Yeast Chemical Genetics

For Identifying Regulators of Late Secretory Traffic Pathways

By

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Abstract

The intracellular transport of proteins and membrane lipids to the cell surface or between organelles is a fundamental process in eukaryotic cells. This process is required for the biogenesis and maintenance of organelles, as well as for traffic to the cell surface for cell growth and proliferation. The transport routes in the late secretory pathways are branched and complex, and their regulation requires sensing and responding to environmental conditions for proper control of cell growth. Both the transport and regulatory mechanisms are robust, so that defects can be overcome by alternate mechanisms. This complexity has made it difficult to identify the late exocytic transport machinery and its regulators. The goal of my thesis work was to use a yeast chemical genetic strategy to identify components of the exocytic transport machinery, and to generate useful chemical tools that will help us to understand how the machinery functions. I analyzed the effects of small molecules that we obtained in two similar high-throughput screens of large libraries of drug-like compounds, in order to identify compounds that cause a block in the late exocytic pathway. Several of our new compounds cause exocytic defects and are selectively toxic to yeast mutants in which one of numerous transport pathways are blocked. The design of the high-throughput screen strategy was based on that of an earlier mutant screen that led to the discovery of a novel component of the transport machinery, Avl9, a conserved eukaryotic protein that has not yet been well characterized. Some of our new compounds are expected to target Avl9 or proteins with functions related to that of Avl9. In order to identify proteins and processes affected by our compounds, I screened for genes which, when overexpressed, can suppress the toxic effects of our compounds. I found that highly-expressed *GTR2*, which encodes a Ras-family small GTPase, can suppress the effects of one of our compounds. Gtr2 and its paralog and binding partner, Gtr1, as well as their metazoan orthologs, signal nutrient availability to regulate both traffic and the activity of TOR (target of rapamycin) kinase, a master regulator of growth. Furthermore, the *gtr1*Δ and *gtr2*Δ mutants share some phenotypes with the *avl9*Δ mutant. Our results indicate that our new compounds will serve as tools to help us understand how Avl9 and Gtr proteins function in cellular response to environmental conditions for proper regulation of protein and membrane transport in the late exocytic pathway.

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Chapter 1

Introduction

The transport of proteins and membranes between organelles and the cell surface is a fundamental process in all eukaryotic cells. Intracellular transport mediated by membrane vesicles and tubules is required to establish and maintain organelle identity and function, and also to regulate the components of the cell surface and mediate cell growth. Traffic in the secretory pathway involves a sequence of processes: proteins enter the secretory pathway by means of translocation into the endoplasmic reticulum (ER) either co-translationally, or, less commonly, after translation in the cytoplasm (36). They are core-glycosylated in the ER and then transported to the Golgi complex where they are further glycosylated. Cargo is then sorted at late Golgi compartments, referred to as the *trans*-Golgi Network (TGN) in most cell types. Sorting involves recruitment into distinct carriers that are targeted to different final destinations: endosomes, lysosomes, and the cell surface (6; 8; 39; 43). Intracellular compartments also receive traffic from the cell surface by the endocytic pathway.

The molecular mechanisms of transport in the secretory pathway have been extensively studied for over 30 years, and much is now known about the basic mechanisms of intracellular transport. Especially well-characterized are the mechanisms of transport from the ER, endocytic uptake at the cell surface, and the mechanisms of transport vesicle fusion at various stages of transport. In my thesis work, I used the popular experimental model, the budding yeast, *Saccharomyces cerevisiae*, to help understand exocytic transport from endosomes and late Golgi compartments. Exocytic traffic from these compartments is still relatively poorly understood, because complex traffic routes have made it difficult to identify the molecular machinery that functions in forming the numerous types of late exocytic vesicles (25-27). However, the fundamental mechanisms of transport at each step of the

secretory pathway are conserved, and in the first part of this introductory chapter I will give an overview of what we know of these mechanisms. Similar mechanisms, and most likely additional processes, are thought to regulate traffic routes in the late exocytic pathway. A long-term goal of my work was to contribute to identifying these mechanisms at the Golgi and endosomes. In addition, it is critical to understand how traffic pathways are regulated. In the second part of this chapter, I describe the traffic and signaling pathways that are most relevant for my thesis work.

MECHANISMS OF VESICLE MEDIATED PROTEIN TRANSPORT

Vesicle Coats Recruit Cargo and Form Vesicles

Cargo exits from donor compartments by the formation of coated vesicles. There are numerous types of coated vesicles, but three types have been characterized in great detail: COPII-coated vesicles, which mediate exit from the ER; COPI (also called coatomer)-coated vesicles, which function at various traffic steps but have been primarily studied in retrograde transport from the Golgi to the ER; and clathrin-coated vesicles, which shuttle between the *trans*-Golgi network ("late Golgi" in yeast), the endsomes, and the plasma membrane (23; 52).

COPII-coated vesicles

Formation of transport vesicles at the ER requires the recruitment of the small GTPase Sar1 by the guanine nucleotide exchange factor (GEF) Sec12 (52; 60). Sec12 is an ER-resident, transmembrane-anchored GEF. Sar1 associates only with ER membranes. After binding of Sar1 to the ER membrane, the GTPase activating protein (GAP) Sec23 and the cargo recruiter Sec24 form a complex with Sar1 and the cargo. There is only one gene for Sec23, and four different Sec24 isoforms are present in mammals (68). Three different Sec24-like genes are encoded in the yeast genome, which suggests that different types of cargo might associate with different Sec24 recruiters, and that different isoforms mediate transport of subsets of cargo (61). Finally, a subcomplex of

Sec13/31 binds to the cargo-associated Sec23/24 complex. The Sec13/31 complex helps to deform the membrane and to stabilize the polymerizing coat. Sec13/31 can even assemble into a cage-like form in the absence of the other components of the COPII coat (62). After the vesicle is formed, the coat is released upon hydrolysis of GTP to GDP by Sar1.

COPI-coated vesicles

The process of COPI vesicle biogenesis is controlled by Arf1, a small GTPase of the Ras superfamily. However, unlike Sar1 in COPII vesicle biogenesis, Arf1 can induce vesicle formation at multiple distinct membranes along the secretory pathway, and it can recruit both COPI coat components and adaptor complexes of the clathrin coat (61). A variety of guanine nucleotide exchange factors (GEFs) are involved in the recruitment of Arf1 to different membranes. All Arf GEFs share a domain with the first Arf GEF that was discovered, Sec7 (11). This domain is required to induce the exchange of bound GDP to GTP in Arf1. GBF1, a large Sec7-domain-containing GEF, is the major exchange factor involved in COPI vesicle biogenesis (40).

Following nucleotide exchange, Arf1-GTP undergoes conformational change, leading to the exposure of a myristoylated N-terminal amphipathic helix that provides stable membrane anchorage (35). Then, Arf1-GTP recruits coatomer to the membrane. Coatomer components in turn recruit cargo proteins. After the formation of a COPI-coated vesicle, the coat has to be released to allow for vesicle fusion with the target membrane. The uncoating reaction depends on GTP hydrolysis by Arf1 (65), catalyzed by Arf1 GTPase-activating proteins (ArfGAPs). In addition to recruiting and releasing coatomer, an Arf- GEF/GAP- mediated cycle of GTP exchange and hydrolysis was proposed to play a role in regulating the uptake of cargo into COPI vesicles (34).

Clathrin-Coated vesicles

Clathrin-coated vesicles can form at different compartments in the late secretory pathway and in the endocytic pathway: the *trans*-Golgi network, endosomes, and the

plasma membrane. The clathrin coat consists of an assembly of clathrin triskelions, which are formed from three clathrin heavy chains and three clathrin light chains. Besides clathrin, the various types of clathrin-coated vesicles contain one of four adaptor protein (AP) complexes (AP1–AP4) and additional adaptor-like proteins, which are involved in the recognition and recruitment of cargoes (45). Similar to COPI, the formation of clathrin-coated vesicles requires the small GTPase, Arf1, for the recruitment of adaptor subunits, which then recruit cargo and clathrin. The best-characterized clathrin-coated vesicles function in endocytic uptake and contain the AP2 adaptin complex. In contrast, the AP1 adaptor-containing clathrin-coated vesicles mediate transport between the Golgi and endosomes, possibly in both directions (45). The functions of the other two adaptin complexes are less clear: AP3 promotes the delivery of proteins to lysosomes, and also appears to function in the formation of regulated secretory granules (2; 58), while AP4 may mediate a pathway to the plasma membrane (55).

The budding and release of clathrin-coated vesicles involves the mechanochemical action of dynamin GTPases (13; 28) as well as various BAR domain proteins that directly modify membrane curvature either at the plasma membrane or at the TGN/endosomes; these include endophilin, amphiphysin, nexins, and epsins (9; 19-20; 64). Actin and its regulators also play critical roles in clathrin coated vesicle formation, both at the plasma membrane and at the TGN (10; 18).

Other vesicle coats

Although COPI, COPII, and clathrin-coated vesicles have been studied the longest and are therefore best characterized, there are other types of vesicles. For example, the retromer is a coat complex that mediates traffic from endosomes back to the Golgi (4). Other coats, especially those that traffic cargo to the cell surface, may be specialized to select only certain types of cargo as a mechanism to regulate what gets transported to the cell surface, as well as when and where transport occurs. These coats likely diverged significantly between distant species or cell types that need to traffic different

cargo to the cell surface. For example, a specialized vesicle coat recognizes and transports chitin synthase Chs3 and a few other proteins to the bud neck region of yeast. This transport is dependent on Arf1p and a protein complex called exomer (51; 66). Metazoans do not contain proteins that are readily identifiable as homologs of the exomer components. It is therefore possible that there are diverse coat complexes with unique cargo specificities and overlapping functions, which makes it difficult to identify the coat proteins involved in transport to the cell surface.

Vesicle Targeting and Fusion

Rab GTPases regulate vesicle targeting

Rab GTPases are central regulators of membrane traffic. Like Arf and Sar1, Rab proteins are members of the Ras superfamily of small GTPases that function as molecular switches. Rab proteins form the largest family of small GTPases, and there are more than 60 Rab GTPases in mammalian cells. As for the other GTPases, Rabs alternate between two conformational states: the GTP-bound "on" form and the GDP-bound "off" form. The exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors (GEFs), which therefore regulate the activation of the GTPases. The primary function of Rab proteins is to ensure that cargoes are delivered to their correct destinations. They do this by controlling various aspects of transport, including vesicle budding, uncoating, motility, and fusion. Rabs play a role in regulating a wide range of effector proteins such as sorting adaptors, tethering factors, kinases, phosphatases, and cytoskeletal motors (42; 63). The effectors are usually activated or recruited by the GTP-bound form of the Rab protein. Conversion from the GTP- to the GDP-bound form is driven not only by the intrinsic GTPase activity of the Rab protein but is also catalyzed by GAPs (***G***TPase ***a***ctivator ***p***roteins). Rab GDI (***G***DP ***d***issociation ***i***nhibitor) prevents release of GDP from Rab, thus stabilizing the GDP –bound form (63).

The first characterized function of Rab proteins was the regulation of membrane vesicle fusion with the appropriate target membrane. This conclusion was made from

the observation that yeast cells with a mutant allele of the Rab GTPase, Sec4, accumulate post-Golgi exocytic vesicles that cannot fuse with the plasma membrane (49). This phenotype suggested that Rab GTPases might mediate vesicle docking or fusion. It was later shown that key effectors for Rab proteins are tethering complexes that form contacts between the transport vesicle and the acceptor membrane. In its GTP-bound form, Sec4 interacts with Sec15, a subunit of the octameric exocyst tethering complex on the plasma membrane (24). Tethering complexes on other target membranes are effectors for Rab proteins that regulate targeting to those membranes. For example, EEA1 is an effector for Rab5 in regulating fusion with early endosomes (12). Different Rab GTPases are localized to distinct organelles, which is another reason that Rab GTPases were initially suspected of playing a role in regulating membrane targeting. In some cases, in particular early endosomes, different Rab proteins are located on the same organelle but reside in distinct membrane microdomains (63).

SNARE proteins mediate specific membrane fusion

Both to maintain organelle identity and to regulate the specificity of traffic, it is critical for membrane fusion to occur in a controlled manner. The key proteins responsible for membrane fusion also play a role in specifying membrane identity. These are the SNARE proteins. The initials stand for ***s***oluble ***N***-ethylmaleimide-sensitive factor ***a***ttachment protein ***re***ceptors). SNAREs were identified in a search for membrane receptors that bind SNAPs (***s***oluble ***N***SF ***a***ttachment ***p***roteins) and NSF (***N***ethylmaleimide-***s***ensitive ***f***actor) (37; 59). SNAREs have been classified as v- and t-SNAREs, because they operate on opposing membranes, usually on a transport vesicle (v-SNARE) and a target membrane (t-SNARE). Tethering factors such as the exocyst act over a longer distance to "capture" the transport vesicles, and this facilitates the subsequent pairing of the v-SNARE with the cognate t-SNARE. The assembly of specifically matched v-/t-SNAREs between two membranes generates SNARE complexes that bring the lipid bilayers close together and drive membrane fusion (1;29; 31; 69). In order to allow subsequent rounds of transport, the SNARE complex needs to

be disassembled. This is catalyzed by the combined action of alpha-SNAP and NSF (46; 72).

Specific SNARE proteins are present on specific membranes, and this helps to regulate proper targeting and fusion. However, SNARE's can potentially pair inappropriately, so there are additional regulators of SNARE-mediated fusion. Key regulators of SNARE complex assembly are Sec1/Munc18-like (SM) proteins (54) . In regulated exocytosis such as synaptic vesicle fusion, SM proteins are important targets of regulatory proteins. Additional regulators of membrane fusion include the Ca^{2+}-binding synaptotagmins (73), and complexins, which either inhibit or facilitate SNARE action (38).

Membrane Lipids Regulate Traffic

The control of membrane lipid composition is critical for regulating essentially all aspects of membrane trafficking. For example, compartment-specific recruitment of components of the traffic machinery, including both coat components and tethering factors, involves localized generation of specific phosphoinositides (PIs) (14). These various PIs are generated by the phosphorylation of the hydroxyl groups at the 3, 4 and 5 carbon positions of the inositol ring of phosphatidylinositol (PtdIns). This process is mediated by various PI kinases and PI phosphatases (57). An additional mechanism by which membrane composition regulates traffic is cargo recruitment into sterol-rich "lipid rafts" (33; 56). Furthermore, lipid modification is important for deforming membranes during vesicle budding and pinching off (5).

Summary

Exocytic trafficking from the Golgi and endosomes is expected to involve all of the mechanisms reviewed here: 1) recruitment of cargo proteins via lipid-mediated enrichment and coat proteins; 2) shaping of the vesicle by coats, lipid-modifying enzymes and the cytoskeleton; 3) pinching off the vesicles by the action of dynamin

GTPases, the cytoskeleton, and motor proteins, and lipid-modifying enzymes; 4) specific targeting and fusion with the plasma membrane, at least in some cases via an endosomal intermediate. The results and tools generated from my thesis work promise to help identify components of this machinery in the late secretory pathway.

TRAFFIC PATHWAYS AND SIGNALING PATHWAYS IN POST-GOLGI TRANSPORT

Defining the traffic routes in the late exocytic pathways, and understanding the intracellular signaling mechanisms that regulate these pathways, is essential for a fundamental understanding of intracellular transport. The amount and direction of traffic from the Golgi and endosomes must be modulated, and the regulation of this process is not well understood. Much of this regulation occurs at the level of endosomes, which receive signals that indicate the nature of the extracellular environment as well as signals that indicate the cell's state, for example the amount of various nutrient reserves. The cell integrates these signals for optimizing the type of cargo, and cargo quantity, to be trafficked to the cell surface, maintained in the endosomes/Golgi, or trafficked to the lysosome either for a function there or for degradation. In particular, regulation of traffic to the cell surface is critical for proper control of cell growth and proliferation. The following review on traffic pathways in yeast will provide background to help explain the rationales behind the design of my thesis work, while a section on signaling will provide background for interpreting some of my results.

Branching in the Late Secretory Pathway

In both yeast and mammalian cells, secretory cargoes are transported to the cell surface by at least two pathways (25; 39). In yeast, secretory vesicles that accumulate in the late *sec* mutants, such as *sec6-4*, can be separated into two vesicle species based on their different buoyant densities and unique cargoes (25). The lighter vesicles transport surface proteins, such as the cell wall protein Bgl2p, which contribute to cell growth,

while the denser vesicles transport invertase and other enzymes secreted into the periplasm or growth medium (25). The existence of two pathways helps to explain why it is difficult to isolate mutants that are defective in the late secretory pathway. A mutation that causes a defect in just one of the transport pathways from the Golgi may not be sufficient to display an obvious phenotype, because cargoes can be diverted from the blocked route so that cells are still viable. Therefore, it is necessary to screen for mutants with defects in the late exocytic pathway by using a mutant strain background that already has a defect in one pathway, so that an additional mutation can generate a screenable phenotype (26-27). As described at the end of this chapter, my thesis work included a chemical-genetic version of a successful mutant screen that was performed in such a strain background. The mutant screen and chemical screen have identified genes and chemical tools that will help us to understand the mechanisms and regulation of late exocytic transport (27; 71).

Multiple Traffic Pathways Require VPS (Vacuolar Protein Sorting) Gene Function

VPS proteins function in traffic to the vacuole

The yeast vacuole is analogous to mammalian lysosomes, but it has additional functions. It is involved in many processes, including protein turnover, osmolarity regulation, pH homeostasis, and nutrient storage. The VPS (vacuolar protein sorting) proteins are involved in the transport of cargo from the Golgi to the vacuole (reviewed in (7)). A model cargo in this pathway, carboxypeptidase Y (CPY), is sorted at the Golgi by being diverted away from the exocytic pathway and recruited into vesicles that deliver it to endosomes called the prevacuolar compartment (PVC), from which it is transported to the vacuole. The proper sorting and processing of CPY is used in assays to study VPS protein function. The *vps* mutants were isolated by screening for mutants that secrete CPY from the cell (3; 44; 48). The mutants were divided into six classes (A to F) based on a number of criteria including vacuolar morphology and the secretion of CPY. For example, mutants in Class E accumulate an exaggerated form of the PVC

endosomes in which most CPY gets trapped. Most of the VPS proteins, including those in the Class E, have mammalian homologs with similar functions.

Among the VPS proteins, only a few have been implicated in the formation of vesicles from the Golgi. One of these proteins, which is very relevant to my thesis work, is Vps1. The *VPS1* gene encodes a homolog of the metazoan protein, dynamin (41; 47). Unlike some dynamins, Vps1 appears not to be involved in endocytosis, but is instead proposed to function at the Golgi and/or endosomes. In the *vps1*Δ mutant, CPY is secreted, and vacuolar membrane proteins are transported to the plasma membrane rather than the vacuolar membrane (41). Clathrin has also been found to be involved in the formation of the vesicles that transport vacuolar proteins from the Golgi (16). As in *vps* mutants, a temperature-sensitive mutant of clathrin results in the mislocalization of vacuolar proteins to the cell surface (53).

VPS proteins function in an exocytic pathway

In addition to functioning in traffic to the vacuole, VPS proteins also play a role in traffic in one branch of the exocytic pathway (26). For example, in the *vps1*Δ mutant, exocytic cargo such as invertase is missorted from high-densitiy secretory vesicles and is instead trafficked in low-density vesicles along with cargo normally in low-density vesicles, such as Bgl2. Thus, only one of the known exocytic pathways appears to be functional in the mutant, and all cargo is transported in this pathway (26). Similarly, clathrin is involved in transporting the cargo secreted by dense vesicles (21; 26). These findings suggest that exocytic cargo normally transported by high-density exocytic vesicles are transported together with vacuolar hydrolases from the Golgi to an endosomal compartment by clathrin-coated vesicles, and from endosomes, cargo is sorted either to the vacuole or to the cell surface. When the Golgi-to-endosome route is blocked in *vps* mutants, both invertase and CPY are missorted into light-density exocytic vesicles. These finding were crucial for the design of genetic screens aimed at identifying components of the late exocytic transport machinery.

A Classical Genetic Screen Strategy for Identifying Genes Involved in Post-Golgi Transport

As I discussed earlier, the branching of the post-Golgi pathway makes it important to screen for mutants involved in the late secretory process using a strain background that already has a secretion defect. Blocking a remaining pathway by an additional mutation in a second gene is then expected to result in lethality. The genetic interaction between the two genes that together are required for viability is called "synthetic lethality." In yeast, ~ 70% of the genes are non-essential under optimal growth conditions, but when these genes are deleted in combination with a deletion of another non-essential gene, together the two deletions can lead to cell death. Such synthetic lethal interactions between two genes usually indicate that they function in the same process, or in parallel, partially redundant, processes. This concept was used in an attempt to identify genes required for exocytic transport from the Golgi. Based on the known role of VPS proteins in one branch of the exocytic pathway, a *vps1*Δ mutation combined with a mutation that results in the block of the remaining transport pathway should result in synthetic lethality. However, this strategy was unsuccessful in identifying novel components of the exocytic transport machinery, indicating that transport routes are more complex than simply two possible pathways. Therefore, an additional mutation, *apl2*Δ, was added to the screen strain background, to screen for mutations in a third gene that would result in lethality (27).

APL2 encodes β-adaptin, which is a large subunit of the AP-1 clathrin adaptor complex. As mentioned in the previous section, the AP-1 adaptor is thought to functions in the sorting of cargo molecules into some classes of clathrin-coated vesicles. However, the exact function of AP-1 is unclear, and there is evidence that it functions either at the TGN, early endosomes, or both (45: 67). The *apl2*Δ mutation was chosen because both the *vps1* and *apl2* mutations perturb the invertase-transporting pathway, but they appear to block transport at different steps along this pathway (27). A screen for synthetic lethality in a *vps1*Δ *apl2*Δ double-mutant background led to the

identification of a novel gene involved in late exocytic transport, *AVL9* (27).

Phenotypic characterization of the *avl9*Δ mutant indicates that it plays a role in transport from the Golgi, most likely in both of the known exocytic pathways (27). When Avl9 was depleted in a *vps1*Δ *apl2*Δ strain, the cells had an exocytic defect and accumulated abundant structures that resemble aberrant Golgi membranes seen in mutants that block the exit from the Golgi, such as *sec14* and *sec7*. The *avl9* mutation alone or *vps1Δ apl2Δ* mutations do not generate such phenotypes (27). Characterization of the *avl9Δ* mutation by itself (so that Vps1 and Apl2 are present) by both subcellular fractionation and electron microscopy showed that the mutation perturbs the formation of secretory vesicles (27). In addition, over-expression of Avl9 is highly toxic to otherwise wild-type cells and results in the accumulation of heterogeneous membrane compartments, as well as a transport defect that appears to be specific for the late exocytic pathway (27). These results indicate that the screen strategy is successful in identifying novel components of the late exocytic pathway. However, the screen, and gene cloning, was very laborious due to the requirement of triple-mutant lethality. My thesis work used a very similar rationale as the AVL mutant screen, but rather than screening for mutants, my work involved high-throughput screens for small molecules that generate the AVL (***a***pl2 ***v***ps1 ***l***ethal) phenotype. Targets of some of the small molecules that generate the AVL phenotype could be genes that are involved in a process related to Avl9 function, or they could target Avl9 itself. Alternatively, the small molecules could target additional novel components of the late transport machinery, or regulators of this machinery.

The TOR Signaling Pathway regulates cell growth

The main goal of my thesis research was to characterize the effects of the compounds that we identified in two high-throughput screens for secretory inhibitors (described in Chapters 2 and 3). In addition, I pursued the identification of compound targets, and/or proteins that are relevant to the functions of the compound targets (described in

Chapter 2). One suppressor that I identified for our best secretory inhibitor was over-expressed *GTR2*. This gene encodes a Ras-type small GTPase, which functions as an amino acid-dependent upstream regulator of TORC1 (**T**arget **o**f **R**apamycin **C**omplex 1) (17; 22; 50). In this final section of my introductory chapter, I will briefly describe the TORC1 signaling pathway, with a focus on the upstream regulation of this pathway, including regulation by Gtr proteins and their metazoan homologs, the Rag proteins. In Chapter 4, I will speculate more on how the TORC1 signaling pathway and Gtr proteins tie in with regulation of transport in the late exocytic pathway.

There are two complexes that contain TOR: TORC1, which is sensitive to rapamycin, and TORC2, which is not, at least in most assays. The TOR protein in these complexes is a highly-conserved Ser/Thr kinase that regulates cell growth and metabolism in response to environmental factors (30). TORC1 is stimulated by nutrients to positively regulate cell growth by up-regulating anabolic processes, including translation and ribosome biogenesis. Activation of TORC1 also negatively regulates catabolic processes by down-regulating stress-responsive gene transcription and autophagy. In contrast, the mechanisms that regulate TORC2 are less-well understood, and they do not appear to be directly connected to nutrient levels (15).

In metazoan cells, TORC1 regulates cell growth by integrating various upstream signals, whereas in yeast, nitrogen source level and quality is the primary signal that controls TORC1 (reviewed in (70)). The major inputs that regulate TORC1 signaling in metazoan cells are growth factors, nutrients, energy, and stress. The best-understood pathway upstream of TORC1 is the pathway mediated by growth factors. TOR is activated in response to growth factor signals via PI3K-mediated activation of the kinase Akt/PKB. The Akt/PKB kinase inhibits Tsc1/2 (tuberous sclerosis proteins), which form a GTPase-activating protein (GAP) complex for the GTPase, Rheb1. Inactivation of Tsc1/2 by Akt/PKB leads to the activation of Rheb1, which in turn stimulates TOR (70). Other signals such as stress and energy also regulate Tsc1/2, and this is through other kinases: LKB, AMP-activated kinase, and MAPK (Mitogen-

activated protein kinase). However, Tsc1/2 is not affected by a major regulator of TOR, which is amino acid level (70). Until recently, TOR regulation by amino acids and other nitrogen sources was not understood in either yeast or metazoan cells.

A clue for amino-acid dependent regulation of TORC1 first came from work by Duboluoz et al., in a yeast study aimed at identifying stimulators of TORC1 following recovery from starvation (17). That study identified a complex containing the small GTPase, Gtr2, as a key regulator required for amino acid-dependent and TOR-dependent recovery from starvation. Gtr2, along with its paralog and binding partner, Gtr1, was also identified in a similar complex by a yeast genetic screen aimed at identifying nitrogen-source-dependent regulators of amino acid permease transport (22). The strongest evidence for Gtr proteins as amino acid-dependent upstream regulators of TORC1 and traffic came from more recent studies in *Drosphila* (32) and mammalian cells (50). The metazoan orthologs of Gtr1 (RagA/B) and Gtr2 (RabB/C) regulate TORC1 activity by regulating its localization to compartments that contain its activator, Rheb1; furthermore, this function of Rag proteins is dependent on amino acids and is required for the amino acid-dependent regulation of cell growth (32; 50).

My identification of *GTR2* as a gene-dosage suppressor of a small-molecule inhibitor of the late exocyic pathway further implicates Gtr2 in the regulation of protein traffic. Furthermore, based on the known functions of Gtr2, this regulation may involve TOR kinase, implicating this kinase in the regulation of cell growth by regulating traffic to the cell surface. These concepts are discussed in more detail in the final chapter of my thesis.

Summary

The discovery that VPS proteins function in one branch of the exocyitc pathway was critical in the design of classical genetic (mutant) screens and chemical genetic (small molecule) screens aimed at discovering genes and molecular tools that will help us to understand the mechanisms of late exocytic transport. Furthermore, the results of my

experimental work, especially the identification of Gtr2 function as a suppressor of the inhibiting effects of one of our new compounds, indicate that some of the compounds we identified in the chemical genetic screen will serve as tools for understanding the signaling pathways that regulate traffic in the late exocytic pathway.

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Chapter 2

A chemical genetic screen for modulators of exocytic transport identifies inhibitors of a transport mechanism linked to *GTR2* function

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Introduction

Cell growth and proliferation as well as the regulation of cell surface composition are achieved by an intracellular transport machinery that delivers proteins and membrane to the cell surface. The transport machinery is regulated by environmental sensing and signaling pathways that are integrated for the fine-tuned control of transport to the cell surface. The mechanisms that regulate cell growth and proliferation are highly robust; therefore, they can function in a wide range of environmental conditions and even when some components of the transport or signaling machinery fail. In eukaryotic cells, this robustness is achieved in part by a complex network of membrane and protein traffic routes to the cell surface (17, 33). Defects in a transport pathway can result in cargo transport by an alternate route, making transport defects difficult to detect in mutant screens (17, 18). Therefore, relatively little is known about the mechanisms by which protein and membrane cargo is transported from late exocytic sorting compartments, the Golgi and endosomes, and we have yet to identify most of the components that mediate and regulate this process.

Complex processes are more readily understood in relatively simple organisms. For this reason, the budding yeast *Saccharomyces cerevisiae* has become one of the most powerful experimental models for understanding intracellular transport, and most of the conserved components of the exocytic traffic machinery were first discovered using

yeast genetic strategies (27). We used a yeast genetic screen to identify a novel component of the late exocytic transport machinery, Avl9, a member of an ancient eukaryotic protein superfamily (18). Avl9 is essential in a mutant strain lacking Vps1, a dynamin homolog that functions in transport vesicle formation at a late Golgi compartment (26, 34), and also lacking Apl2, a large subunit of the adaptor protein-1 (AP-1) complex, which is required for forming certain classes of clathrin-coated vesicles at the Golgi and endosomes (18, 19, 31, 42). The *apl2*Δ and *vps1*Δ mutants have defects in an exocytic pathway(s), but these mutants, as well as an *apl2*Δ *vps1*Δ double-mutant, grow well because cargo is rerouted into a remaining pathway(s) (18). Mutations such as *avl9*Δ, which are lethal in a *apl2*Δ *vps1*Δ strain but not in a wild-type strain, are expected to cause defects in a branch of the exocytic pathway that remains functional in the *apl2*Δ *vps1*Δ strain. Analogous to using mutagenesis to screen for a secretory block in the *apl2*Δ *vps1*Δ mutant, we performed a high-throughput screen of a large library of small molecules to identify compounds that inhibit the growth of the *vps1*Δ *apl2*Δ mutant but which have relatively little effect on wild-type cells. The targets of these compounds are potential components of the secretory machinery, and some of the compounds may interfere with an Avl9-related function. The biochemical function of Avl9 and related proteins is still unknown, and the inhibitors identified by our screen strategy could be valuable tools in understanding the role of Avl9 in both yeast and mammalian cells.

Our high-throughput screen was successful in identifying novel exocytic transport inhibitors, and we describe the phenotypic effects of one structurally similar group of compounds in detail. Furthermore, we show that the toxic effects of this group of compounds is inhibited by highly expressing *GTR2*, which encodes a Ras-like small GTPase that plays a role in regulating nutrient-responsive TORC1 (Target Of Rapamycin Complex 1) kinase signaling, exocytic cargo sorting at endosomes, and

epigenetic control of gene expression (7, 11, 14, 25, 37). Therefore, the small molecules identified by our chemical-genetic approach are promising tools for understanding how signaling pathways that respond to environmental conditions regulate the traffic pathways that mediate cell growth and proliferation.

Materials and Methods

Reagents, plasmids, and yeast strains

Minimal medium for growing plasmid-carrying yeast strains was CSM (complete synthetic medium) lacking a nutrient for plasmid selection, with amino acid mixes from Q-Biogene. All other growth media components were from Difco, and were prepared following recipes described in (39). Rich medium was YPD (yeast extract, peptone, 2% glucose) or YPGal (yeast extract, peptone, 2% galactose) unless otherwise stated. Culture growth was monitored by measuring OD600 in a Genesys 5 spectrophotometer (Thermo-Fisher). Rapamycin was from Sigma-Aldrich and prepared as described in (6). Hit compounds from high-throughput screening were reordered from ChemBridge (KU#1-11) or ChemDiv (KU#12-15).

Our "wild-type" yeast strains are EHY46 and EHY47 (18). EHY361 is a *vps1*Δ mutant strain in the EHY47 background (18). EHY644 is an *apl2*Δ *vps1*Δ mutant strain (obtained from EHY361 crossed to GPY1783-10A; (18)). EHY644 was transformed with pEH227, which is pRS316 with *VPS1* (18) to generate EHY658; with pEH331, which is pRS316 with *APL2* (18) to generate EHY1166; or with an "empty" vector, pRS316, which is a *URA3 CEN* plasmid (40) to generate EHY707. EHY807 is an *apl2*Δ strain, generated from EHY47 by integrating a PCR product containing *apl2*Δ*::kanMX4* (obtained from Y12725 using primers EH130, EH131; (18)). EHY1325 is a wild-type diploid generated by crossing EHY47 to EHY46. *GTR2* was deleted in this diploid to generate EHY1326 by integrating a PCR fragment containing *gtr2*Δ*::LEU2* (using primers EH212: GGAAAGGACCGTTTCCGGAC and EH213: CGACCCCCATCGTGAGTGCT), obtained from strain NBW5Δgtr2 (a kind gift of Takeshi Sekiguchi; (25)). EHY1326 was sporulated, and *gtr2*Δ progeny (LZY260) was crossed to EHY644 to obtain the following

haploid progeny: *gtr2*Δ *vps1*Δ *apl2*Δ (LZY253); *gtr2*Δ *apl2*Δ (LZY256); and *gtr2*Δ *vps1*Δ (LZY257).

Plasmids containing mutant alleles of *GTR1* and *GTR2* that encode proteins restricted to the GDP- or GTP-bound conformations were the generous gift of Takeshi Sekiguchi (Kyushu University), and are pL146, pL148, pL264, and pL263 (described in (25, 43). We switched the *TRP1* auxotrophic marker in these plasmids to *URA3* for use in our strains, using the pTU marker-swapper plasmid (9). Plasmid pLZ43 contains *GTR2* (20 upstream and 61 downstream bp) under the control of the *GAL1* promoter, and was isolated from a cDNA library (22). Plasmid pLZ44 contains *GTR1* under the control of the *GAL1* promoter and was generated by cloning a genomic PCR fragment (using primers LZP48: GTAATGTCGTCAAATAATAGGA and LZP26: AAACACTCAATTGCCGAATGT) into pCR-BluntII-TOPO, using the Zero-Blunt TOPO kit (Invitrogen). The *GTR1*-containing insert was then subcloned into pRS316-GAL (22) using the PstI and SacI restriction enzyme sites.

High-throughput screen

Our high-throughput screen for compounds that selectively inhibit the growth of an *apl2*Δ *vps1*Δ yeast strain was performed at the University of Kansas High Throughput Screening Laboratory, which has a collection of over 100,000 compounds selected from the ChemBridge, ChemDiv, and Prestwick Libraries. 101,376 screening compounds were distributed in 384-well plates. Each plate had 352 wells for compounds and 32 wells for positive control (no cells) and negative control (DMSO without screening compound). The plates were seeded with an overnight (18-hour) culture of EHY644 (*apl2*Δ *vps1*Δ)grown at 24°C in YPD to OD600 0.05 (early log phase). 80 μl of culture was mixed with 20 μl of compound dissolved in 2.5% DMSO, for a final concentration of 5 μg/ml compound, 0.5% DMSO, in 100 μl per well. The plates were

then stacked (but not sealed) and incubated at room temperature for 15-17 h to a final average OD600 of 0.8 (close to late exponential growth under these conditions). Plates were read on an Envision multilabel plate reader (PerkinElmer, Wellesley, MA). We defined hits as those compounds that gave an OD600 that was 60% lower than that of the negative control. We identified 279 hits from screening with the *apl2*Δ *vps1*Δ mutant. These hits were then tested with both the *apl2*Δ *vps1*Δ mutant (EHY644) and the corresponding wild-type strain (EHY47), in a 6-point dose-response assay (0.15 μg/ml - 5 μg/ml) in 96-well plates. Of the 279 primary screen hits, 15 compounds inhibited the growth of the *apl2*Δ *vps1*Δ mutant strain but were significantly less toxic to the wild-type strain.

Assays for drug effects

All liquid cultures for growth assays and secretion assays were grown at 24°C, with the exception of the pulse-chase assays, which were performed at 30°C. (Growth at 24°C and 30°C was compared for the enzymatic invertase assay in selected samples, and no difference in results was observed for the two temperatures.) Yeast on agar plates were incubated at 30°C. For shaking-culture exponential-phase growth assays, cells were grown overnight to early exponential phase in CSM (minus uracil) medium to maintain plasmids. They were then diluted to OD600 0.07, and compounds dissolved in DMSO were added at the indicated concentrations, with final DMSO concentration of 0.25% in each case. Cultures were placed on a rotating platform for aeration, and OD600 readings were taken every 2 hours at least five times to generate an exponential growth curve. Rates were calculated from an exponential curve fit equation using Kaleidagraph 3.6 (Synergy Software; Reading, PA). The correlation coefficient (Pearson's R) was >0.9 in each case.

The invertase, Bgl2, and CPY transport assays were performed as described previously (18). For the invertase assay, we grew cells overnight to exponential phase in either YPD or CSM (to select for CEN plasmids), then shifted them to fresh YPD with 5% glucose for 2-3 hours. Compound or DMSO control was then added to this medium at the indicated concentration and cultures were grown for an additional 15 min, followed by shifting cells to YPD with 0.1% glucose (to derepress invertase expression) plus compound or DMSO control for 90 min prior to performing the invertase secretion assay as described previously (18). Results from at least three independent cultures grown on different days were averaged, and variability is indicated as SEM (standard error of the mean). Statistical significance (using a student t-test) was calculated using Kaleidagraph 3.6 software. Pulse-chase analysis of transport kinetics was performed as in (18). Briefly, exponential-phase cells were inoculated into CSM, -Cys, -Met at 4 OD600/ml, shaken at 30°C for 5 min, and compound or DMSO control was added for 20 min prior to a 4-min pulse with 25 μCi labeling mix/OD600 cells. Chase for 2-20 min was with excess cold amino acids, and cells were processed for immunoprecipitation and detection of Bgl2, invertase and CPY as described (18). Cells for thin-section electron microscopy were grown at 30°C in YPD, and prepared as described previously (18).

Screen for gene overexpression suppressors of drug effects

To screen for genes that, when overexpressed, can suppress the toxic effects of our drugs, we used both a 2μ (high copy) genomic library (5) and a *GAL*-promoter-driven cDNA library (22). Only the cDNA library yielded a suppressor clone. For that library, we screened for suppressors in strain EHY644 (*apl2*Δ *vps1*Δ). The strain was transformed with library DNA using the method of Schiestl and Gietz (36), and cells were plated on CSM -Ura, 2% galactose plates with compound (1 μM KU#7 or 2 μM KU#4). Plasmids were recovered from colonies that could grow on drug plates and re-

transformed and re-tested for suppression in EHY644. Plasmids that retested were sequenced at the insert junctions to identify the suppressing gene.

Results

A high-throughput screen for compounds that are selectively toxic to a vps1Δ apl2Δ mutant strain

We performed a high-throughput screen of a library of drug-like molecules for compounds that inhibit the growth of a yeast strain with *apl2*Δ and *vps1*Δ mutations but which have relatively small effects on a corresponding background strain. Our goal was to identify compounds that generate an AVL phenotype (*apl2 vps1* lethal), analogous to the phenotype of an *avl9*Δ mutant (18). Of 101,376 compounds screened, 279 significantly inhibited the growth of a *vps1*Δ *apl2*Δ mutant (less than 40% of growth without drug). These compounds were then screened in dose-response growth assays to eliminate compounds that inhibited the growth of both wild-type and mutant strains. Of our 279 initial hits that inhibited the growth of a *vps1*Δ *apl2*Δ mutant, we identified 15 hit compounds that selectively inhibited the growth of the mutant strain.

Seven of the 15 hit compounds could be grouped into two groups based on similar structures, whereas the rest of the structures were unique (Figure 1). We identified four similar compounds that we named Group A, and three compounds that we named Group B. A fifth Group A compound, KU#7f, was not identified in our screen but was purchased as a substitute for KU#7 when that compound was no longer available from the supplier (KU#7f is somewhat more active than KU#7 in our assays). Dose-response growth assays in 96-well plates for representative Group A compounds and a Group B compound are shown in Figure 2a. Piperazine rings are common in drug-like molecules, but the published bioactive piperazine derivatives that most resembled our Group A structures (12, 44) did not have growth-inhibiting activity for our mutant strains, when tested at up to 10 μM. In particular, we found that the N-benzoyl

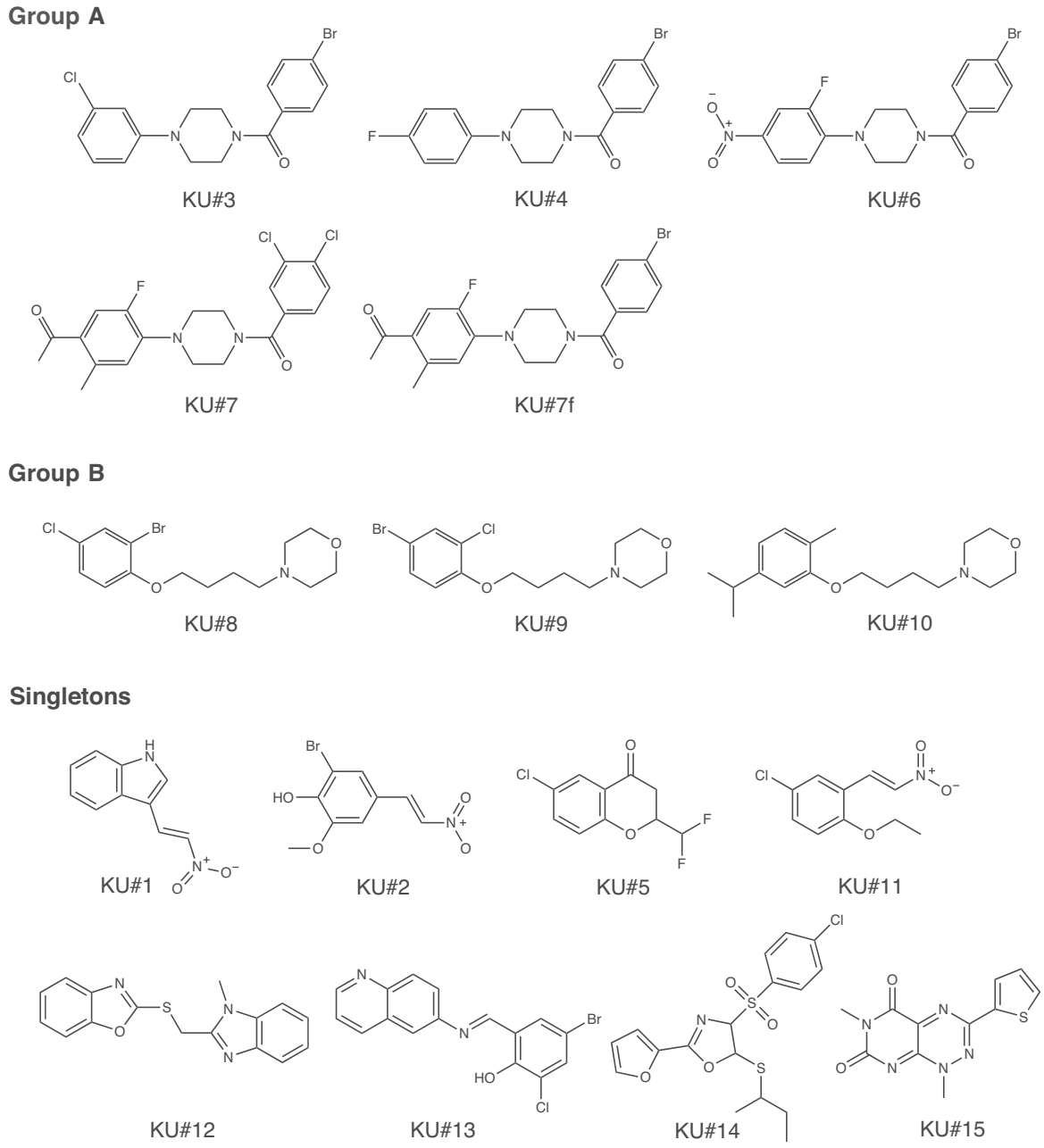


Figure 1. Structures of all 15 hit compounds identified in a high-throughput phenotypic screen of a 101,376-compound library for small molecules that have an AVL (*apl2*Δ *vps1*Δ lethal) effect. Some of the confirmed hit compounds grouped into two structural groups, Group A and Group B, whereas other hits had unique structures ("sinlgetons").

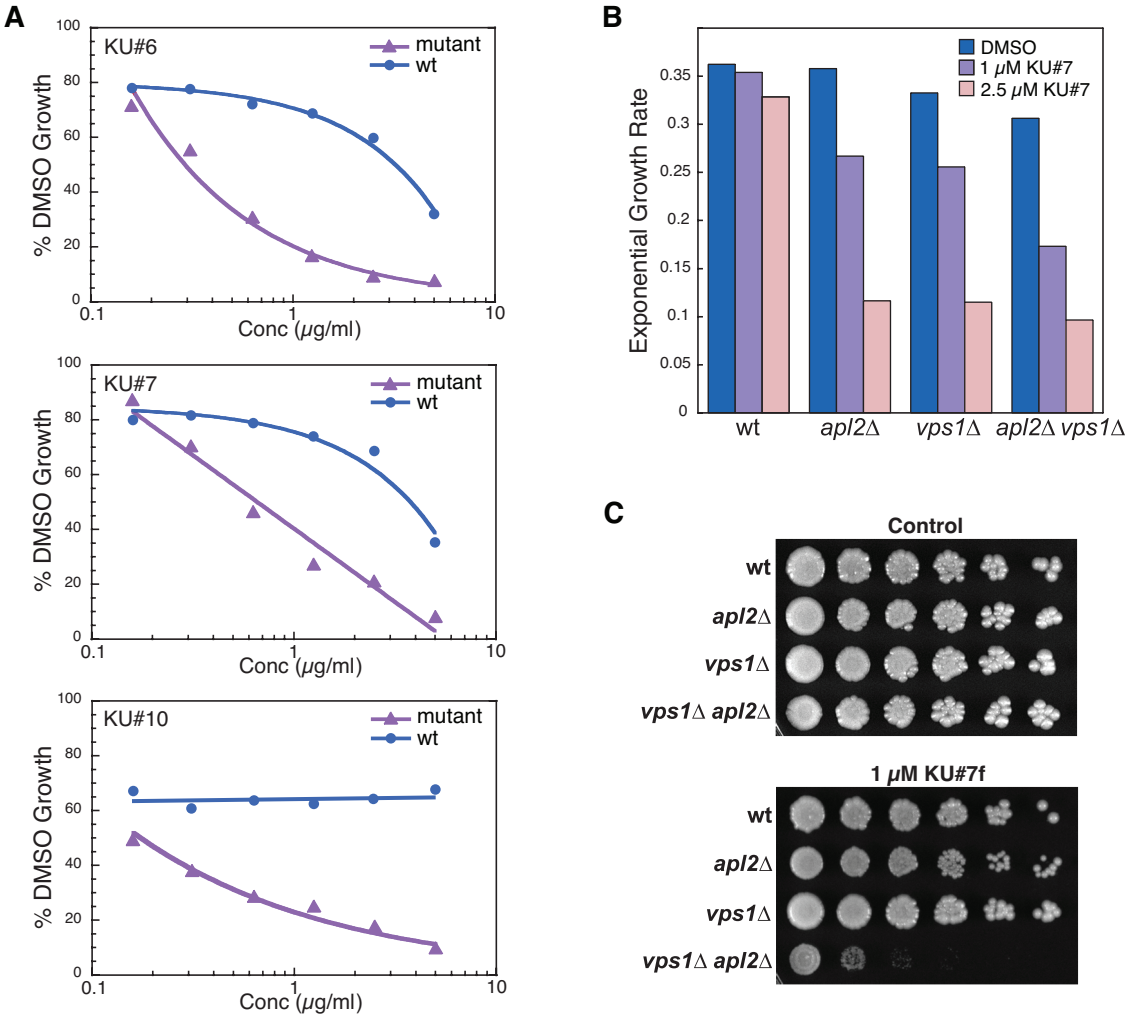
substituent was essential for the activity of our Group A compounds, because similar piperazine compounds that did not have this substituent (12) had no effect in our assays (our unpublished observations). We did not find published examples of bioactive compounds that resembled our Group B compounds.

Group A compounds selectively inhibit the growth of apl2Δ and vps1Δ mutants

We tested all of our 15 hit compounds in exponentially growing shaking cultures for growth-inhibiting activity and found that the Group A compounds had the most rapid (within 30 min), dramatic effect on the growth of either the *apl2*Δ or *vps1*Δ or *apl2*Δ *vps1*Δ mutant strains, with relatively much smaller effect on the growth of our wild-type strain (Figure 2b). However, when grown on solid medium, the toxic effects of the compounds were specific to the *apl2*Δ *vps1*Δ strain; thus, the Group A compounds have an AVL (***a****pl2*Δ ***v****ps1*Δ **l**ethal) effect (Figure 2c). It is possible that rapidly-growing cells in shaking cultures, in which membrane trafficking occurs at a greater speed than it does in cells grown on plates, are more sensitive to Group A compounds. Alternatively, our results could reflect the time scale of the assays, a few hours in shaking cultures at exponential growth, compared to several days on plates, in which time the *vps1*Δ and *apl2*Δ single-mutants could possibly adapt to the effects of the compounds.

In contrast to Group A compounds, most of our hit compounds initially did not appear to inhibit growth of exponentially growing shaking cultures. This included the Group B compounds, which consistently strongly inhibited the growth of the *apl2*Δ *vps1*Δ strain but not the wild-type strain when the growth assay was performed in 96-well (non-shaking) liquid cultures (Figure 2a) or agar plates (not shown). However, we found that after growing cells for a longer time (>4 hours), growth inhibition in shaking liquid cultures became apparent in Group B compounds (results not shown). The delay in growth defect was not due to a need for build-up of compound in cells, because we

Figure 2 (following page). Group A and Group B compounds have mutant-specific effects on growth. (A) Dose-response growth assays for KU#6 (Group A), KU#7 (Group A) and KU#10 (Group B) compounds. Wild-type and *apl2*Δ *vps1*Δ ("mutant") cells were grown in the presence of compound or DMSO control in 96-well plates. Compounds were at the following concentrations, prepared by 2-fold serial dilutions: 5, 2.5, 1.25, 0.625, 0.31, and 0.15 μg/ml. The graphs show [(OD600 in compound)/OD600 in DMSO]x100, after ~18 h growth. (B) Growth rates of exponential-phase cells in shaking cultures. Overnight exponential-phase cultures were diluted to OD600 0.07, and compounds or DMSO control were added at the indicated concentrations with final DMSO concentration of 0.25% in each case. Cultures were grown with aeration, and OD600 measurements were taken over ~9h of growth to generate growth curves. Rates were determined from an exponential curve fit equation (correlation coefficients >0.9 in each case). (C) Growth on solid media in presence of Group A compounds. Cultures were grown to OD600 1.0 and spotted on a YPD plate containing 1 μM KU#7f and on a control plate, after 4-fold serial dilutions. Mutant strains have an EHY47 wild-type background and are identical except for the plasmids they contain: EHY658 (*apl2*Δ), EHY1166 (*vps1*Δ), and EH707 (*apl2*Δ *vps1*Δ).


A
KU#6
mutant
wt
% DMSO Growth
Conc (μg/ml)
KU#7
KU#10
B
DMSO
1 μM KU#7
2.5 μM KU#7
Exponential Growth Rate
wt
apl2Δ
vps1Δ
apl2Δ vps1Δ
C
Control
wt
apl2Δ
vps1Δ
vps1Δ apl2Δ
1 μM KU#7f

noticed the same time delay in lower and higher concentrations of the drug. It is possible that these compounds affect gene expression or signaling pathways that regulate growth.

Group A compounds inhibit exocytic transport

It is difficult to interpret results with slow-acting growth inhibitors, so we focused on analyzing the effects of Group A compounds, which rapidly inhibited the growth of our mutant strains. To determine whether the toxicity of these compounds is due to a transport block, we assayed the effects of the compounds on exocytic cargo transport. In wild-type yeast, two pathways can transport exocytic cargo. One route transports the cell wall protein Bgl2, whereas another route transports the periplasmic enzyme invertase (16). These two cargoes are sorted into one pathway in a *vps1*Δ mutant, in which the normal invertase pathway appears to be blocked (17). Bgl2 transport can be conveniently assayed, because at steady state most of this protein is in the cell wall, which can be removed by enzymatic digestion (spheroplasting). Wild-type cells have very little Bgl2 after spheroplasting, which represents intracellular Bgl2 in transit to the cell surface. We found that 15 min after adding compound, intracellular accumulation of Bgl2 was detected in the *vps1*Δ and *apl2*Δ mutants but not in the corresponding wild-type strain (Figure 3). This accumulation was very dramatic after 90 min, indicating a significant defect in Bgl2 transport.

We also assayed the transport of a periplasmic enzyme, invertase, in the presence of Group A compounds. Invertase expression is repressed when cells are grown in 2% glucose, and expression is derepressed when cells are shifted to 0.1% glucose (8). There is no detectable invertase secretion prior to derepressing expression. We added compounds to cells 15 min prior to derepression, and then shifted cells into low glucose with compound for 90 min, followed by enzymatic assays for invertase secretion

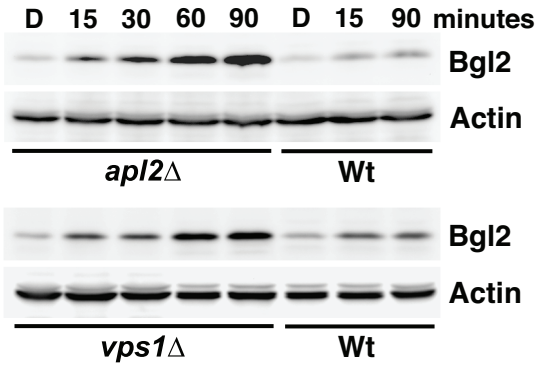


Figure 3. Group A compounds cause internal accumulation of the cell wall protein, Bgl2, in *apl2*Δ and *vps1*Δ mutant strains. Cells were grown to exponential phase, and compound KU#4 (Group A) was added at 10 μM for the indicated times. DMSO controls ("D") represent 60-min time-point samples. The cell walls were digested to remove external Bgl2, and internal Bgl2 was assayed by western blotting. Actin is shown as loading control. Strains are as in Figure 2.

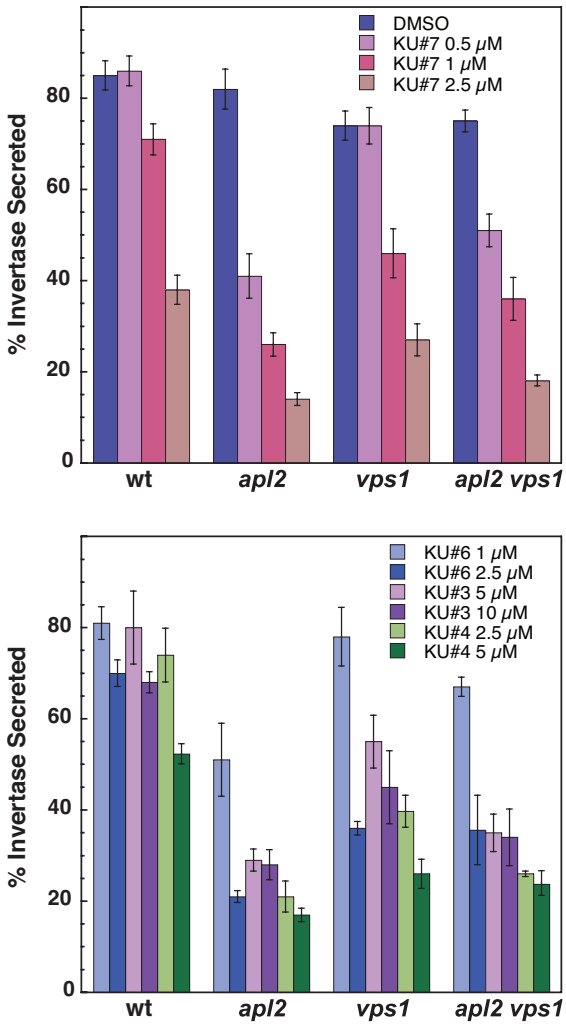


Figure 4. Group A compounds cause a mutant-specific defect in invertase secretion. Cultures were grown to exponential-phase in high-glucose medium, and compound was added for 15 min before shifting to low glucose with compound (to derepress invertase expression). Growth in compound was continued for 90 min. External invertase was compared to total invertase by an enzymatic assay to calculate the percent of invertase secreted. Strains are as in Figure 2. The means of three experiments (from three independent cultures) are shown. Error bars, SEM.

(Figure 4). We observed a very significant secretory defect for the *apl2*Δ mutant in as low as 0.5 μM compound KU#7, the lowest concentration that we assayed. We found similar effects on secretion for all of our Group A compounds, and the *apl2*Δ mutant was consistently the most sensitive to compound in this assay (Figure 4). The mutant strains were identical except for the plasmids that they contained (double mutant with either empty vector, or vector with *VPS1* or *APL2*). It is possible that the double mutant adapts to the transport defect in some way that makes it less sensitive to compound in this assay. Interestingly, the relative invertase secretion defects in the different mutants did not correspond with the relative growth decrease in compounds (Figure 2b,c).

The secretory assays described above have the advantage of being very sensitive for detecting transport defects. However, the assays show cargo accumulation over time, rather than an immediate effect on transport kinetics. Therefore, we also performed metabolic labeling and pulse-chase analysis of secretory cargo to determine whether the compounds had observable effects on transport kinetics in this assay (Figure 5). In the absence of inhibitors, about half of the Bgl2 is exported within 5 min, while transport of invertase is faster (Figure 5 a,c shows results for *apl2*Δ; results are similar for wild type). Both wild-type and *apl2*Δ cells show a defect in Bgl2 transport kinetics in compound KU#4 and KU#7 (Figure 5b), and the defect is more substantial in *apl2*Δ cells. The slower transport kinetics was most easily observed at early chase time points, but there was not a complete transport block, and most Bgl2 was secreted by 20 min (not shown). KU#4 and KU#7 also slowed invertase transport in the *apl2*Δ mutant, but not in wild-type cells (Figure 5d). However, invertase transport is normally very fast, so it is difficult to detect a partial transport block by pulse-chase analysis. Wild-type cells did show a defect in the enzymatic invertase secretion assay (Figure 4), but internal invertase in that assay represents accumulation over 90 min. Furthermore, the enzymatic assays also show an incomplete transport block for wild-type and even

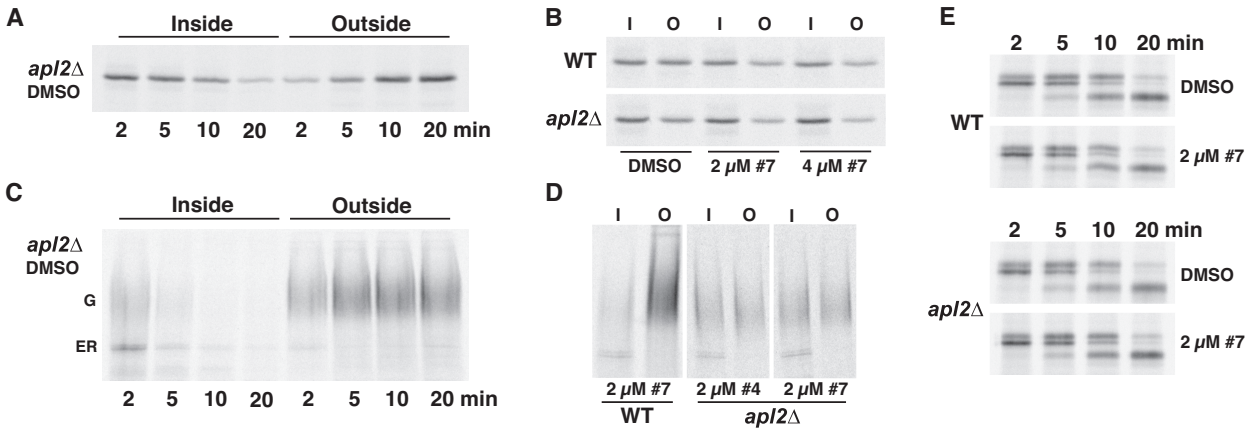


Figure 5. Pulse-chase analysis shows a kinetic lag of Bgl2 and invertase exocytic transport in the presence of Group A compounds. Cells were pre-incubated with compound or DMSO control for 20 min and metabolically labeled with 35S cysteine and methionine for 4 min, followed by addition of excess unlabeled amino acids for 2-20 min chase times (A, C, E) or for a 5 min chase (B and D). The inside (I) fraction was separated from the cell wall and media fraction (O), and Bgl2 (A, B), invertase (C, D) and CPY (E) were immunoprecipitated and detected by phosphorimaging. Bgl2 is not visibly modified, whereas invertase has an ER form and a Golgi-modified (G) heterogeneously glycosylated form. CPY has a 67 kDa ER form, a 69 kDa Golgi form, and a 61 kDa vacuole form. Group A compounds do not cause a defect in ER-to-Golgi or Golgi-to-vacuole transport of CPY.

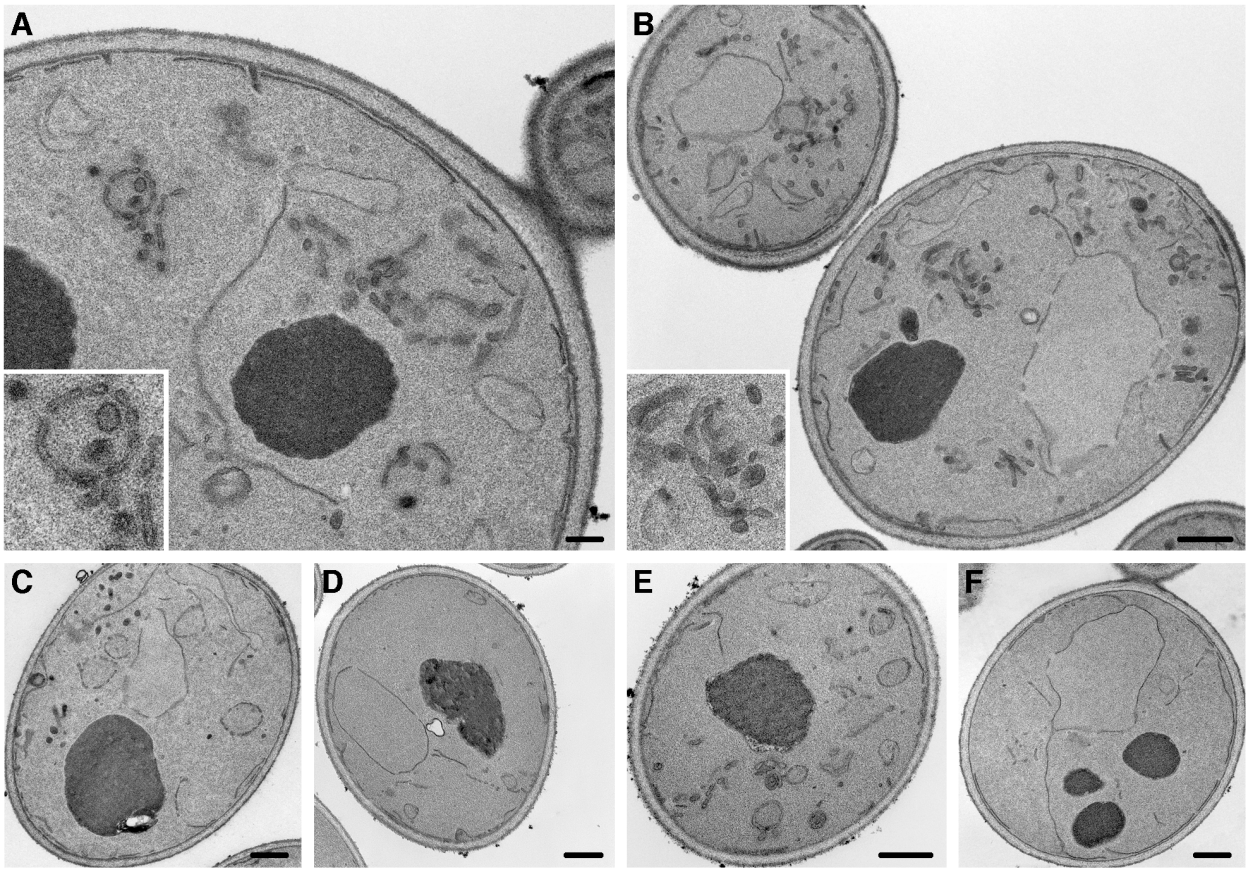


Figure 6. Group A compounds cause accumulation of Golgi or endosome-like membranes. An *apl2*Δ strain (EHY807; A, B) or wild-type (EHY47; E) were grown in the presence of 2.5 μM compound KU#7 for 30 min. Similar results were obtained for *apl2*Δ cells grown in 10 μM KU#3 (a less-potent Group A compound) for 60 min (C). DMSO controls without compound are shown in D (*apl2*Δ) and F (wild type). Cells were prepared for thin-section electron microscopy as described (18). The scale bars represent 200 nm (A), or 500 nm (B-F).

mutants, at the drug concentrations we tested (for comparison, the *sec6-4* conditional secretory mutant secretes <5% invertase at restrictive temperature in this enzymatic assay; (27) and our unpublished observations). The transport defects caused by our compounds appear to be specific to the late exocytic pathway, because pulse-chase analysis of transport of a vacuolar protein, CPY, showed no detectable defect in ER-to-Golgi transport or transport from the Golgi to vacuole (Figure 5e).

Group A compounds cause accumulation of Golgi-like membranes

To further define the membrane traffic defects caused by our Group A compounds, we examined the ultrastructure of cells grown in the presence of KU#7 or KU#3 by thin-section electron microscopy (Figure 6). The *apl2*Δ strain showed dramatic accumulation of membranes when grown in KU#7 for just 30 min (Figure 6a, b). Most of the membranes were clusters of vesicles and/or tubules (tubules and fenestrated membranes could appear as clustered vesicles in the plane of a thin section). Another common and striking structure was a ring of discontinuous membranes (enlarged inset in Figure 6a; also seen in Figure 6b,c,e). Similar rings were observed in cells grown in the presence of KU#3 for 60 min (Figure 6c; KU#3 is our least-effective Group A compound). Because the rings were consistently circular, they likely represent fenestrated spheres, structures also observed in *sec14-ts* mutants after a short shift to a restrictive temperature (32) and in an *arf1*Δ mutant (15). Gaynor et al. (15) observed both very large and smaller rings in *arf1*Δ cells, and from complementing immunolocalization studies by light microscopy, they concluded that the large rings likely represent aberrant endosomes, whereas the smaller rings represent Golgi membranes. In our samples, we did not observe the very large rings seen in the *arf1*Δ mutant. However, the *arf1*Δ and *sec14-ts* mutants have defects in transport from both Golgi and endosomes (15, 23), and the invertase-transporting exocytic pathway likely transits endosomes (17), so our Group A compounds could perturb exit from either or

both the Golgi and endosomes. Wild-type cells had a similar but less abundant membrane-accumulation phenotype when grown in the presence of KU#7 (Figure 6e). We did not observe these abnormal membranes in either *apl2*Δ cells or wild-type cells grown in DMSO control (Figure 6d, f), although the *apl2*Δ cells had occasional small continuous rings which represent cup-shaped structures, similar to but much smaller than Berkeley bodies, thought to represent aberrant Golgi, in *sec7-ts* and *sec14-ts* mutants (27)). We also observed small continuous rings in cells grown in KU#7, but these were likewise smaller and rarer than the structures accumulated by *sec7* and *sec14* mutants. Cells grown in KU#7 for 1h had a similar phenotype (not shown), so the fenestrated spheres did not progress to Berkeley bodies at the drug concentrations we tested. It is possible that the fenestrated spheres and tubules represent an incomplete block in exit from the Golgi (as is the case with the *arf1*Δ mutant described in (15)). Alternatively, the different abnormal membranes may represent different molecular defects.

Toxicity of Group A compounds can be suppressed by overexpressed GTR2

A relatively simple strategy for identifying potential drug targets, or genes that are relevant for the function of a drug target, is a screen for gene-dosage suppression of the drug effects. We screened both a 2μ (multicopy) genomic library and a *GAL*-promoter-driven cDNA library (22) for genes that, when overexpressed, can suppress the toxicity of Group A compounds. We obtained no suppressors from the 2μ library and only one strong suppressor gene from the cDNA library. We identified the suppressor gene as *GTR2* (Figure 7). Gtr1 and Gtr2 are homologous Ras-like GTP binding proteins that form hetero- and homo-dimers (14, 25). They function as nutrient-responsive regulators of the TORC1 signaling pathway (7, 11), exocytic sorting of the Gap1 general amino acid permease at endosomes (14), and epigenetic control of gene expression (37). Although Gtr1 and Gtr2 are thought to function as obligate heterodimers, Gtr1 had no

suppressing effect when highly expressed from the *GAL* promoter (Figure 7). A 2μ (high-copy) plasmid containing *GTR2* under its native promoter could not suppress toxicity either on glucose or galactose (our unpublished observations).

A dominant-negative form of the Gtr complex and decreased TORC1 signaling suppress the secretory defect caused by Group A compounds

Our invertase secretion assay is not compatible with growth on galactose-containing media, so we could not use our *GAL-GTR2* construct to test whether overexpression of *GTR2* can suppress the effects of Group A compounds on invertase secretion. Instead, we used mutant alleles of the *GTR* genes expressed from their native promoters in CEN (low-copy) plasmids. The Gtr1/Gtr2 dimer, as well as the metazoan counterpart, RagAB/RagCD, are active in positively regulating TORC1 signaling when Gtr1 (or RagA or RagB) is GTP-bound and Gtr2 (or RagC or RagD) is GDP-bound. Furthermore, in the opposite conformations (Gtr1/RagAB in a GDP-bound state and Gtr2/RagCD in a GTP-bound state) have a dominant-negative effect on TORC signaling (7, 11, 20, 35). Likewise, the Gap1 permease is sorted from a vacuolar pathway to the exocytic pathway when Gtr1 is GTP-bound and Gtr2 is GDP-bound, but not when the GTPases are restricted to the opposite conformations (14). We tested *GTR* alleles that express either GTP-restricted or GDP-restricted forms of the proteins for effect on invertase secretion in *apl2*Δ *vps1*Δ cells grown in presence of KU#7f (Figure 8a). We found that both Gtr1 and Gtr2 can partially suppress the effects of KU#7f on secretion, and only when the GTPases are restricted to the conformations that are expected to form a dominant negative complex. Although the suppression of the secretory defect was not dramatic, the Gtr proteins were not overexpressed in these experiments, and the wild-type proteins were also present. Furthermore, the results were consistent in each repeat of the experiment and statistically significant (P = 0.001 for secretion with empty vector compared with secretion when Gtr2-GTP is expressed). In contrast, the

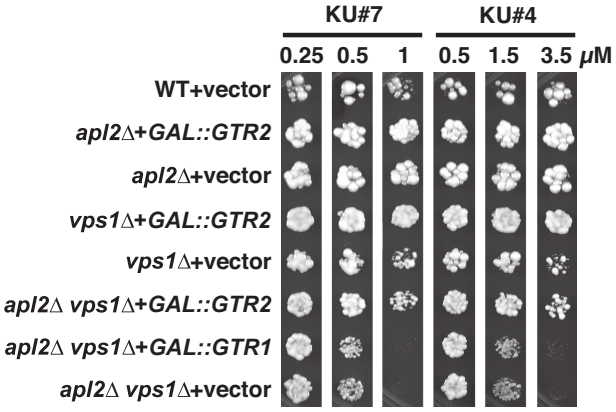


Figure 7. Overexpression of *GTR2* on galactose-containing medium suppresses the toxic effect of Group A compounds. The strains are described in *Materials and Methods* and are as follows: EHY47 with pRS316, EHY807 with pLZ43; EHY807 with pRS316, EHY361 with pLZ43, EHY361 with pRS316, EHY644 with pLZ43, EHY644 with pLZ44, and EHY644 with pRS316.

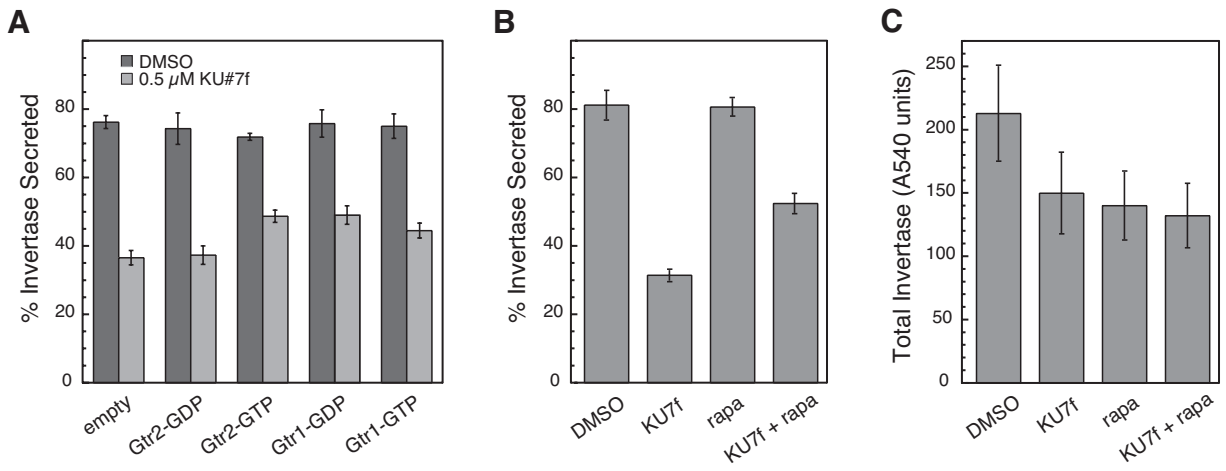


Figure 8. Dominant-negative Gtr proteins and sub-inhibitory rapamycin suppress the invertase secretion defect caused by Group A compounds. (A) An *apl2*Δ *vps1*Δ mutant strain (EHY644) was transformed with empty vector (pRS316) or pRS-based (CEN) vectors expressing GDP- or GTP-restricted mutant forms of Gtr1 and Gtr2, from their native promoters. The strains were tested for invertase secretion after growth in 0.5 μM KU#7f, as described for Figure 4. For each strain, the means were calculated from 7 repeats (for KU#7f) or 3 repeats (for DMSO). Each repeat was an independent culture. (B) An *apl2*Δ *vps1*Δ mutant strain (EHY644) culture was divided into four samples, which were treated with either DMSO (control), or 0.5 μM KU#7f, or 10 ng/ml rapamycin, or 0.5 μM KU7f plus 10 ng/ml rapamycin together. The means from three independent (pre-drug) cultures are shown. (C) Total invertase activity (secreted plus internal) from the cultures used in (B). Invertase is expressed as A540 units per OD600 cells from the invertase enzymatic assay. Error bars, SEM.

alleles that are expected to form the TORC1-activating form of the Gtr complex (Gtr2-GDP and Gtr1-GTP) had less or no effect on invertase secretion (Figure 8a).

Because the "active" Gtr complex positively regulates TORC1 signaling, whereas the "inactive" complex has a dominant negative effect on TORC1 activity, we asked whether decreasing TORC1 activity also suppresses the secretory defect in the presence of KU#7f. We found that 10 ng/ml rapamycin (which is the amount typically used as a sub-inhibitory level, while 200 ng/ml is used to inhibit TORC1; (6)) suppressed the secretory defect caused by KU#7f to an extent similar to that of the dominant-negative Gtr proteins (Figure 8b). A nearly identical result was obtained with 20 ng/ml rapamycin (not shown), so our results with both the Gtr proteins and rapamycin may represent the maximum possible suppression. Rapamycin alone, either 10 ng/ml (or 20 ng/ml, not shown) had no effect on invertase secretion in the *apl2*Δ *vps1*Δ strain when no Group A compound was present (Figure 8b).

TORC1 positively regulates translation (45), so we speculated that suppression by reducing TORC1 activity may be due to a decrease in cargo flow through the secretory pathway, such that the reduced secretory competence due to effects of our Group A compound is less deleterious to the *apl2*Δ *vps1*Δ mutant. Cells are grown in the drugs for 15 min prior to derepression of invertase expression, followed by 90 min more in drug, so some reduction in protein synthesis might eventually occur in the time course of our experiments. However, for the identical samples shown in Figure 8b, we found that total invertase (secreted plus internal) was essentially the same when just KU#7f was added to cells and when both KU#7f and rapamycin were added (although total invertase level was somewhat lower compared to DMSO control, whether or not rapamycin and KU#7f were combined or each was present alone). These results suggest that TORC1 activity may regulate membrane traffic in a way that is deleterious

when traffic is perturbed in the *apl2*Δ *vps1*Δ mutant by Group A compounds. We were unable to detect suppression of the growth-inhibitory effect of Group A compounds, by either the mutant alleles of Gtr proteins or by sub-inhibitory levels of rapamycin. This could be because the invertase assay measures a relatively short-term effect, and the long-term effect on growth is not as readily suppressed.

GTR2 is not a likely direct target of Group A compounds

Suppression by overexpressed Gtr2 could indicate either that Gtr2 is a target of our Group A compounds, or that the function of the drug target is relevant for Gtr2 activity. Because Group A compounds are toxic to an *apl2*Δ *vps1*Δ mutant, a defect in the drug target could likewise be deleterious to the *apl2*Δ *vps1*Δ strain. We found that crossing a *gtr2*Δ mutation into our *apl2*Δ *vps1*Δ strain did not noticeably reduce the growth of the *gtr2*Δ *apl2*Δ *vps1*Δ triple-mutant progeny (Figure 9). It is still possible that Group A compounds have a dominant effect on Gtr2, or target a regulator of its activity. However, this is unlikely, because in such a case, the deleterious effects of the compounds would require the presence of *GTR2*, and we found that KU#7f is at least as toxic to a *gtr2*Δ *apl2*Δ *vps1*Δ mutant as it is to an *apl2*Δ *vps1*Δ mutant (Figure 9). Although we have not yet identified the molecular target of our comounds, we can conclude that modulation of TORC1 activity is critical when the effects of Group A compounds are combined with the *apl2*Δ and *vps1*Δ mutations, and our results are valuable in suggesting additional target candidates.

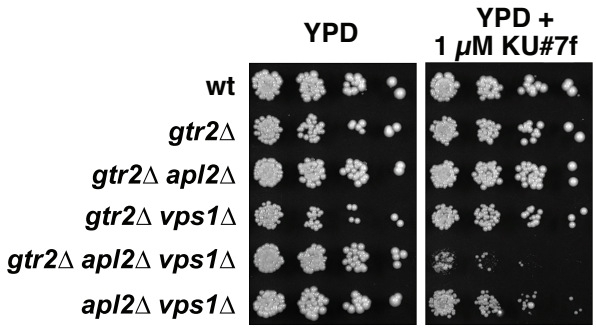


Figure 9. Gtr2 is not a likely target of our Group A compounds. A *gtr2*Δ mutation (in EHY47 background) was crossed into an *apl2*Δ *vps1*Δ strain (EHY644). All double- and triple-mutant progeny were viable; therefore, unlike Group A compounds, *gtr2*Δ does not produce an AVL phenotype. The *gtr2*Δ *apl2*Δ *vps1*Δ strain was at least as sensitive to 1 μM KU#7f as was an *apl2*Δ *vps1*Δ strain, indicating that the drug does not interact with Gtr2 for a dominant effect. These results show that Group A compounds do not have a direct effect on Gtr2 function.

Discussion

We screened a 101,376-compound library for small molecules that perturb the late exocytic pathway, with the goal of identifying novel molecular probes to help us understand the mechanisms and regulation of late exocytic transport. We identified 15 compounds that had selective toxicity towards a yeast mutant, *apl2*Δ *vps1*Δ, in which exocytic cargo is rerouted from a defective pathway to an alternative exocytic route. Thus, the compounds are expected to perturb a remaining exocytic pathway in this mutant, and cause an AVL (***a****pl2* ***v****ps1* **l**ethal) phenotype, analogous to the phenotype of the *avl9*Δ mutant identified in a classical yeast mutant screen (18). Some of our hits fell into two groups of similar structures, and one of these groups, which we named Group A, was characterized in detail for effects on membrane trafficking.

The Group A compounds inhibit the growth of the *apl2*Δ *vps1*Δ strain at a low (<1 μM) concentration, and cause an exocytic defect at <0.5 μM both in this double-mutant and the *apl2*Δ and *vps1*Δ single-mutants. The transport defect appears to be specific to the late exocytic pathway, because the compounds have no detectable effect on transport to the vacuole/lysosme in either *apl2*Δ or wild-type cells. Furthermore, the compounds cause rapid (within 30 min) accumulation of membranes that resemble the aberrant membranes in other mutants with exocytic blocks at the Golgi. With higher concentrations of compound (>2 μM), we detected secretory defects and membrane accumulation in wild-type cells as well. These results indicate that our compounds inhibit exocytic transport from the Golgi and possibly endosomes, and thus target a molecule or molecules that mediate traffic at these steps.

We initiated efforts to identify the molecular target of our Group A compounds by screening for genes that suppress the toxicity of the compounds when overexpressed.

Although Avl9 is a potential target, it is highly toxic when overexpressed (18), and other strategies are needed to explore it as a potential drug target. Our suppressor screen identified just one gene, *GTR2*, which suppressed the toxic effects of our most potent Group A compound, KU7f, when highly expressed from the *GAL* promoter. Gtr2 and its homolog, Gtr1, function as a dimer to regulate numerous nutrient-responsive processes, but the primary function of these GTPases and the corresponding metazoan proteins is thought to be the regulation of TORC1 signaling, and thus regulation of growth (7, 11, 20, 35). Overexpression of *GTR1* could not suppress the toxicity of KU7f, a result that was explained in our subsequent assays with GDP- and GTP-restricted mutant alleles of the GTPases. We found that the dominant-negative alleles of the GTPases, having a GDP-restricted conformation for Gtr1 and GTP-restricted conformation for Gtr2, partially suppressed the invertase secretion defect caused by KU7f in the *apl2*Δ *vps1*Δ mutant. Therefore, the overexpressed wild-type proteins are likely more abundant in a GTP-bound conformation.

The alleles of the GTPases that best suppressed the secretory defect caused by KU7f are dominant-negative regulators of TORC1 signaling, and, correspondingly, sub-lethal concentrations of rapamycin, a TORC1 inhibitor, likewise suppressed the invertase secretory defect caused by KU7f. This result was not due to a reduction in invertase production. Therefore, TORC1 signaling may regulate a pathway that needs modulation in response to secretory transport blocks. Invertase in wild-type cells likely transits endosomes en route to the cell surface (17), and endosomes are the most likely site where the Gtr/Rag complex functions in regulating transport in response to amino acid signals in both yeast and mammalian cells (14, 35). Furthermore, TORC1-mediated signaling functions at endosomes or the Golgi for sorting the high-affinity tryptophan and histidine permeases, Tat2 and Hip1, from an endosome-mediated vacuolar pathway to the exocytic pathway (6). It is possible that both Group A compounds and

TORC1 signaling enhance sorting into an endosome-mediated exocytic transport route that is defective in the *apl2*Δ *vps1*Δ mutant. TORC1 signaling and our compounds may in addition, or instead, downregulate an alternative transport route for invertase that is utilized by the *apl2*Δ *vps1*Δ mutant.

In addition to linking exocytic transport to signaling mechanisms that regulate growth, the identification of *GTR2* as a suppressor of our Group A compounds was an exciting result, because it suggests that our chemical-genetic strategy may lead to insights about Avl9 function. The *gtr* and *avl9* mutants were among the top-ranked mutants in a genome-wide screen of the non-essential yeast gene deletion collection for mutants that are hypersensitive to both high hydrostatic pressure and cold temperature (3). The reason for this growth phenotype of *gtr* an *avl9*Δ mutants is not clear, but it could reflect defects in traffic due to reduced membrane fluidity under these conditions. Other mutations in non-essential genes that cause defective transport from the Golgi were also identified in the screen (3). Furthermore, at high pressure and cold temperature, the Tat2 permease is sorted to the vacuole even though TOR signaling seems unaffected (2), suggesting that the TORC1-regulated exocytic route is especially sensitive to conditions that reduce membrane fluidity.

Transcriptional profiles of S. ce*revisiae* subjected either to high hydrostatic pressure or to cold temperature indicate highly upregulated expression of genes involved in remodeling of the cell surface (plasma membrane and cell wall) under anaerobic growth conditions (1, 4, 10, 13, 28). Traffic pathways or mechanisms that involve Avl9 and Gtr function may be involved in cell surface remodeling, and a defect in this process may lead to pressure- and cold-sensitivity. Alternatively or in addition, the high levels of expression of these same set of genes encoding cell surface components may be toxic to the *avl9*Δ mutant. The *avl9*Δ mutant is SLAM (synthetic lethality analysis on

microarrays) with *mot3*Δ (29), and Mot3 is a repressor of the expression of most of these same cell surface components (38). It is also possible that Avl9 and Gtr proteins have a role in regulating plasma membrane homeostasis, or an Avl9- and Gtr-dependent sorting or transport process depends on optimized lipid composition. There is much evidence that regulation lipid composition is critical for cargo sorting and transport carrier formation in the late secretory pathway (21, 23, 24, 30, 41).

The signaling pathways that regulate cell growth and proliferation are expected to coordinate with regulation of the traffic pathways that deliver cell-surface components and thereby mediate cell growth. Therefore, our result that modulation of a TORC1-mediated process is critical in the presence of our Group A compounds suggests that these compounds may serve as powerful probes for understanding the molecular mechanisms by which late exocytic transport is regulated in response to environmental factors. Furthermore, a possible link to Avl9 function via Gtr2-mediated suppression of the drug effects, as well at the AVL phenotype conferred by the compounds, indicate that our new exocytic transport inhibitors may help us to discover the biochemical and biological functions of the Avl9 protein family.

Acknowledgments

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Chapter 3

A high-throughput screen for chemical inhibitors of exocytic transport in yeast

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Introduction

The signaling pathways that control cell proliferation must regulate the membrane and protein traffic pathways that are required for cell growth and cell division. How these membrane traffic pathways are regulated, in particular at the late sorting steps of the exocytic pathway, is poorly understood. Both the signaling pathways and exocytic routes are highly complex, resulting in numerous alternate mechanisms by which transport to the cell surface can be regulated. The budding yeast, *Saccharomyces cerevisiae*, has proven to be a powerful model for understanding complex cellular processes, due both to the relative simplicity of this organism and the many tools available that take advantage of its genetic manipulability. Yeast genetic studies played a major role in elucidating the basic signaling pathways by which cells respond to nutrient and other environmental conditions to regulate cell growth and proliferation [1, 2]. Furthermore, many of the components of the membrane traffic machinery were first discovered and characterized in yeast [3, 4]. However, the mechanisms by which cargo is sorted and packaged into transport vesicles in late exocytic compartments (Golgi and endosomes) is still not known. This is in large part because we still need to identify the molecular machineries that carry out exocytic transport from the Golgi and endosomes, and we need to define the signaling mechanisms that regulate traffic from these organelles. The goal of the present study was to identify molecular probes that hold promise to serve as powerful tools for both identifying and understanding the machineries that regulate and carry out late exocytic transport.

The difficulty in identifying components of the late exocytic transport machinery is due to multiple exocytic traffic routes in both yeast and mammalian cells [5, 6], which allow cargo transport by alternate routes when a pathway is blocked [7]. Such ability of cells to overcome defects in late exocytic transport steps has made it difficult to identify the genes involved in mediating and regulating late exocytic transport, necessitating complex and laborious genetic screens [8]. An alternative and potentially more efficient strategy is a chemical genetic version of a successful classical yeast genetic screen that identified a novel component of the late exocytic transport machinery, Avl9 [8]. Here we describe such a chemical genetic screen, in which we identified small molecules that are novel secretory inhibitors. Identifying the molecular and biological targets of these inhibitors will facilitate our understanding of late exocytic transport mechanisms, and how these mechanisms are regulated.

Materials and Methods

Yeast strains and reagents

The construction or origin of yeast strains used in this study are shown in Table 1. Strains were generated by crosses using standard yeast genetic techniques [26]. In some cases, sequential crosses were needed using progeny from the indicated parents in order to obtain the desired combined mutations. The primary screen strains were JTY2953 (*snq2*Δ *pdr5*Δ) and an *snq2*Δ *pdr5*Δ *vps1*Δ *apl2*Δ strain derived from it, LZY35 (AID 738, 739). These strains are adenine auxotrophs (due to the *ade2-101* allele, common in many laboratory strains). In order to improve strain growth and to back-cross mutant strains into our wild-type background, new strains having wild-type *ADE2* were constructed for use in all subsequent screening steps and assays. These strains have primarily an

Table 1. Yeast strains used in this study.

Strain	Genotype	Source
GPY1783-10A	*MATα apl2Δ::TRP1 his3-Δ200 leu2-3,112 trp1-Δ901 ura3–52 suc2-Δ9*	Yeung et al. 1999
JTY2953	*MATa pdr5::TRP1 snq2::hisG ade2-101 his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3–52*	Egner et al. 2000
NY10	*MATα ura3–52*	Salminen and Novick, 1987
EHY644	*MATa vps1Δ::LEU2 apl2Δ::TRP1 trp1 leu2–3,112 ura3–52 his3-Δ200*	Harsay and Schekman, 2007
EHY1172	*MATα vps1Δ::LEU2 apl2Δ::KAN trp1 leu2-3,112 ura3–52 ade2–101 ade3*	Harsay and Schekman, 2007
LZY35	*MATa vps1Δ::LEU apl2Δ::KAN pdr5::TRP1 snq2::hisG ade2-101 his3-Δ200 leu2 trp1-Δ63 ura3–52*	JTY2953×EHY1172
LZY40	*MATα vps1Δ::KAN apl2Δ::TRP1 leu2–3,112 his3-Δ200 trp1-Δ901 ura3-52 suc2-Δ9*	GPY1783-10A, deleted *vps1*
LZY50	*MATα apl2Δ::TRP1 pdr5::TRP1 snq2::hisG his3-Δ200 leu2 trp1 lys2–801am ura3–52*	JTY2953×GPY1783-10A
LZY53	*MATα pdr5::TRP1 snq2::hisG his3-Δ200 leu2 lys2-801 trp1 ura3–52*	JTY2953×GPY1783-10A
LZY80	*MATα apl2Δ::KAN pdr5::TRP1 snq2::hisG his3-Δ200 leu2 trp1 ura3–52*	LZY35×LZY53
LZY81	*MATa vps1Δ::LEU2 pdr5::TRP1 snq2::hisG his3-Δ200 leu2 trp1 ura3–52 lys2–801am*	LZY35×LZY53
LZY82	*MATa vps1Δ::LEU apl2Δ::KAN pdr5::TRP1 snq2::hisG his3-Δ200 leu2 trp1 ura3–52 lys2–801am suc2-Δ9*	LZY35×LZY53
LZY96	*MATa vps1Δ::LEU2 apl2Δ::TRP1 snq2::hisG leu2 his3-Δ200 trp1 ura3–52*	LZY40×LZY35
LZY104	*MATα vps1Δ::LEU2 apl2Δ::TRP1 pdr5::TRP1 his3-Δ200 leu2 lys2–801am trp1 ura3–52*	LZY53×EHY644
LZY108	*MATa pdr5::TRP1 his3-Δ200 leu2 lys2–801am trp1 ura3–52*	LZY53×EHY644
LZY109	*MATa snq2::hisG his3-Δ200 leu2 lys2–801am trp1 ura3–52*	LZY53×EHY644
LZY113	MATa *vps1Δ::LEU2 snq2::hisG leu2 his3-Δ200 trp1 ura3–52 lys2–801am*	LZY53×EHY644
LZY115	MATa *apl2Δ::TRP1 snq2::hisG leu2 his3-Δ200 trp1 ura3–52*	LZY53×EHY644
LZY119	MATa *vps1Δ::LEU2 pdr5::TRP1 leu2 his3-Δ200 trp1 ura3–52*	LZY53×EHY644
LZY120	MATa *apl2Δ::TRP1 pdr5::TRP1 leu2 his3-Δ200 trp1 ura3–52*	LZY53×EHY644

S288c background [27]. Additional strains were constructed to cross out a *suc2*Δ mutation (deletion of the gene for invertase) to enable us to perform invertase secretion assays. Strains were grown in YPAD medium: Difco Bacto yeast extract (1%), Difco Bacto peptone (2%), and glucose (2%), supplemented with adenine hemisulfate (20 mg/L). Screening compounds (a ~97,000 compound library) were from the Molecular Libraries Small Molecule Repository. All compounds were provided by BioFocus DPI in dimethyl sulfoxide (DMSO, 100%) at a concentration of 10 mM.

High-Throughput Screens

Yeast strains were maintained as frozen glycerol stocks or on YPD agar plates (kept at 4°C for up to two weeks). Two days before screening, ~4 colonies were inoculated into YPAD (50 mL) and grown at 30°C with aeration on a rotating platform (260 rpm) for 18-24 h to mid-exponential phase (OD_{600} 0.5-0.9, read in a Genesys 5 spectrophotometer, Thermo-Spectronic Instruments). This primary culture was inoculated into YPAD such that the secondary culture, grown at 30°C for ~20 h, was OD_{600} 0.04 (strain JTY2953) and 0.1 (strain LZY35) at the time of plating for the screen. This was done by first determining the growth rate of each screen strain, so that the secondary culture would not need to be diluted prior to plating, thus minimizing a lag in growth at the start of the screen. The initial plating density of cells resulted in cultures nearing the end of exponential growth after ~18h growth in plates.

For the primary screens (AID 738, 739), compound (7.5 nL) was added to YPAD medium (1 μL) in 1536-well black, clear bottom plates (Corning), and secondary yeast culture (7 μL) was added to the plates for a final compound concentration of 10 μM and a final DMSO concentration of 0.1%. Each plate contained control wells, with YPAD medium containing DMSO (0.1%) serving as negative control and KU#7 (1.25 μg/mL), DMSO (0.1%) as positive control [16]. Liquid handling steps were performed with a

Biomek FX liquid handler (Beckman Coulter), a Multidrop Combi dispenser (Thermo Scientific), or an Echo 550 liquid handler (Labcyte). After adding the yeast culture to all wells, the plates were incubated with lids (un-sealed), and inverted, at 30°C for 18 h in a humidified atmosphere. Incubating the plates inverted maximized cell growth due to better access to oxygen, as the cells settled at the surface of the medium. Following incubation, the plates were slowly shaken before the reading at OD_{615} in an EnVision multilabel plate reader (PerkinElmer).

For the dose response screens (AID 788, 789, 790), 320 compounds that selectively inhibited the growth of the *vps1*Δ *apl2*Δ mutant in the primary screen were retested in a 10-point dose response assay. Compounds were diluted in YPD medium to prepare a 10x concentrated dosing solution (250 μM) from which a 10-concentration, 2-fold dilution series was prepared in YPD medium and added to 384-well clear plates (Corning) in 5 μL. The yeast cultures (strains LZY53, LZY80, LZY81 and LZY82) were grown as for the primary screen to generate an early-exponential phase culture, and yeast culture (45 μL) was added to the plates with compounds for final compound concentrations between 0.05 μM and 25 μM, and a final DMSO concentration of 0.25%. Positive and negative controls were as for the primary screen. The plates were incubated at 30°C in a humidified atmosphere for 18 h, and OD_{615} was read in an EnVision multilabel plate reader.

Data analysis for primary and dose-response screens

All data from primary screens and dose-response assays were imported into ActivityBase (IDBS) data management software for analyses. For the primary screens, percent inhibition for both the control and mutant strains was calculated as: 100 x (1-(Median of test compound – Median of positive control)/Median of negative control – Median of positive control)). Active compounds were those that showed ≥30% inhibition (30% is

the average compound % inhibition plus three times the standard deviation of compound % inhibition). Among the compounds chosen as having ≥30% inhibition, a hit was determined to be any compound that was significantly more toxic to the mutant test strain than to the control background strain. Compounds that showed less than 30% inhibition were defined as Inactive. Compounds that were screened more than once and had both Active and Inactive outcomes were labeled Inconclusive. For the dose-response screens, the percent inhibition of growth rate was calculated as: 100 x ([growth rate DMSO-treated cells]-[growth rate compound-treated cells])/[growth rate DMSO-treated cells].

Secondary screens

For secondary screening, 24 compounds from the 93 dose-response hits were reordered from the original suppliers and subjected growth and secretion assays in mutant and background strains. The first assay was a shaking-culture growth rate assay to determine growth rates during exponential growth were measured. Strains were grown in YPD overnight to exponential growth phase, and culture densities were adjusted to OD_{600} 0.07 in YPD (8 mL), in 25x150 mm glass culture tubes. Compounds were added at the indicated concentrations for a final volume of 10 mL and final DMSO concentration of 0.25%, and tubes were placed on a roller drum (Bellco) rotating at 50 rpm. OD_{600} readings were taken every two hours, for at least 4 readings to generate a growth curve. Rates were calculated from an exponential curve fit equation. The correlation coefficient (Pearson *r*) was >0.9 in each case. Secondary growth assays to generate growth curves were also performed in 96-well plates. OD_{595} was read with a Model 680 microplate reader (Bio-Rad) controlled by Microplate Manager software (Bio-Rad).

We tested selected hit compounds in two established secretion assays. The first was an invertase secretion assay [3]. Cells were grown overnight to exponential phase,

then grown in YPD with high glucose (5%) for 2-3 hours on a roller drum at 50 rpm. Compound was added at the indicated concentration (or DMSO control, 0.25% final concentration), and cultures were grown for 15 min, followed by shifting cells to YPD with low glucose (0.1%) and compound or DMSO control for 90 min prior to performing the invertase secretion assay as described previously[8]. For slow-acting compounds (Figure 4E), the cells were grown in compound for 3h rather than 15 min prior to derepressing invertase expression. Invertase expression is repressed in high-glucose media, but upon shifting to low glucose, its expression is rapidly derepressed. Therefore, all invertase expression and secretion occurs after compound addition. As a second secretory assay, we assayed for internal accumulation of an abundant cell wall protein, Bgl2, as described previously[8].

Results and Discussion

Most of the genes involved in membrane traffic were discovered by perturbing their functions either by mutations or with drugs [3, 9, 10]. We used a triple-synthetic-lethal yeast mutant screen to identify a novel eukaryotic gene involved in exocytic transport, *AVL9* [8]. This gene is essential in a mutant strain lacking a dynamin homolog, Vps1 [11, 12], and also lacking a subunit of the adaptor protein-1 (AP-1) adaptin complex, Apl2, which is required for forming certain classes of clathrin-coated vesicles at the Golgi and endosomes [8, 13-15]. The *apl2*Δ *vps1*Δ double mutant has a block in an exocytic pathway but grows well because cargo is rerouted into a remaining pathway [8]. Mutations or drugs that are lethal for the *apl2*Δ *vps1*Δ mutant but have little or no effect on wild-type cells are expected to cause a block in a remaining exocytic pathway. We performed a high-throughput phenotypic screen of a large library of drug-like compounds to identify small molecules that have such a mutant strain-specific effect. An earlier screen produced only a few hits, likely due to the ability of yeast cells, like other fungi, to efficiently pump out a very broad range of structurally and functionally unrelated

compounds due to an abundance of ATP-binding cassette (ABC) transporters [16, 17]. *S. cerevisiae* has at least nine ABC pumps that are localized to the plasma membrane, and at least three of these, Pdr5, Snq2, and Yor1, are involved in pleiotropic drug resistance [17]. The most important of these are Pdr5 and Snq2, which have overlapping but also distinct specificities [18]. To increase the number and diversity of compounds in our phenotypic screen, we added the *pdr5*Δ and *snq2*Δ mutations to our *vps1*Δ *apl2*Δ strain.

High-throughput screens for mutant-specific growth inhibitors

Before screening the full compound library, we screened a subset of 10,000 compounds for inhibition of growth of the test strain, *vps1*Δ *apl2*Δ *snq2*Δ *pdr5*Δ (LZY35). The screen was performed in duplicate, in both 384- and 1536-well formats, to check reproducibility of the assay and to confirm the appropriate screening parameters (data not shown, see PubChem Assay ID's 636 and 637). The Pearson's correlation between the two runs (all 10,000 compounds) was a moderate 0.4, and the Minimum Significant Difference (MSD, denoting the variability between the two assays) was an acceptable 15.2% inhibition. We then screened 97,519 compounds from the NIH Molecular Libraries Small Molecule Repository for compounds that selectively inhibit the growth of a *vps1*Δ *apl2*Δ *snq2*Δ *pdr5*Δ strain, when compared to the growth of the *snq2*Δ *pdr5*Δ background strain (Figure 1). The screens were run in several batches on different days, with failed plates repeated. Actives in the screen were defined as compounds that at 10 μM showed ≥30% inhibition in the assay (see details in *Experimental Section*). Out of the compounds with an active outcome for the mutant strain, 320 that had an apparent selective activity towards the *vps1*Δ *apl2*Δ *snq2*Δ *pdr5*Δ strain over the *snq2*Δ *pdr5*Δ control strain were reordered for a confirmatory dose response screen.

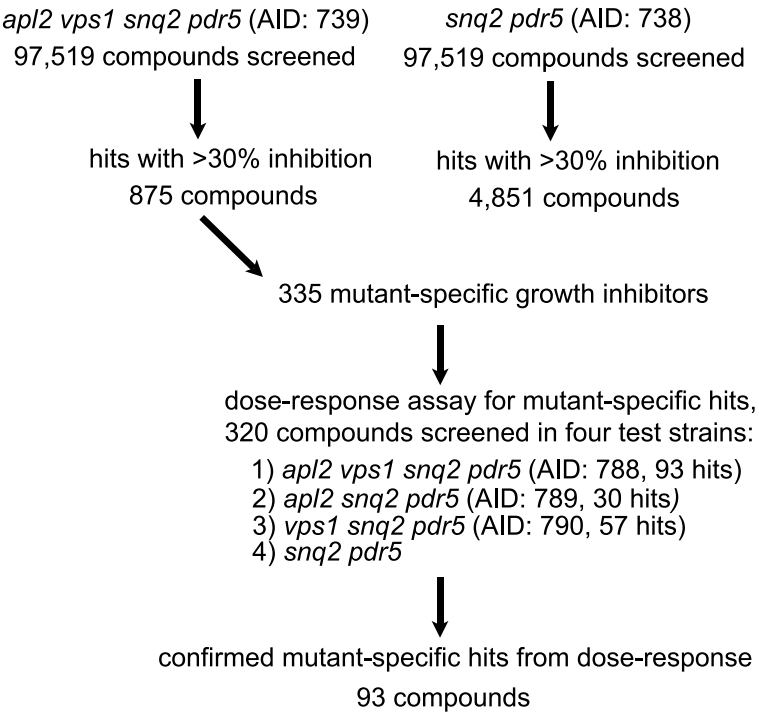
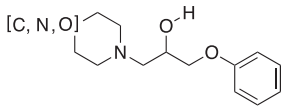
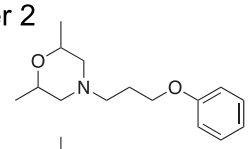
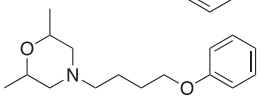
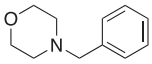
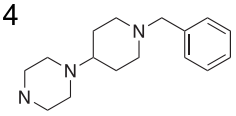
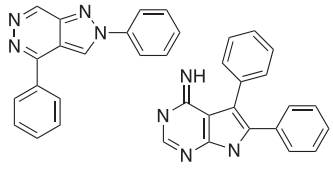
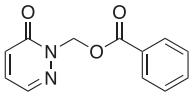


Figure 1. Summary of a high-throughput screen for identifying compounds that preferentially inhibit the growth of a mutant strain having *apl2*Δ and *vps1*Δ mutations. PubChem Assay ID's (AID) are indicated for each screen step.

Figure 2 (next page). Summary of dose-response end-point growth assays for identifying 93 hit compounds. A) Structural clustering of compounds from the 93 hits. PubChem SID's for our designated SR numbers are shown in Table 2. The SR number corresponds to the rank from the dose-response assay, with SR2 being the top-ranked compound. B) Data from the dose-response assays allowed grouping of compounds according to specificity for mutants (PubChem AID 788, 789, 790). The majority of our hit compounds were specific for the *apl2*Δ *vps1*Δ double mutant. Among the top 56 confirmed compounds (ranked according to dose response growth assay), 13 compounds caused inhibition due to the *vps1*Δ mutation and were similarly toxic for the *apl2*Δ *vps1*Δ mutant; 12 compounds inhibited primarily the *apl2*Δ *vps1*Δ mutant but also had significant effect on the *apl2*Δ mutant; and 31 compounds inhibited the growth of primarily the the *apl2*Δ *vps1*Δ double mutant with little or no effect on the *apl2*Δ or *vps1*Δ mutants. None of the top 56 compounds inhibited growth the *apl2*Δ *vps1*Δ mutant due only to the *apl2*Δ mutation.

A)

	# in 93 Hits	# in 97 k Library	Hit Compounds
Cluster 1			
	4	149	SR5, 13, 25, 60
Cluster 2			
	2	9	SR21, 25
	3	4	SR2, 4,12
Cluster 3			
	7	411	SR3, 10,16, 30, 34, 43, 53
Cluster 4			
	2	17	SR17, 46
Cluster 5			
	3	5	SR41, 42, 50
	2	7	SR75, 81
Cluster 6			
	6	8	SR45, 73, 76, 90, 92, 93

B)

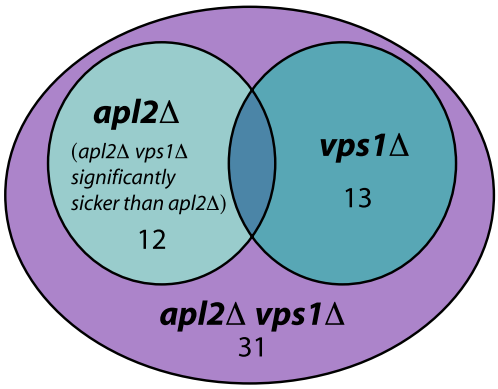


Table 2. PubChem Substance ID for compounds referenced in this study.

SR#	SID #	SR#	SID #	SR#	SID #
SR2	14 730 770	SR21	79 77 933	SR42	65 201 738
SR3	846 694	SR22	79 77 576	SR43	17 415 595
SR4	147 311 747	SR23	79 74 278	SR45	17 402 823
SR5	3 716 857	SR25	37 13 939	SR46	852 608
SR6	17 414 883	SR26	17 408 707	SR50	17 386 484
SR7	17 388 421	SR27	14 739 448	SR52	846 638
SR8	17 414 779	SR28	17 406 960	SR53	863 029
SR9	14 735 884	SR29	79 72 891	SR60	14 732 364
SR10	848 590	SR30	858 784	SR73	14 729 820
SR11	17 411 920	SR31	79 76 228	SR75	14 731 482
SR12	14 731 751	SR34	42 64 952	SR76	14 729 194
SR13	42 52 400	SR37	79 78 229	SR81	7 975 000
SR14	14 73 1776	SR38	14 740 626	SR90	17 402 824
SR15	14 739 450	SR39	79 76 613	SR91	7 973 601
SR16	850 821	SR40	17 414 870	SR92	17 402 821
SR17	14 720 508	SR41	17 406 691	SR93	14 729 821
SR20	14 730 765				

Dose-response and secondary assays

A 10-point dose-response assay with compound concentration ranging between 0.05 μM and 25 μM was performed for the 320 compounds obtained from the primary screens, for each of the following strains, with the test-mutations indicated in bold: ***vps1Δ apl2Δ*** *snq2*Δ *pdr5*Δ, ***vps1Δ*** *snq2*Δ *pdr5*Δ, ***apl2Δ*** *snq2*Δ *pdr5*Δ, and the *snq2*Δ *pdr5*Δ background strain (PubChem Assay ID's 788, 789, and 790). Of the 320 compounds tested in the dose-response assays, 93 confirmed as being selective for one of the three traffic mutant strains over the control strain. Some of these compounds grouped into structural clusters (Figure 2A). More than half of our 56 top-ranked hits from the dose-response growth assays required both the *vps1*Δ and *apl2*Δ mutations for significant growth-inhibitory activity, consistent with the rationale for the design of our chemical genetic screen (Figure 2B). However, some compounds had activity due to the *vps1*Δ mutation alone (with no increase in effect on the *apl2*Δ *vps1*Δ strain). Other compounds significantly affected the growth of the *apl2*Δ mutant and not the *vps1*Δ strain; however, all of these had greater activity on the *apl2*Δ *vps1*Δ strain, indicating more robustness in Apl2-mediated processes. The different mutant-specificities of our hit compounds indicate that they have unique molecular and biological targets.

Of our 93 hit compounds from the dose-response growth assay, 24 compounds were chosen for follow-up studies based on: 1) significant selectivity towards the *vps1*Δ *apl2*Δ strain over the background strain; 2) representatives of major structural clusters among the identified hits; 3) preference for being active for the *vps1*Δ *apl2*Δ mutant over one of the single traffic mutants, but with representatives of each class selected; and 4) chemical tractability. We named these compounds "SR#," and they are listed, along with other compounds referenced here, in Table 2.

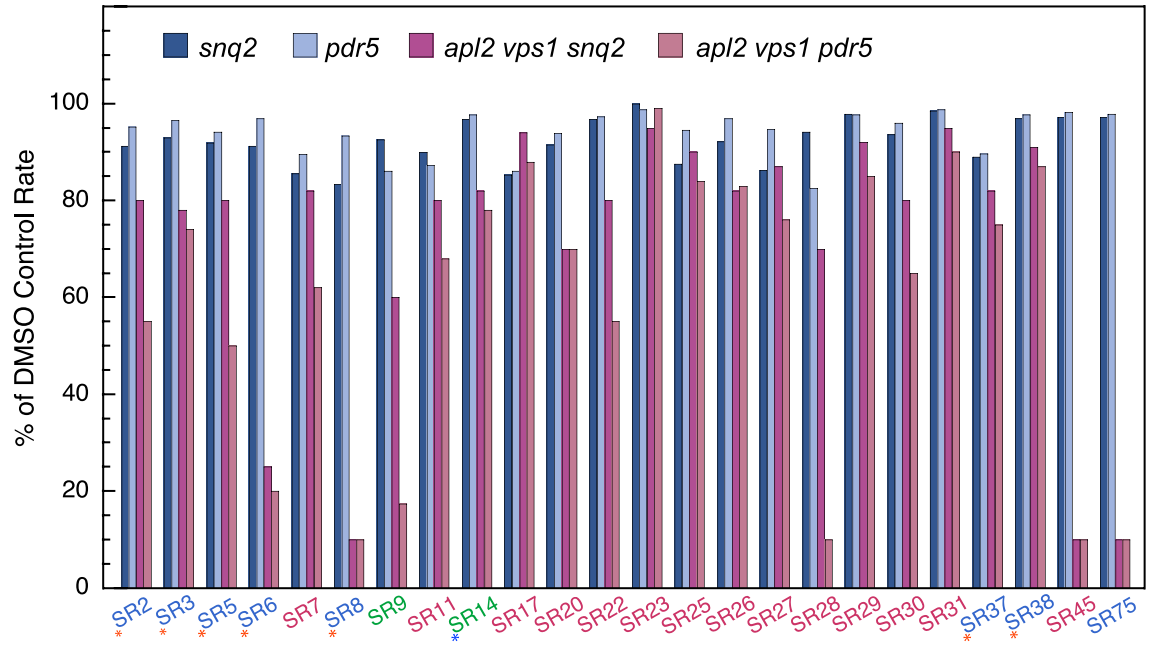
As our first secondary assay, we retested growth in shaking exponential-phase cultures, in presence of compound compared to DMSO control. Whereas the dose-

response end-point growth assays were performed in 384-well plates, non-shaking, over ~18 h of growth, the shaking cultures had greater aeration, allowing faster growth, and were assayed only during ~9 h of exponential growth phase (see *Experimental Section*). Some of the compounds did not show confirmed activity in the shaking-culture assay, likely because they were slow-acting, and therefore would have observable activity over 18 h, with just one end-point measurement taken, but not in the first few hours of exponential growth. Alternatively, some compounds could be more active in lower-oxygen conditions or on slower-growing cells in plates. The shaking-culture secondary screen was repeated with strains having only either the *pdr5*Δ or only the *snq2*Δ mutation, to test which drug pump mutation was more critical for compound effect (Figure 3A). Effectiveness of compounds in the presence of just one of these pump mutations was desired because we noticed a drop in growth rate of the *vps1*Δ *apl2*Δ mutant when both drug pump mutations were crossed into the strain. In contrast, effect on growth was much less when just one ABC transporter was deleted (Figure 3B), indicating that including just one of these deletion mutations would minimize growth defects or other phenotypes due to pump mutations in our subsequent assays. We found that the *pdr5*Δ mutation was the more critical one for sensitizing our strain to almost all of the 24 compounds we tested (Figure 3A). This mutation had no effect on the growth of an *apl2*Δ or a *vps1*Δ strain, which had a similar growth profile as the *pdr5*Δ background strain; however, the *apl2*Δ *vps1*Δ *pdr5* triple mutant did have some growth defect (Figure 3C). We performed our remaining secondary assays using just the *pdr5*Δ mutation in all strains (wild-type for *SNQ2*).

Our largest structural group from the 93 compounds identified in the dose-response assays is shown in Figure 4A. Two of these compounds fall into Cluster 1 (Figure 2A), and three-dimensional overlay of all five compounds in this group shows that they have very similar structures (Figure 4B). The dose-response growth assays for these compounds indicated that they were all highly specific for the *vps1*Δ *apl2*Δ mutant, with

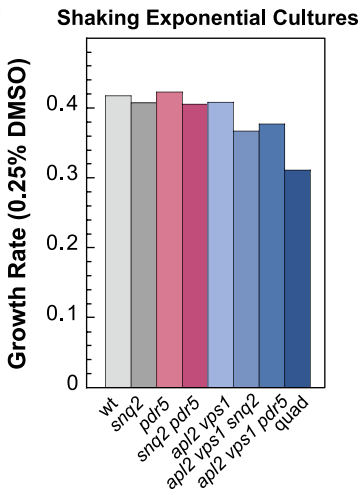
Figure 3 (next page). Exponential-growth-rate assays of mutant and background strains. A) Shaking-culture growth assays showed that the *pdr5*Δ mutation is sufficient for sensitizing yeast strains to most of the compounds (at 20 μM) identified as hits in our dose-response assay. Rates were determined from the equation of an exponential curve fitted to the exponential growth curve of each strain (correlation coefficient >0.9) and shown as a percent of the rate without compound added. Each compound is at 5 μM. The sequence of SR numbers corresponds with ranking in the dose-response growth assay, with SR2 being the highest-ranked hit. SR numbers are color-coded according to mutant specificity, as determined by dose-response assays (PubChem AID 788, 789, 790). Corresponding PubChem SID's are shown in Table 2. B) The *pdr5*Δ and *snq2*Δ mutations confer a growth defect on a *vps1*Δ *apl2*Δ strain, which is most pronounced if both *pdr5*Δ and *snq2*Δ mutations are present and least-pronounced with *pdr5*Δ. Rates were determined as in (A). C) Microwell-plate exponential growth curves of strains having a *pdr5*Δ mutation. Strains for A-C are as follows: LZY109 (*snq2*Δ); LZY108 (*pdr5*Δ); LZY96 (*apl2*Δ *vps1*Δ *snq2*Δ); LZY104 (*apl2*Δ *vps1*Δ *pdr5*Δ); NY10 (wild-type = "wt"); LZY53 (*snq2*Δ *pdr5*Δ); EHY644 (*apl2*Δ *vps1*Δ); LZY82 (*apl2*Δ *vps1*Δ *snq2*Δ *pdr5*Δ = "quad"); LZY119 (*vps1*Δ *pdr5*Δ) and LZY120 (*apl2*Δ *pdr5*Δ).

A)



apl2 specific, *vps1* specific, *apl2 vps1* specific, **apl2 vps1* most inhibited, but significantly inhibited for *apl2*
**apl2 vps1* most inhibited, but significantly inhibited for *vps1*

B)



C)

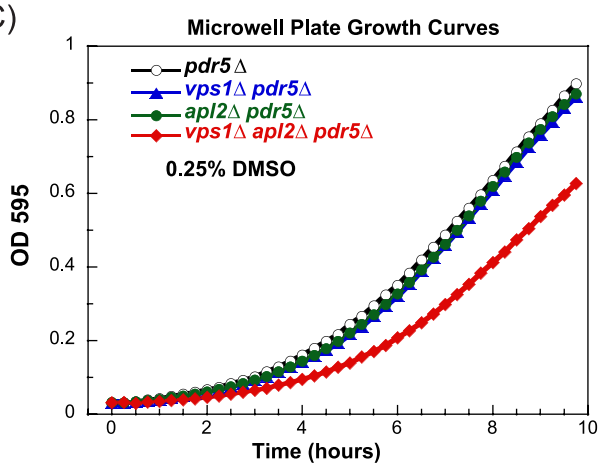
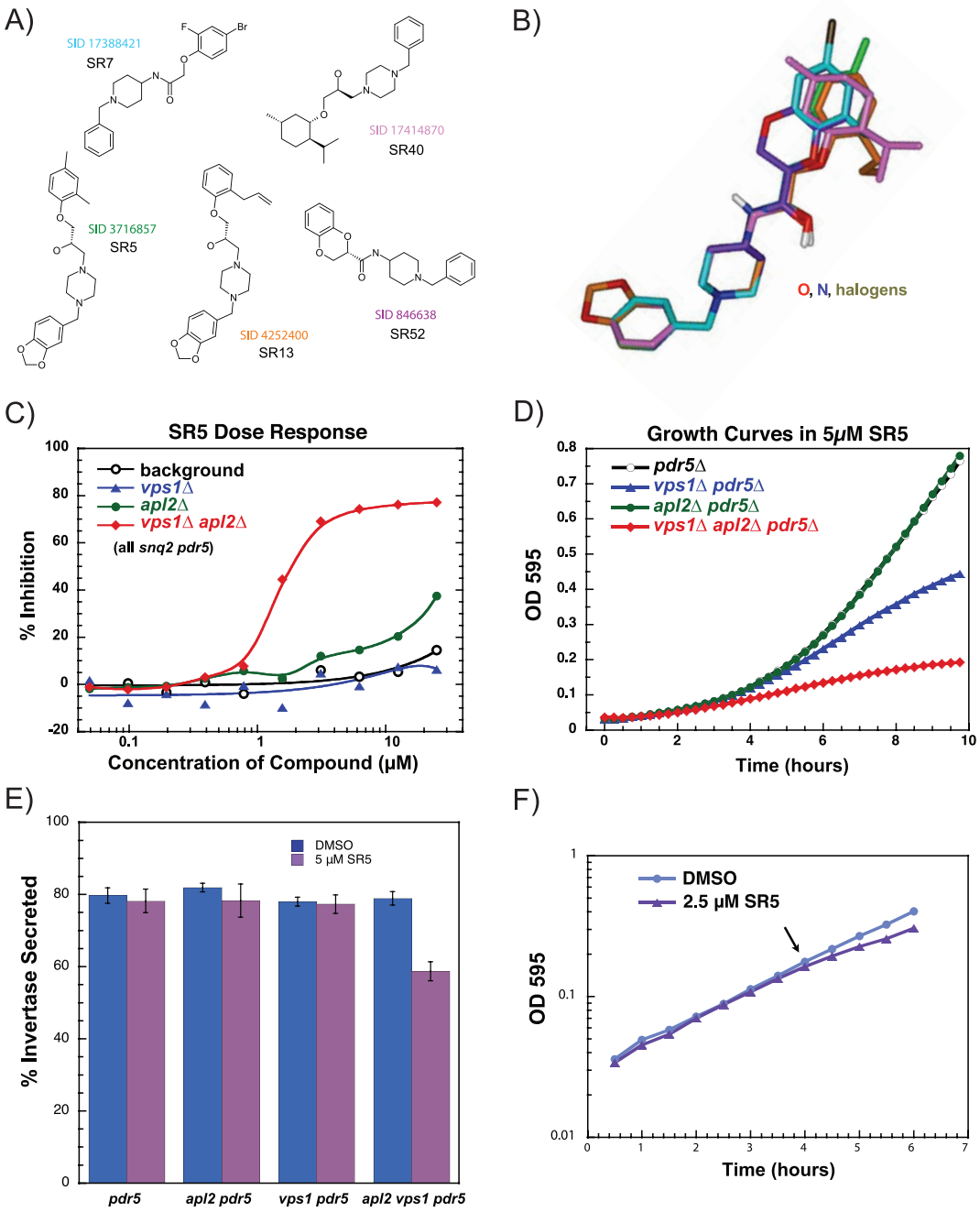


Figure 4 (next page). The largest structural group of hit compounds had greatest specificity for the *apl2*Δ *vps1*Δ mutant for inhibiting growth and secretion. A) Compound structures with SR# and PubChem SID. B) 3-dimensional overlay of the five compounds shown in (A), with carbons in each compound colored as the SID #. C) Dose-response end-point growth assay for SR5. The yeast strains are LZY53 (background); LZY81 (*vps1*Δ); LZY80 (*apl2*Δ); and LZY82 (*vps1*Δ *apl2*Δ); all four strains have *snq2*Δ *pdr5*Δ mutations. D) Microwell plate growth curves for SR5 (compare to Figure 3C). E) Invertase secretion assay for SR5 shows a secretion defect specific for *vps1*Δ *apl2*Δ. Strains are as in Figure 3C. The means of three experiments (from three independent cultures) are shown. Error bars, SEM. F) Strain LZY104 (*apl2*Δ *vps1*Δ *pdr5*Δ) grown in microwell plates, in either 2.5 μM SR5 or DMSO control. There is no growth defect until ~4h after adding compound.


A)
SID 17388421
SR7
SID 17414870
SR40
SID 3716857
SR5
SID 4252400
SR13
SID 846638
SR52
B)
O, N, halogens
C)
SR5 Dose Response
background
vps1Δ
apl2Δ
vps1Δ apl2Δ
(all snq2 pdr5)
% Inhibition
Concentration of Compound (μM)
D)
Growth Curves in 5μM SR5
pdr5Δ
vps1Δ pdr5Δ
apl2Δ pdr5Δ
vps1Δ apl2Δ pdr5Δ
OD 595
Time (hours)
E)
DMSO
5 μM SR5
% Invertase Secreted
pdr5
apl2 pdr5
vps1 pdr5
apl2 vps1 pdr5
F)
DMSO
2.5 μM SR5
OD 595
Time (hours)

smaller effect on the growth of *vps1*Δ or *apl2*Δ mutants. Figure 4C and D show growth assays for SR5, the highest-ranked compound in this group. Furthermore, an assay for invertase-secretion (see *Experimental Section*) showed that SR5 has an inhibitory effect on secretion that was specific for the *vps1*Δ *apl2*Δ mutant and not detected in the other strains (Figure 4E). However, we also found that these compounds were relatively slow-acting, with little effect on growth until ~4h of exponential growth (Figure 4F). This suggests that these compounds may affect signaling pathways that regulate gene expression, or they may need to build up a toxic effect (although higher and lower concentrations of compound showed a similar delay in effect; not shown).

Another compound of interest is SR28 (Figure 5A), which did not fall into a structural group from our 93 hits. Dose-response and growth-curve analyses indicated that this compound affects primarily *vps1*Δ (and therefore also the *vps1*Δ *apl2*Δ mutant). This compound is of significant interest because it causes a very severe, rapid block in invertase secretion (Figure 5D). Although the greatest effect on secretion is seen in the *vps1*Δ strain, at higher concentrations there is also significant effect on the *apl2*Δ and background strains (Figure 5D). Likewise, SR28 caused a significant internal accumulation of the cell wall protein, Bgl2, which at steady-state is normally almost entirely in the cell wall (Figure 5E). Interestingly, the background strain also had slight internal accumulation of Bgl2 at the 30 min time point after adding compound, but this accumulation did not increase, and even decreased slightly, with longer time points, suggesting that the background strain was able to adapt to the transport defect, perhaps by upregulating an alternate transport route that is blocked in the mutant strain.

Another compound that had a significant and rapid effect on invertase and Bgl2 secretion is SR9 (Figure 6). We tested structurally similar compounds, and found one compound with very similar activity, having PubChem Compound ID 656067 (Figure 6A). The dose-response end-point growth assay for SR9 indicated that all growth ef-

fects were due to the *vps1*Δ mutation, and this was consistent both in our exponential growth curve assays and invertase secretion assays, which likewise showed *vps1*Δ-specific effects (Figure 6B-D). SR9 was of interest also because unlike the majority of the other hits that we tested, it had a significant effect on secretion even in *PDR5 SNQ2* strains (wild-type for drug pumps; not shown), making it more promising in future genome-wide screens for identifying the molecular and biological targets of the compound.

A group of compounds with similar structures that also did not require pump mutations for effects on growth is shown in Figure 7A. Some of these compounds fell into Cluster 2 (Figure 2A). Additional hit compounds that are similar to these are shown in Figure 7B. The compounds in Figure 7 were among the highest-ranking hits from our dose-response growth assays for potency and selectivity for the *vps1*Δ *apl2*Δ strain, with some activity for the *apl2*Δ strain, and little or no effect on either *vps1*Δ or the background strain (Figure 7C, D). The lack of effect on the *vps1*Δ strain differentiates these compounds from others that we analyzed in detail (Figures 5-7), so it is likely that they have a different molecular and biological target than those of the other hits. Interestingly, these compounds were effective primarily in rich media (YPD), with little effect in minimal (synthetic) media (such as CSM) (our unpublished observations). This effect of media is interesting because it resembles the phenotype of yeast *ras* mutants as well as mutants of the yeast synaptobrevin homologs, which function in targeting exocytic vesicles to the plasma membrane[19]. We also found that the compounds were slow-acting, and therefore difficult to analyze in our transport assays. Like the compounds in Figure 4, they may affect signaling pathways that respond to transport blocks by regulating gene expression.

Many of the 24 compounds selected for secondary assays did not block invertase secretion in our assays. Some of these compounds may have a delayed effect and block

Figure 5 (next page). SR28 was the most potent secretory inhibitor obtained from the screen. A) Structure of SR28. B) Dose-response end-point growth assay for SR28. C) Microwell plate growth curves for strains grown in 5 μM SR28. D) Invertase secretion assay for strains grown in 2 μM or 5 μM SR28. All strains had a secretory defect in this compound, and the defect was most severe for the *vps1*Δ and *vps1*Δ *apl2*Δ mutants. The means of three experiments (from three independent cultures) are shown. Error bars, SEM. E) Western blots showing the accumulation of internal Bgl2 in 5 μM SR28 at the indicated times. Bgl2 is almost entirely in the cell wall at steady state, so the internal accumulation of this protein can be detected by removing the cell wall. PGK (a cytoplasmic protein) is shown as a loading control. Strains are as in Figure 3C.

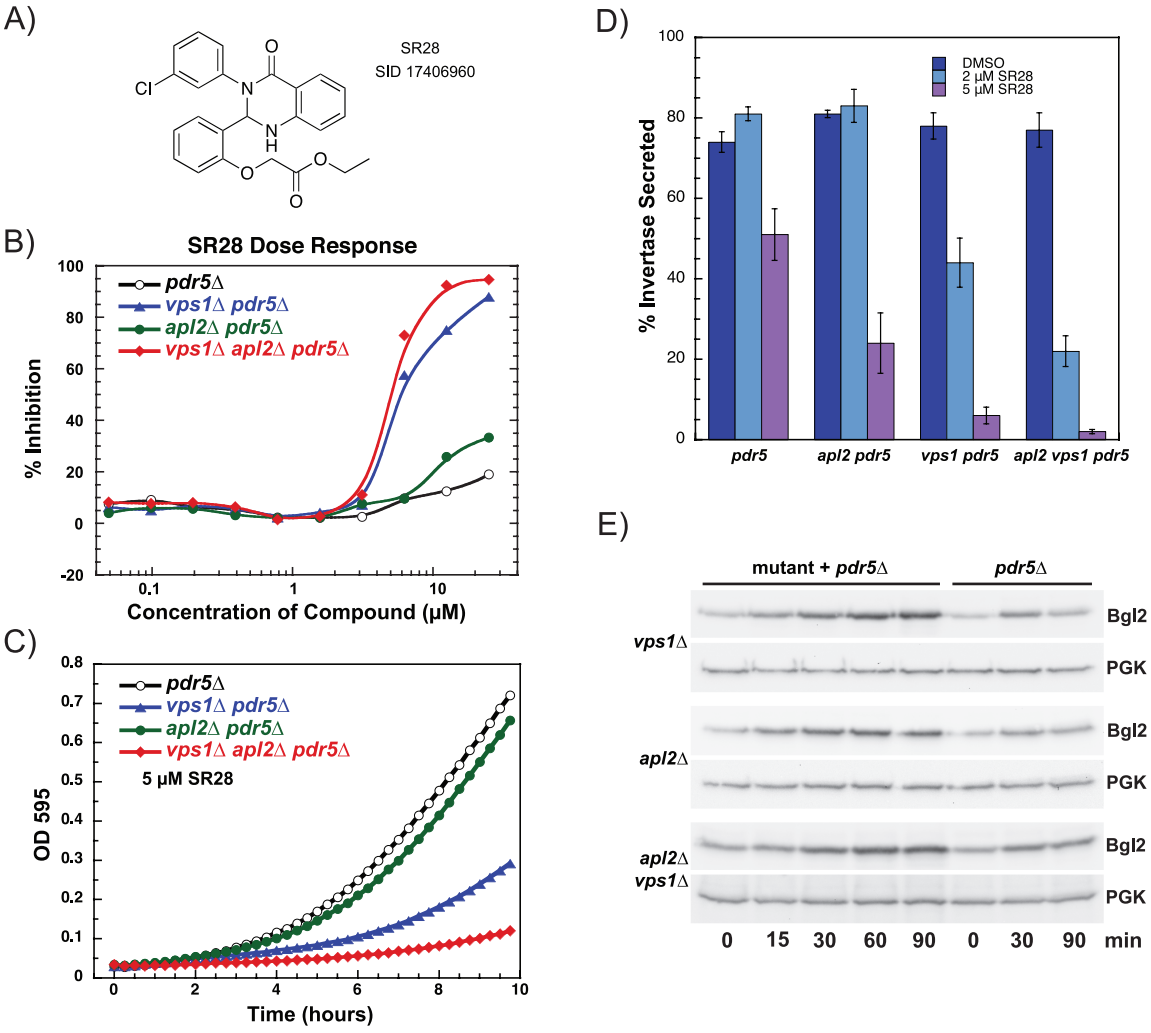
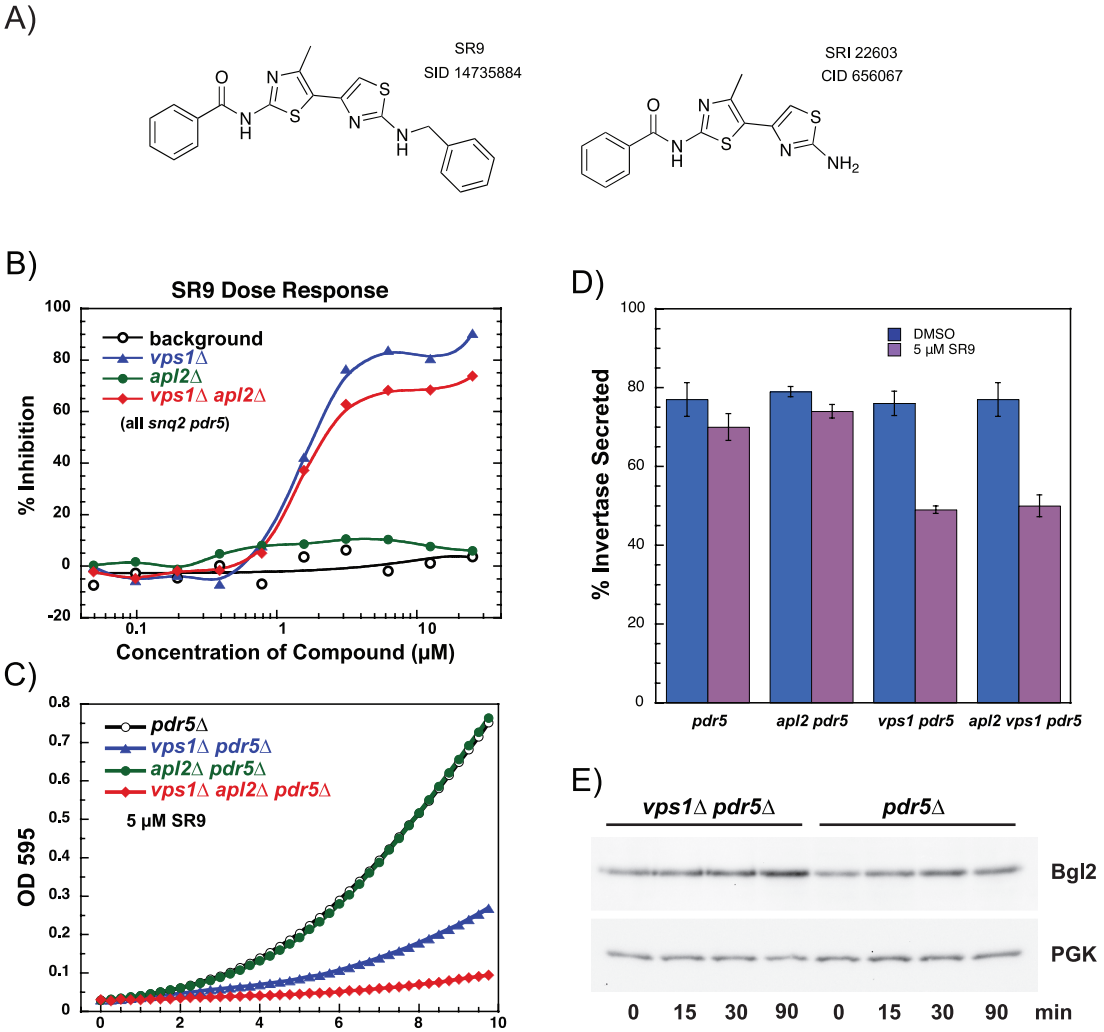

A)
SR28
SID 17406960
B)
SR28 Dose Response
pdr5Δ
vps1Δ pdr5Δ
apl2Δ pdr5Δ
vps1Δ apl2Δ pdr5Δ
% Inhibition
Concentration of Compound (μM)
C)
5 μM SR28
OD 595
Time (hours)
D)
DMSO
2 μM SR28
5 μM SR28
% Invertase Secreted
pdr5
apl2 pdr5
vps1 pdr5
apl2 vps1 pdr5
E)
mutant + pdr5Δ
pdr5Δ
vps1Δ
apl2Δ
Bgl2
PGK
0 15 30 60 90 0 30 90 min

Figure 6 (next page). SR9 is a *vps1*Δ-specific growth and secretory inhibitor. A) Structure of SR9 (left) and an analog (right) with similar activity. B) Dose-response end-point growth assay for SR9 (strains as in Figure 4C). C) Microwell plate growth curves for strains grown in 5 μM SR9 (strains as in Figure 3C). D) Invertase secretion assay for strains grown in 5 μM SR9. The secretory defect is dependent entirely on the *vps1*Δ mutation. The means of three experiments (from three independent cultures) are shown. Error bars, SEM. E) Western blots showing accumulation of internal Bgl2 after growing strains in 5 μM SR9 for the indicated times. PGK is a loading control.


A)
SR9
SID 14735884
SRI 22603
CID 656067
B)
SR9 Dose Response
background
vps1Δ
apl2Δ
vps1Δ apl2Δ
(all snq2 pdr5)
% Inhibition
Concentration of Compound (μM)
C)
pdr5Δ
vps1Δ pdr5Δ
apl2Δ pdr5Δ
vps1Δ apl2Δ pdr5Δ
5 μM SR9
OD 595
Time (hours)
D)
DMSO
5 μM SR9
% Invertase Secreted
pdr5
apl2 pdr5
vps1 pdr5
apl2 vps1 pdr5
E)
vps1Δ pdr5Δ
pdr5Δ
Bgl2
PGK
0 15 30 90 0 15 30 90 min

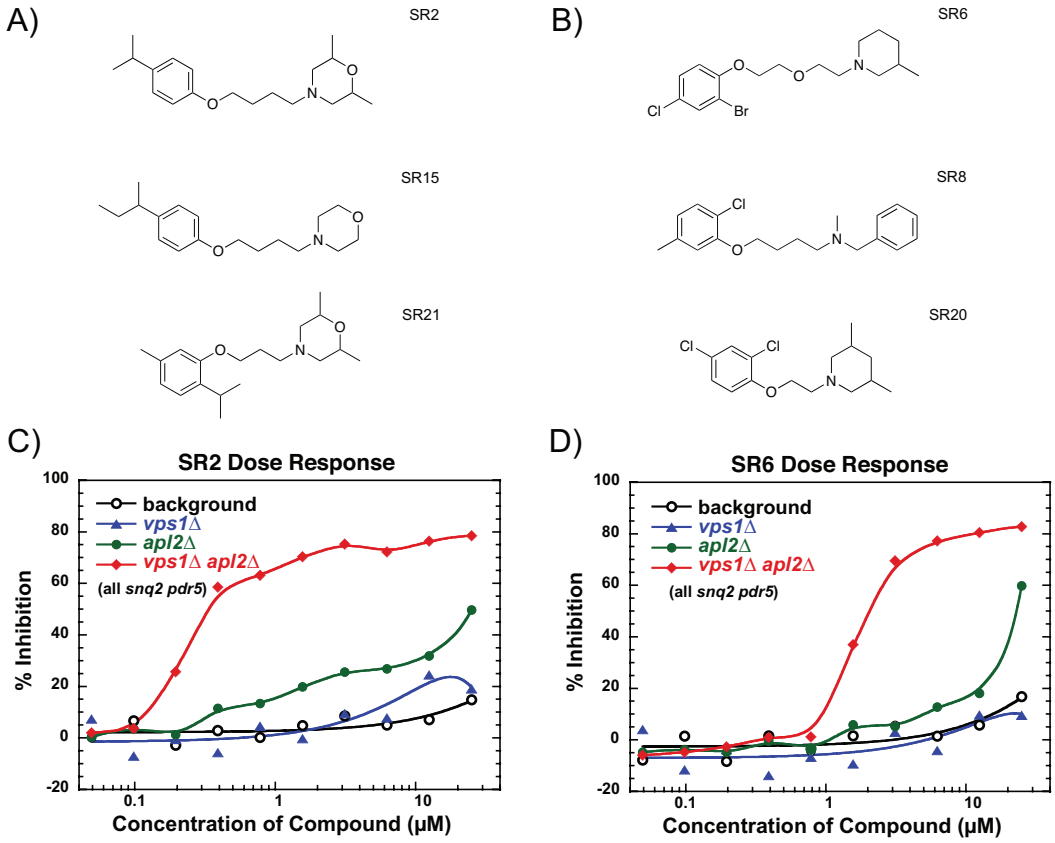


Figure 7. Cluster 2 and similar compounds were highly selective for the vps1Δ apl2Δ double mutant. A) Structures for two Cluster 2 and one closely related compound. B) Additional compounds that resemble Cluster 2 compounds. C) The Cluster 2 compound, SR2, ranked highest in our dose-response screen. It is highly selective for inhibiting the growth of the *apl2Δ vps1Δ* double mutant in microwell plates (shown) and agar plates (not shown). D) Compounds shown in (B) were also specific for the *vps1Δ apl2Δ* mutant. Yeast strains are as in Figure 3C.

secretion at a later time point. Alternatively, they may have *vps1*Δ *apl2*Δ mutant-specific toxicity that is not due to blocking exocytic transport. Recent studies have suggested that traffic mutants are particularly prone to drug hypersensitivity, perhaps because they are defective in trafficking of drug transporters that function in detoxification either at the cell surface or at the vacuole [20, 21]. However, we would expect a larger number of hits if our screen merely identified toxic substances due to a general drug-sensitivity of our test strain. Analysis of screen results submitted by others to PubChem suggests that our compounds were highly specific for our screen, in particular at the dose-response or confirmatory steps, again arguing against general drug-sensitivity of our screen strain. Furthermore, because both the *vps1*Δ *apl2*Δ mutant and control background strain had a significant defect in detoxification due to the *pdr5*Δ *snq2*Δ mutations, neither test nor control strain could efficiently eliminate drugs, so most of our hits likely target a process that is relevant to membrane traffic function. Therefore, even compounds that do not rapidly block secretion are of potential future interest. For example, they could trigger signaling mechanisms that shut down growth in response to traffic defects.

Conclusions

Using a high-throughput phenotypic screen, we identified 93 compounds that specifically affect the growth of a mutant strain blocked in one branch of the exocytic pathway. Our analysis of selected compounds from these hits indicates that at least some of our mutant-specific growth inhibitors are novel secretory inhibitors that likely affect a transport pathway that remains functional in the mutant in the absence of drugs. Therefore, further analysis of our hits is expected to identify additional traffic inhibitors.

Identifying the molecular targets of our inhibitors, as well as biological effects in genome-wide fitness assays [22, 23], will contribute to a better understanding of the mechanisms that mediate late exocytic transport. Some of our compounds are expected to target Avl9 or an Avl9-related process, since they generate an AVL (***a****pl2*Δ ***v****ps1*Δ **l**ethal) phenotype, similar to that of *avl9*Δ [8]. Avl9 is a conserved, ancient eukaryotic protein and a member of a novel superfamily of ancient paralogs, most of which have not been studied in any organism [8]. Therefore, the hits identified in our HTS assay may serve as useful tools for discovering a potentially novel function mediated by Avl9 and its homologs. Prior to seeking the targets of our new inhibitors, we may need to optimize our compounds by testing analogs for potency as well as specificity for the traffic mutant strains. The possibility of non-specific effects is common to all small molecule screens, whether the screens involve *in vitro* assays or a cell-based phenotypic assay such as ours [24]. However, low-specificity compounds would be expected to be similarly toxic to the background control strain in our assays. Therefore, some of our hits should have sufficient specificity for simple screens that could help to identify potential target(s), for example a gene-dosage screen [25].

Although some of our hit compounds may have pleiotropic effects, mutations likewise can have pleiotropic effects, and specificity of effect is not necessarily a greater concern with small molecules than it is with mutants as tools to study gene function. For example, most bioactive small molecules do not primarily affect protein target levels, whereas many mutations do affect protein abundance, causing pleiotropic effects especially in cases where the protein is normally a part of a complex [24]. Furthermore, our chemical genetic strategies are likely to identify genes that would be difficult or impossible to identify by the classical genetic mutant screen that identified Avl9. For example, gene mutations that result in very severe growth phenotypes even in the presence of *APL2* and *VPS1*, or which have only sickness rather than lethal phenotypes when their functions are blocked in the screen strain background, would not be so read-

ily identified in the mutant screen. Therefore, our chemical genetic strategy is a valuable complement to classical genetics for understanding the mechanisms and regulation of late exocytic transport.

Acknowledgments

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Chapter 4

Conclusions and Future Directions

Despite the enormous advances in the membrane traffic field in the past three decades, the mechanisms of exocytic transport from the Golgi and endosomes have remained elusive. This is due primarily to the complexity of the post-Golgi traffic pathways (9-10). The goal of my doctoral thesis was to contribute towards understanding how cargo is transported to the cell surface, and how this process is regulated. My strategy involved chemical genetic screens, in which we identified novel inhibitors of the late exocytic pathway (23-24). I discovered that one of these inhibitors, KU7, can be suppressed by over-expressing *GTR2*. This gene encodes a small GTPase that regulates the traffic of an amino acid permease and the activity of a major regulator of growth, TORC1 (23). My results indicate that the chemical genetic strategy is useful for discovering components and regulators of the late exocytic transport machinery.

Another contribution of my thesis work is helping to establish a link between Gtr2 and Avl9 function in the late secretory pathway. The *avl9*Δ mutation is lethal in an *apl2*Δ *vps1*Δ background (11). Likewise, our secretory inhibitors have an "AVL" phenotype, meaning that they are especially toxic or lethal in an *apl2*Δ *vps1*Δ background (23-24). Therefore, some of the inhibitors could inhibit Avl9, or they could inhibit a process that is somehow connected to Avl9 function. Prior to my work, there was already a hint that Avl9 and Gtr2 may have a related function, because both *GTR2* and *AVL9* had been identified together in a genome-wide screen for genes important for survival under high pressure and in the cold (2). *AVL9* and *GTR2* were among the very top-ranked mutants in that screen, and very few other phenotypic screens have identified either *AVL9* or *GTR2*. However, the cold/pressure sensitivity screen identified genes in a

wide range of functional classes, so that screen alone did not indicate a strong functional link between the genes. Because increased *GTR2* expression suppresses a traffic-related defect caused by KU7, and KU7 causes a traffic defect that is related to Avl9 function, it is possible that both Avl9 and Gtr2 function together to regulate traffic by a mechanism that is sensitive to cold and high pressure. Alternatively, Avl9 and Gtr2 may regulate the traffic of cargo that protect cells from environmental stress such as cold and high pressure.

Only a few traffic-related genes were identified in the cold/pressure sensitivity screen. Gtr2 is part of a complex that sorts an amino acid permease at endosomes depending on nutrient conditions (7). Other members of this complex are encoded by genes that were also identified as cold/pressure sensitive (2). In addition, the cold/pressure sensitivity screen identified *DRS2*, which encodes for an aminophospholipid translocase (flippase) important for the formation of clathrin-coated vesicles (4; 6). This suggests that regulation of membrane lipid composition may be the key traffic-related factor that is sensitive to the physical environment. Many genes that regulate lipid composition are either highly redundant or essential for viability. Essential genes would not have been identified in the screen by Abe and Minegishi, because that screen made use of the haploid collection of yeast gene deletion mutants, so this could explain why they did not identify many lipid regulators in their screen. They also did not include mutants that were very sick even under normal growth conditions. Although Avl9 and Gtr2 function may be sensitive to lipid composition, neither protein is likely to be directly involved in regulating membrane lipids. This is because a large genome-wide genetic interaction study did not link either Avl9 or Gtr2 closely to this function (5). So, Avl9 and Gtr2 may regulate a traffic pathway that is especially sensitive to membrane composition. The proper sorting of some nutrient permeases is very sensitive to high pressure as well as to altered membrane lipid composition (1; 20). In the future, we plan to examine whether Avl9 has a function in trafficking nutrient

permeases, as has already been shown for Gtr2 (7).

Another function of Gtr2, along with Gtr1, is regulating the activity of TORC1 in response to amino acids (14; 19). TORC1 is a major regulator of cell growth, and cell growth requires the exocytic pathway for transporting membranes and proteins to the cell surface. Therefore, Gtr2 and possibly Avl9 may link TORC1 activity to regulating the late secretory pathway for growth. When nutrient conditions are poor, it is important to downregulate an exocytic pathway that promotes growth, and when nutrient conditions are optimal, then growth-promoting exocytosis should be increased. Under nutrient-poor conditions, another traffic pathway, autophagy, is increased in order to recycle nutrients, and this is in part regulated by TORC1 (reviewed in (21)). Recent evidence from our laboratory showed that Avl9 has a regulatory role in autophagy (unpublished results). Therefore, a future research direction is to determine whether Avl9 and Gtr proteins regulate the balance between the exocytic and autohagic pathways, both of which require traffic from late Golgi compartments and possibly endosomes (8; 15).

The biochemical function of Avl9 is still not known, so we do not know exactly how it is related to Gtr2 function or traffic regulation. Avl9 is a member of an ancient eukaryotic protein family (11), one branch of which has recently been shown to encode for Rab GEF's (3; 22). However, Avl9 was not found to function as a GEF for any Rab tested (22). This suggests the possibility that the Avl9-branch of the superfamily also functions as a GEF but for another type of small GTPase. The link of Avl9 and Gtr2 suggests that perhaps Avl9 is a GEF for Gtr2. However, my work suggests that this is very unlikely. *AVL9* and *GTR* mutants have different phenotypes: I found that unlike *gtr2*Δ or *gtr1*Δ, the *avl9*Δ mutant is not sensitive to the TORC1 inhibitor rapamycin (my unpublished results). This is in contrast to the result from genome-wide analysis by others, which indicates that *avl9*Δ homozygous diploid is rapamycin sensitive, but we

found that there is a problem with the *avl9*Δ mutant used in many genome-wide studies. Also, as shown in Chapter 2, I found that the *gtr2*Δ mutation is not lethal in combination with *vps1*Δ *apl2*Δ double-mutation. More recently, I found that *gtr1*Δ *vps1*Δ *apl2*Δ as well as the quadruple mutant, *gtr1*Δ *gtr2*Δ *vps1*Δ *apl2*Δ, likewise have only a small growth defect and are viable (my unpublished results). In contrast, the *avl9*Δ *vps1*Δ *apl2*Δ triple mutant is not viable (11). Furthermore, overexpression of *AVL9* is highly toxic (11), and I found that this toxicity is not rescued by the *gtr2*Δ mutation. Although my results do not entirely rule out the possibility that Avl9 has GEF activity for Gtr2, this cannot be the primary function of Avl9. Most likely, Avl9 functions as a regulator of another GTPase, and future experimental analysis of Avl9 function will help to identify that GTPase.

Discovering the targets of some of our new secretory inhibitors promises to reveal more about Avl9 and Gtr function, and this is an important area for our future research. One very promising strategy for target identification, which can indicate both the biological function being targeted and hint at the specific molecular target, is parallel analysis of fitness, and this is the method we plan to use. In this method, pools of yeast gene deletion mutants are grown in the presence of a compound, and the cultures are then assayed to determine which mutants have reduced fitness under these conditions, compared with fitness in the absence of compound (12; 17). In the heterozygous gene deletion strain collection, the compound target may be revealed, because if there is only one copy of the gene for the target, the mutant would likely have reduced fitness. In a haploid or homozygous diploid collection (nonessential genes only), the biological function being targeted can be revealed. Our compounds are somewhat toxic even in wild type cells, so it should not be necessary to include the *vps1*Δ or *apl2*Δ mutations to see effects on fitness, which is assayed after many generations of growth and does not require a high level of toxicity. There are additional strategies for compound target identifications, including more direct methods that assay for physical interaction

between compounds and targets, but such methods generally require strong, stable interactions and are often not successful (13; 16; 18). These methods could be used in combination with the chemical genomics strategy described above.

In summary, my thesis work shows that our new compounds promise to serve as tools to study the exocytic transport process, and they will help us to learn more about Avl9 and Gtr2 function. My work also provides an example of how chemical genetic strategies can serve as helpful complements to classical genetics (mutant screens and genetic interactions) for understanding complex cellular processes. Similar yeast chemical genetic screens should be useful in other areas of cell biology. Our work will contribute to future screens because we have optimized compound library screening with yeast cells.

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