1p has been shown to localize to Golgi membranes at steady-state.[15] These studies point to a role for Yip1p in regulating Rab proteins or serving as a membrane receptor for Rab proteins during Golgi membrane tethering (**Fig. 2**). Yip1p may carry out these functions within a complex containing Yif1p, Yos1p, Yip3p, Yip4p, Yip5p and Yop1p, with which it physically associates in vitro.[16,20,22,23,36-38]

However, Yip1p has been implicated in the formation of COPII-derived vesicles at the ER, with no role in tethering or fusion of those vesicles to the Golgi membrane (**Fig. 2**).[39] Antibodies against the hydrophilic N-terminus of Yip1p inhibited vesicle transport from the ER to the Golgi complex in cell-free assays and inhibition was specific to the COPII dependent budding stage.[39] Furthermore, yeast cells depleted of Yip1p amassed large quantities of ER membranes.[15] Other data suggested that Yip1p functions in establishing the docking or fusion competence of ER transport vesicles,[40] possibly by recruiting Rab proteins or Golgi SNAREs into nascent vesicles. However, any role for Yip1p in the formation of vesicles is independent of its interaction with Ypt1p, since *ypt1* thermosensitive mutant yeast accumulated elevated amounts of transport vesicles at nonpermissive temperatures, demonstrating that Ypt1p is not required for vesicle formation.[41]

Mammalian Yip1 Proteins and the Role of Yipf6 in Intestinal Homeostasis

Yip1p has two mammalian homologs, Yipf5 and Yipf7, each of which shares about 31% sequence identity with Yip1p. Yip1p is an essential gene in yeast, and the lethality of a yeast Yip1p deletion can be rescued by expression of human Yipf5, demonstrating a conservation of function across species.[20] However, the mammalian Yipf5 and Yipf7 did not interact with Rab1 (homolog of yeast Ypt1p), suggesting that they interact with a distinct group of Rabs or function Rab-independently in mammals. Yipf5 colocalized with Sec31 and Sec13, components of the COPII coat protein complex found at ER exit sites. These data are consistent with a role for Yipf5 in COPII-dependent vesicle budding from the ER.[17] Yipf5 may also affect vesicle trafficking from the ER through an ability to regulate ER organization. siRNA knockdown of Yipf5 in HeLa cells resulted in the deformation of ER structure into large densely packed clusters of stacked membranes in concentric whorls, the presence of which correlated with slowed exit of cargo from the ER.[42] Yipf5, Yipf3 and Yipf4 have also been implicated in the maintenance of Golgi structure.[42-44]

Yipf5 is expressed in a wide variety of tissues (heart, brain, spleen, lung, liver, kidney and testis) whereas Yipf7 is mainly expressed in the heart, suggesting that Yip1 family proteins might have tissue specific functions.[17] Our studies revealed high levels of Yipf6 throughout the gastrointestinal tract, particularly in the colon. When expressed exogenously in HEK293 cells, Yipf6 colocalized with Sec31a, a component of the COPII coat complex at ER exit sites.

In the gastrointestinal tract several transmembrane proteins are important for sensing and responding to microbes. These include the TLRs, EGFR, and amphiregulin and epiregulin (EGFR ligands), which cause susceptibility to DSS induced colitis when mutated in mice. If Yipf6 indeed regulates COPII vesicle formation or ER to Golgi transport similarly to Yip1p and other yeast Yip1 proteins, one might predict that numerous transmembrane and GPI anchored proteins important for colonic homeostasis fail to be transported to their proper membrane sites and are inadequately expressed in *Klein-Zschocher* mutants. In addition, the increased intestinal permeability observed in *Klz* mice suggests that the transport of junctional proteins to epithelial intercellular junctions may be compromised, consistent with a key role for vesicle trafficking in the assembly of epithelial junctions.[45-47] However, it is not clear whether Yipf6 might be responsible for general expression of membrane and junctional proteins or for expression of a restricted group of such proteins.

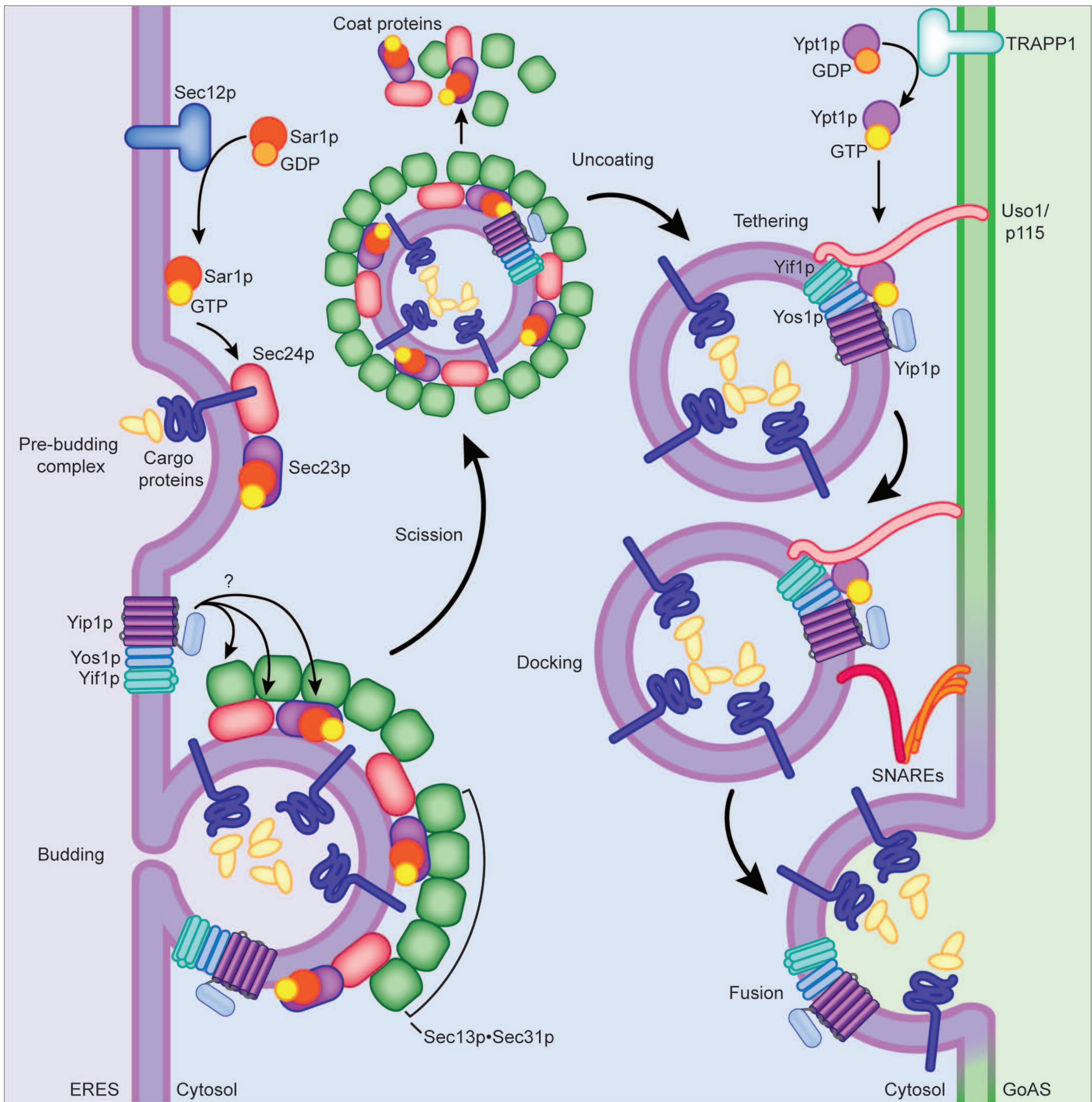

Figure 2. Hypothesized involvement of Yip1p in COPII vesicle formation and fusion. The COPII coat assembles by the stepwise deposition of Sar1p·GTP, Sec23p·Sec24p, and Sec13p·Sec31p at ER exit sites (ERES). Cytosolic Sar1p·GDP is converted to membrane bound Sar1p·GTP by the transmembrane GEF Sec12p. Sar1p·GTP then recruits the Sec23p·Sec24p subcomplex by binding to Sec23p, forming the pre-budding complex. Transmembrane cargo proteins are concentrated in COPII-coated buds by binding to Sec24p using a variety of ER export signals. Sec13p·Sec31p polymerizes onto Sec23p·Sec24p to form a mesh-like scaffold and crosslinks the pre-budding complex. Yip1p is thought to cycle between the ER and golgi compartments. Yip1p may promote the process of vesicle budding by interacting with Sec13p, Sec31p, Sec23p or Sec24p. As COPII vesicles travel toward the Golgi, they are thought to become uncoated. They approach Golgi arrival sites (GoAS) and become tethered by the combination of Ypt1p, which is activated by the GEF complex TRAPP1, and the tethering factor Uso1/p115. v- and t-SNARES then assemble into a four-helix bundle to mediate vesicle docking, and fusion of the lipid bilayers of the vesicle and Golgi membranes occurs. Yip1p forms part of a Ypt1p-interacting complex with Yif1p and Yos1p, and may promote Ypt1p-dependent vesicle tethering to the Golgi membrane.[18]

Secretory proteins traverse the ER to Golgi route, and Yipf6 might therefore also be important for the secretion of antimicrobial proteins. Several mouse models have demonstrated lack of antimicrobial molecules and increased intestinal inflammation.[5,7,48,49] Mammalian Yipf5 has been shown to interact with the Sec23/24 complex of COPII vesicles.

Sec24p has an important role in cargo selection[50,51] and one might predict that Yipf6 also influences cargo selection through interaction with COPII components. Further studies will be important to determine the mechanism of Yipf6 function in the intestine.

As *Klein-Zschocher* mice with a mutation in *Yipf6* develop spontaneous disease, we suggest that the X-linked human *YIPF6* should be regarded as a susceptibility locus when searching for causes of inflammatory bowel disease in humans, particularly males.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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