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New Tools and Approaches for Studying the Role of the Saccharomyces cerevisiae phosphatidylinositol 4-kinase Pik1 in the Yeast Nucleus

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New Tools and Approaches for Studying the Role of the *Saccharomyces cerevisiae* phosphatidylinositol 4-kinase Pik1 in the Yeast Nucleus

by

Evguenia S. Klimenko

A dissertation in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular and Cell Biology in the Graduate Division of the University of California, Berkeley

Committee in charge:

Professor Jeremy W. Thorner, Chair
Professor Karsten Weis
Professor Jamie H. D. Cate
Professor Jay T. Groves

Spring 2011
New Tools and Approaches for Studying the Role of the *Saccharomyces cerevisiae* phosphatidylinositol 4-kinase Pik1 in the Yeast Nucleus

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by

Evguenia S. Klimenko
Abstract

New Tools and Approaches for Studying the Role of the Saccharomyces cerevisiae phosphatidylinositol 4-kinase Pik1 in the Yeast Nucleus

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Jeremy W. Thorner, Chair

Phosphoinositides are a specialized type of glycerophospholipids found in all eukaryotes and their levels undergo dynamic temporal and spatial changes mediated by the actions of dedicated phosphatidylinositol kinases, phosphatases, and lipases. The Saccharomyces cerevisiae genome encodes two distinct Type III PtdIns 4-kinases that share sequence homology to each other: PIK1 and STT4. Together, Pik1 and Stt4 account for synthesis of more than 90% of the PtdIns4P detectable in yeast extracts, each is essential for viability, and the inactivation of either Pik1 or Stt4 via conditional alleles results in distinct non-overlapping phenotypes. It is thought that the independent functions of Pik1 and Stt4 result from their localization to specific cellular compartments. In the budding yeast, Stt4 localizes exclusively to the plasma membrane, whereas Pik1 localizes primarily to the Golgi body, but is also found in the cytoplasm and in the nucleus.

For the first part of my dissertation research (Chapter 3), I have characterized a novel potential specific inhibitor of Pik1. Compound ST016598, dubbed optimistically “pikostatin”, specifically inhibits the growth of cells with reduced dosage of PIK1, without affecting Pik1 stability, in vivo protein-protein interactions, or localization of fluorescently-tagged Pik1. Although ST016598 (pikostatin) is only a weak inhibitor of the lipid kinase activity of Pik1 in vitro, pikostatin treatment nevertheless results in a specific, rapid, and reversible depletion of the Pik1-dependent Golgi body-specific pool of PtdIns4P, without affecting the separate pool of PtdIns4P created at the plasma membrane by Stt4. I also found that a subset of pik1ts alleles exhibit hypersensitivity to pikostatin even under permissive conditions, with subsequent implications for the mechanism by which pikostatin might inhibit Pik1 activity at the Golgi body.
Previous work from this lab used two differentially-localized Pik1 constructs to demonstrate that localization of Pik1 both to the Golgi and to the nucleus is required for viability. However, so far, no attempts have been made to separate the function of Pik1 in the nucleus from its now well-characterized essential function in the Golgi compartment. For the second part of my dissertation research (Chapter 4), I have tested directly whether Pik1 is responsible for generating nuclear PtdIns4P that might serve as the precursor for production of a soluble cofactor (InsP$_6$) that is necessary for mRNA export. I also attempted to apply an unbiased genetic selection for dosage suppressors of the lack of nuclear Pik1 as a means to identify potential phosphoinositide- or inositol-polyphosphate-binding effectors in the yeast nucleus.
To my wonderful family:

Sergey G. Klimenko
Tatiana I. Klimenko
Maria S. Klimenko

Thank you for unconditional love and support.
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1.1 Phosphatidylinositol derivatives in cell compartmentalization and signaling.

Phosphatidylinositol (PtdIns) and its phosphorylated derivatives (referred to, collectively, as phosphoinositides) are a specialized type of glycerophospholipids found in all eukaryotes (Strahl and Thorner, 2007). The inositol headgroup of PtdIns has a total of five available hydroxyls, three of which (positions 3, 4, and 5) undergo modification by a super-family of lipid kinases and phosphatases specific for PtdIns (Figure 1.1). These hydroxyls can be phosphorylated combinatorially, creating a variety of different phosphoinositide species, including PtdIns4P, PtdIns3P, PtdIns4,5P$_2$, PtdIns3,4P$_2$, PtdIns3,5P$_2$, and PtdIns3,4,5P$_3$, depending on the organism and cell type. Although they constitute less than 5-10% of membrane phospholipids, phosphoinositides play a key role in defining distinct cellular compartments (Strahl and Thorner, 2007) and in localizing various effector proteins that associate with target membranes via specific phosphoinositide-binding domains (Lemmon, 2003, 2007, 2008).

Phosphoinositides, therefore, can serve as signaling molecules and, indeed, the level of any given phosphoinositide undergoes dynamic temporal and spatial changes mediated by the actions of dedicated phosphatidylinositol kinases and phosphatases. Moreover, PtdIns4,5P$_2$ (phosphatidylinositol-4,5-bisphosphate) can be hydrolyzed by several different classes of phospholipase C to release second messengers diacylglycerol (DAG) and soluble inositol 1,4,5-triphosphate (InsP$_3$). In animal cells, InsP$_3$ triggers the release of Ca$^{2+}$ ion from intracellular reserves (reviewed in Berridge, 2009).

Moreover, InsP$_3$ can be phosphorylated on its remaining free hydroxyls to create a variety of soluble inositol-polyphosphates (IP$_x$s) as well as specific inositol-pyrophosphate stereoisomers (Tsui and York, 2010; York, 2006). These higher phosphorylated derivatives of IP$_3$ have generated significant excitement, as recent genetic analyses have implicated them in various cellular processes, particularly inside the nucleus (Monserrate and York, 2010). However, many open questions remain about the mechanism of action of inositol-polyphosphates and inositol-pyrophosphates, the regulation of their synthesis and break-down, and also whether their local production is important for eliciting their effects optimally.

**Figure 1.1. The stereochemistry of myo-D-inositol.** Left panels, schematic representations of myo-D-inositol chemical structure (upper panel) and stereochemistry (lower panel) are shown, with the six carbons in the inositol ring numbered in red. In PtdIns, the inositol moiety is conjugated to diacylglycerol via a phosphodiester bond between C3 in glycerol and C1 in the inositol ring. PtdIns 4-kinases phosphorylate the inositol ring in PtdIns on position C4. Right panel, Agranoff’s turtle analogy for myo-D-inositol stereochemistry is shown, with C4 represented by the turtle’s left hind flipper. The figure was adapted from Irvine and Schell (2001).
1.2 Phosphatidylinositol 4-kinases in the budding yeast *Saccharomyces cerevisiae*. A phosphatidylinositol 4-kinase (PtdIns 4-kinase) phosphorylates the hydroxyl group at the 4 position of the inositol ring in PtdIns, producing PtdIns4P, which serves as a membrane recruitment signal for PtdIns4P-specific binding proteins and as a precursor for the synthesis of PtdIns4,5P₂ (and all its downstream metabolites). The *S. cerevisiae* genome encodes three PtdIns 4-kinases: PIK1 (Flanagan and Thorner, 1992; Flanagan *et al.*, 1993; Garcia-Bustos *et al.*, 1994); STT4 (Yoshida *et al.*, 1994; Audhya *et al.*, 2000; Audhya and Emr, 2002); and, LSB6 (Han *et al.*, 2002; Shelton *et al.*, 2003).

Pik1 and Stt4 are Type III PtdIns 4-kinases that share sequence homology with each other and with the catalytic kinase domain of Type I PtdIns 3-kinases. Pik1 corresponds to mammalian PI4KIIIβ (Meyers and Cantley, 1997) and Stt4 to mammalian PI4KIIIα (Wong and Cantley, 1994) (reviewed in Fruman *et al.*, 1998; Strahl and Thorner, 2007).

Lsb6 is the sole Type II PtdIns 4-kinase in yeast and has been implicated in the regulation of actin cytoskeletal organization (Han *et al.*, 2002) and possibly in endosome motility (Chang *et al.*, 2005; Kim *et al.*, 2006); yet, Lsb6 is not essential for viability under a wide variety of different conditions, stresses, and developmental stages that have been tested (Han *et al.*, 2002), unlike Pik1 and Stt4 (Flanagan *et al.*, 1993; Audhya *et al.*, 2000).
1.3 Pik1 and Stt4 are functionally distinct yeast Type III PtdIns 4-kinases. Together, Pik1 and Stt4 account for synthesis of more than 90% of the PtdIns4P detectable in yeast extracts (Audhya et al., 2000) and each is essential for viability (Flanagan et al., 1993; Garcia-Bustos et al., 1994; Cutler et al., 1997). Although both enzymes perform the same chemical reaction, conjugating a phosphate group to the hydroxyl at position 4 on the inositol ring of PtdIns to generate PtdIns4P, the inactivation of Pik1 or Stt4 via conditional alleles results in distinct non-overlapping phenotypes.

Loss of function stt4 mutants show weakened cell walls, aberrant organization of the actin cytoskeleton, abnormal vacuole morphology (Audhya et al., 2000), and defects in sphingolipid biosynthesis (Tabuchi et al., 2006; Brice et al., 2009). In contrast, pik1 mutants show aberrant Golgi body morphology, reduced transport of various cargoes from the TGN to the plasma membrane, defective protein retrieval from endosomes to the Golgi, delayed vacuolar transport and vacuole fragmentation (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000) as well as defects in meiosis (Rudge et al., 2004) and endocytosis (Walch-Solimena and Novick, 1999; Audhya et al., 2000).

The first evidence suggesting that Pik1 and Stt4 generate discrete pools of PtdIns4P came from the observation that overexpression of PIK1, but not of STT4, could rescue the temperature-sensitive growth of sec14-3ts (Hama et al., 1999) and frq1-1ts mutants (Hendricks et al., 1999), gene products that are associated with secretion through the Golgi body. Later, it was demonstrated directly that overexpression of STT4 is unable to rescue the lethality of pik1Δ cells, and vice versa (Audhya et al., 2000).

Ample additional evidence for independent functions of Pik1 and Stt4 has accumulated over the past ten years, including partial rescue of stt4ts, but not pik1ts, cells by deletion of the phosphoinositide phosphatase Sac1 (Foti et al., 2001), excessive forward transport of Chs3p (chitin synthase) resulting in cell wall defects when PIK1, but not STT4, is overexpressed (Schorr et al., 2001), mislocalization of PtdIns4P-binding protein Kes1/Osh4 in pik1ts, but not stt4ts, mutants (Li et al., 2002), rapid starvation-induced loss of the Golgi compartment pool of PtdIns4P in sac1Δ pik1ts cells, but not in sac1Δ stt4ts cells (Faulhammer et al., 2007); suppression of ypp1ts alleles by STT4, but not by PIK1, overexpression (Zhai et al., 2008); and, conversely, suppression of myo2-12 by PIK1, but not STT4 or LSB6, overexpression (Santiago-Tirado et al., 2011).

Moreover, several genes that showed a negative genetic interaction with stt4ts (Tabuchi et al., 2006) did not show any such interaction with pik1ts (Sciorra et al., 2005) but do show negative genetic interactions with mss4ts using synthetic genetic array (SGA) (Tong et al., 2001) analysis (Audhya et al., 2004), consistent with the roles of Stt4 and Mss4 in a common pathway that regulates actin cytoskeletal organization (Audhya and Emr, 2002, 2003).

It is thought that the independent functions of Pik1 and Stt4 result from their localization to specific cellular compartments. Indeed, in the budding yeast, Stt4 has been shown to localize exclusively to the plasma membrane, where it assembles into
so-called “PIK patches” with the accessory proteins Ypp1 and Efr3 (Baird et al., 2008; Zhai et al., 2008), and possibly also Sfk1 (Audhya and Emr, 2002). These are distinct from other cortical structures in yeast, such as actin patches (Drubin et al., 1988; Michelot et al., 2010), Pti1-based eisosomes (Walther et al., 2006) and the puncta containing the sole yeast PtdIns4P 5-kinase, Mss4 (Yoshida et al., 1994; Audhya and Emr, 2002). In contrast, Pik1 localizes primarily to the Golgi body (Hendricks et al., 1999; Ames et al., 2000; Strahl et al., 2005; Strahl and Thorner, 2007), but is also found in the cytoplasm (Demmel et al., 2008a) and in the nucleus (Garcia-Bustos et al., 1994; Walch-Solimena and Novick, 1999; Strahl et al., 2005).

1.4 Pik1 regulates secretion from the Golgi compartment. Pik1 itself lacks any obvious membrane-targeting motifs and localizes to the Golgi body via its interaction with the Ca\(^{2+}\)-binding N-myristoylated protein Frq1, the yeast homolog of Drosophila frequenin and mammalian neuronal calcium sensor-1 (NSC-1) (Hendricks et al., 1999; Ames et al., 2000; Strahl et al., 2005; reviewed in Strahl and Thorner, 2007).

Both a mutant of Frq1 that cannot be N-myristoylated (Hendricks et al., 1999) and a mutant of Pik1 that is missing its Frq1-binding site (Huttner et al., 2003) are unable to support viability at elevated temperatures, suggesting that both the binding of Pik1 to Frq1 and the ability of Frq1 to interact with membranes, are necessary for the optimal function of Pik1. The Pik1-Frq1 interaction is observed in S. pombe (Lim et al., 2011) and conserved across species, as human NCS-1 binds to yeast Pik1 at the same site as Frq1 and is able to rescue the inviability of frq1\(\Delta\) cells (Strahl et al., 2003, Huttner et al., 2003).

Frq1 is required for optimal activity of Pik1 in vitro (Hendricks et al., 1999) and a recent structural study suggests that Frq1 may enhance Pik1 lipid kinase activity by promoting the association of the N-terminal lipid kinase unique (LKU) domain of Pik1 with its C-terminal catalytic domain (Strahl et al., 2007). In a similar fashion, mammalian NCS-1 has been shown to bind PI4KIII\(\beta\) and to be necessary both for the localization of this PtdIns 4-kinase isoform to the Golgi body and for the activation of its lipid kinase activity in a variety of cell types (Zhao et al., 2001; Pan et al., 2002; Taverna et al., 2002; Kapp-Barnea et al., 2002).

Recently, it has been shown that yeast Pik1 physically interacts with Sec7 (Gloor et al., 2010), one of three guanine nucleotide exchange factors (GEFs) for Arf1, a small GTPase that localizes to the Golgi body and is involved in the formation of both clathrin- and COPI-coated vesicles (Springer et al., 1999; Roth, 1999; Jackson and Casanova, 2000). Although Pik1 localizes at the Golgi body fairly normally in sec7-4\(^{4}\) cells at restrictive temperature, Pik1 produces PtdIns4P much less efficiently at the Golgi cisternae when Sec7 function is compromised (Gloor et al., 2010). Similarly, in mammals, both PI4KIII\(\beta\) (Godi et al., 1999) and NCS-1 (Haynes et al., 2005) are recruited to Golgi membranes by binding to ARF. This interaction is thought to bring PI4KIII\(\beta\) in close proximity to a key activator, protein kinase D (PKD), which also binds to ARF (Pusapati et al., 2010). There is no PKD ortholog encoded in the S. cerevisiae genome, however.
1.5 Phosphoinositide-binding effectors associated with the Golgi body. The Golgi body performs at least two important functions in both yeast and mammalian cells. First, protein cargoes moving through the secretory pathway are sorted into exocytic vesicles at the trans-Golgi network (TGN). Second, the synthesis of complex membrane lipids, which starts in the endoplasmic reticulum (ER), is finished in the Golgi where these lipids are also packaged into vesicles and sent to target membranes. The pool of PtdIns4P created by Golgi body-localized Pik1 is central to both lipid and protein trafficking through the Golgi and functions by recruiting specific PtdIns4P-binding effectors that fall into two major classes: lipid translocation proteins and vesicle budding and transport factors (reviewed in Mayinger, 2009; Graham and Burd, 2011).

Three lipid transport proteins have been characterized in mammalian cells: ceramide transfer protein (CERT) (Hanada et al., 2003), four-phosphate adaptor protein 2 (FAPP2) (Godi et al., 2004), and oxysterol-binding proteins (OSBPs; reviewed in Ridgway, 2010). CERT transfers ceramide from the ER to the Golgi via specialized membrane contact sites (Hanada et al., 2003) and, in a similar way, FAPP2 transports glucosylceramide inside the Golgi stack (Halter et al., 2007; D’Angelo et al., 2007). OSBP1 collaborates with CERT to coordinate sterol and sphingolipid biosynthesis in the Golgi compartments, but it is not clear whether it functions as a sterol transport protein or a sterol sensor (Perry and Ridgway, 2006). However, recent evidence indicates that OSBPs are needed for the establishment and/or maintenance of ER-plasma membrane junctions (Stefan et al., 2011). Curiously, CERT and FAPP2 orthologs are absent in yeast.

Although the OSBP ortholog Kes1/Osh4 is perhaps the best characterized yeast PtdIns4P-binding lipid transport protein (Li et al., 2002), there are six other OSBP orthologs in S. cerevisiae (Beh et al., 2001; Beh and Rine, 2004). Kes1/Osh4 localizes to the Golgi via its PtdIns4P-binding pleckstrin-homology (PH) domain, where it acts in opposition to the major yeast PtdIns/PtdCho-transfer protein, Sec14, thought to deliver PtdIns substrate to Pik1, so that the inactivation of Kes1 function bypasses the essential requirement for Sec14 (Li et al., 2002). Although the specific mechanism of Kes1 action remains unknown, it likely modulates the amount of PtdIns4P available in the Golgi compartment, which in turn may affect the localization and/or activity of other PtdIns4P-dependent effectors.

One such effector was recently identified in yeast: the aminophospholipid flippase Drs2. Drs2 localizes to the trans-Golgi network (TGN) and transfers phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEth) from the luminal leaflet to the cytosolic leaflet, thus establishing phospholipid asymmetry in Golgi-derived vesicles (Muthusamy et al., 2009a, 2009b; Natarajan et al., 2004, 2009). The enzymatic activity of Drs2 strongly depends on its binding to PtdIns4P and the TGN Arf-GEF Gea2 (Natarajan et al., 2009) and Drs2 activity is important for normal secretory function (Sciorra et al., 2005). Interestingly, mutations in the human Drs2 ortholog, ATP8B1, result in liver disease, characterized by the inability of liver cells to secrete bile (Folmer et al., 2009).
The second class of PtdIns4P-binding, Golgi body-localized effectors are involved in sorting cargo into secretory vesicles and vesicle budding from the TGN. It has been shown that adaptor protein complex AP-1 in mammals (Wang et al., 2003; Heldwein et al., 2004) and Golgi body-associated, γ-ear containing, ARF-binding (GGA) proteins in both mammals (Wang et al., 2007) and yeast (Zhdankina et al., 2001; Demmel et al., 2008b) localize to Golgi membranes via their dual binding to PtdIns4P and ARF/Arf1 and regulate cargo sorting into clathrin-coated vesicles in the TGN.

Vesicle budding factors include PtdIns4P-binding effectors that mediate membrane rearrangements (Drs2, FAPP1, FAPP2) and components of the Rab GTPase cascade necessary for vesicle scission (de Graaf et al., 2004; Sciorra et al., 2005). In particular, in yeast, the late Golgi Rab, Ypt32, in conjunction with PtdIns4P recruits Sec2, a GEF for another Golgi body-associated Rab, Sec4, that coordinates the assembly of the exocyst complex onto budding vesicles, which then guides these vesicles to the plasma membrane for exocytosis (Miżuno-Yamasaki et al., 2010).

Interestingly, Myo2, an essential yeast class V myosin necessary for the directional transport of secretory vesicles along actin filaments (Johnston et al., 1991; reviewed in Pruyne et al., 2004), also interacts with the Rabs Ypt31p and Sec4 and is the most recently identified PtdIns4P effector in yeast (Santiago-Tirado et al., 2011). Another unconventional myosin (MYO18A) has been implicated in maintaining the structure of the Golgi cisternae in mammalian cells and is recruited to Golgi membranes via interactions with GOLPH3/GPP34/GMx33 (Snyder et al., 2006), a PtdIns4P-binding protein (Dippold et al., 2009; Wood et al., 2009).

Interestingly, the GOLPH3 ortholog in yeast, Vps74, regulates a different aspect of the biology of the Golgi compartment, the retention of Golgi-resident glycosyltransferases in the Golgi cisternae (Schmitz et al., 2008, Tu et al., 2008; Wood et al., 2009). Nevertheless, mammalian GOLPH3 can suppress vps74Δ phenotypes, suggesting that the function of GOLPH3/Vps74 has been conserved between mammals and yeast (Tu et al., 2008; Wood et al., 2009).

1.6 Pik1 interacts with 14-3-3 proteins in the cytoplasm. In yeast, the Pik1-Frq1 complex rapidly and reversibly dissociates from Golgi membranes when a fermentable carbon source is depleted (Faulhammer et al., 2007; Demmel et al., 2008a). The cytoplasmic Pik1-Frq1 complex is bound by the two yeast 14-3-3 orthologs, Bmh1 and Bmh2 (Gavin et al., 2002; Huttner et al., 2003; Faulhammer et al., 2007; Demmel et al., 2008a). This interaction depends on the phosphorylation of Ser396 in a 14-3-3 consensus motif found in the N-terminal regulatory portion of Pik1 (Demmel et al., 2008a), a site that is also conserved in mammals (Hausser et al., 2006).

In mammalian cells, two members of the protein kinase D family (PKD1 and PKD2) reportedly are responsible for phosphorylating the corresponding residue (Ser294) in the 14-3-3 consensus motif of PI4KIIIβ (Hausser et al., 2005). However,
there is no PKD ortholog in yeast. Thus the kinase that phosphorylates yeast Pik1 on Ser396 remains unidentified.

In yeast, Pik1 phosphorylation on Ser396 leads to its relocalization from Golgi membranes to the cytoplasm, where it is retained via its interaction with Bmh1 and Bmh2 (Demmel et al., 2008a), thereby inhibiting PtdIns4P production in the Golgi body and thus inhibiting secretion. By contrast, interaction of PI4KIIIβ phosphorylated on Ser294 with 14-3-3 proteins in mammalian cells stabilizes PI4KIIIβ catalytic activity and protects Ser294 from dephosphorylation (Hausser et al., 2006). The underlying physiological rationale for this difference in the regulation of PtdIns 4-kinases by 14-3-3 proteins in yeast versus mammals is not clear.

1.7 Pik1 plays an essential, but unknown, role in the nucleus. In addition to their well-characterized localization and function at the Golgi apparatus, both yeast Pik1 (Garcia-Bustos et al., 1994, Walch-Solimena and Novick 1999; Strahl et al., 2005) and mammalian PI4KIIIβ (de Graaf et al., 2002) have been observed in the nucleus and have been shown to undergo nucleocytoplasmic shuttling (de Graaf et al., 2002; Strahl et al., 2005).

The fact that the inviability of a pik1Δ cell can be rescued only when a cytoplasmically-targeted and a nuclearly-targeted derivatives of Pik1 were co-expressed and only when each is catalytically active (Strahl et al., 2005), suggests that Pik1 produces discrete pools of PtdIns4P at the TGN and in the nucleus and, further, that these distinct PtdIns4P pools support separable and essential functions in each compartment.

To date, however, no nuclear function of Pik1 or nuclear PtdIns4P-binding effector has been described in S. cerevisiae or any other organism, although effectors that bind nuclear PtdIns4,5P2 (Yu et al., 1998; Zhao et al., 1998, Rando et al., 2001), PtdIns5P (Gozani et al., 2003; Mellman et al., 2008) and PtdIns3,4,5P3 (Okada et al., 2008) have been reported in mammalian cells.

Moreover, structural studies have identified phosphoinositide-derived inositol-polyphosphates as co-factors for proteins, such as the RNA-editing enzyme ADAR2 (Macbeth et al., 2005) and the complex of the nucleoporin Gle1 with the DEXD/H-box helicase Dbp5 (Weirich et al., 2006; Montpetit et al., 2011). Two members of the nuclear receptor family of transcription factors, SF-1 and LRH-1, also reportedly bind phosphoinositides (Krylova et al., 2005).

One initial mission of my dissertation research was focused on efforts to create new tools for dissecting compartment-specific functions of Pik1 through the use of an isoform-specific inhibitor (Chapter 3) and on both directed and unbiased approaches for discovering essential processes Pik1 may regulate within the yeast nucleus via identifying additional positive regulators and PtdIns-binding effectors that may assist Pik1 in carrying out its essential nuclear function(s) (Chapter 4).
2.1. Yeast strains and growth conditions.

2.1.1. Existing yeast strains. Saccharomyces cerevisiae strains used in this study are listed in Table 2.1. Strains JKY01, JKY02, JKY03, and JKY04 were obtained from the heterozygous diploid collection originally sold by Research Genetics, Inc. and, later, upon the dissolution of this enterprise, by Invitrogen Corp. JTY3350 (pik1Δ/PIK1) was originally retrieved from the heterozygous diploid collection by T. Strahl. Strains containing TRP1-marked integrated conditional pik1ts alleles were generated by E. A. Schnieders (Schnieders, 1996); the method used for integration (p. 30-32) and the mutations present in each conditional allele (p. 88) are described in her thesis (Schnieders, 1996). JTY3125 (mss4ts) and JTY3108 (stt4ts) were obtained from the Emr lab (currently at Cornell University) and crossed into the YPH499 strain background by T. Strahl and F. Roelants, respectively. Strain JTY2308 (YPH499 plc1Δ::LEU2) contains the LEU2 gene in place of nt 277-2333 of the PLC1 open reading frame (Flick and Thorner, 1993).

2.1.2. Integration of in-frame N-terminal tags into the chromosomal PIK1 locus. JKY05 was generated by replacing one copy of the native PIK1 promoter in BY4743 with a PCR fragment, amplified from the pFA6a-kanMX6-pGAL1-GST template (Longtine et al., 1998). Transformants were selected on YPD plates containing the antibiotic G418 and the genotype of drug-resistant transformants was confirmed by PCR with appropriate primers. GST-Pik1 expression upon galactose induction was confirmed by immunoblot analysis. JKY06 was generated using the loxP-cre method for seamless N-terminal tagging of endogenous yeast proteins (Prein et al., 2000). Briefly, loxP-kanMX4-loxP-yeGFP was amplified from the pYGFPgN PCR template and introduced by DNA-mediated transformation into diploid strain BY4743, inserting the amplified fragment between the PIK1 promoter and the PIK1 ORF in one of the two copies of chromosome XIV with the GFP in-frame with the N-terminus of the PIK1 coding sequence. To restore the function of the endogenous PIK1 promoter, the resulting heterozygous diploid was transformed with the CRE expression vector, pSH47, and the production of cre recombinase was induced by galactose addition. The resulting single colonies were screened for visible GFP expression by fluorescence microscopy and concomitant acquisition of sensitivity to G418.

2.1.3. Generation of double mutants for in situ analysis. First, the entire NUP42 reading frame was replaced with the kanMX6 marker amplified from pFAa-kanMX6 PCR template (Longtine et al., 1998) in a YPH499 MATα haploid, to generate strain JKY07. Then, JKY07 was crossed to JTY2308, JTY3125, JTY3108, and JTY2944, the resulting diploids were sporulated, and 10-15 tetrads were dissected for each cross, except in the case of JTY3125, which required additional dissections to isolate the desired double mutant because MSS4 and NUP42 are linked (separated by 23
kb on chromosome IV). Tetrads were genotyped and only full tetratype asci were collected (omitting the wild-type spore) for in situ analysis. Two separate tetrads were collected from the JTY2944 (pik1-83ts) cross. Mutants were stored on plates at 4°C between the three biological replicates and later made into glycerol stocks. The same strategy was used to generate JKY13 (W303 plc1Δ nup42Δ) from JTY3158 (W303 MATa plc1Δ::LEU2) and JKY12 (W303 MATα nup42Δ::KanMX6).

2.1.4 Generation of tester strains for the genetic selection for dosage suppressors. Strains JTY2943 (pik1-63ts::TRP1) and JTY2944 (pik1-83ts::TRP1) were transformed with pRR1 (HO^R-pPIK1-GFP-pik1-CCaaX-hisG-URA3-hisG-HO^L), digested with NolI, and transformants were selected on medium lacking uracil. Integration at the HO locus was confirmed by colony PCR. To obtain Ura- derivatives of the two tester strains, cells were streaked out on medium containing 5-fluoro-orotic acid (5-FOA). The temperature-sensitive growth phenotype of Ura- tester derivatives was identical to that of the original Ura+ testers (R. Rajaii undergraduate honors thesis, UC Berkeley, 2010).

2.1.5 Growth conditions. Unless otherwise indicated, strains were cultured at 30°C in standard rich (YP) medium or defined (SC) medium (Burke et al., 2000) containing 2% glucose (D or Dex). All temperature-sensitive strains (plc1Δ, pik1ts, mss4ts, stt4ts) were cultured at 25°C. For galactose induction, cells were grown first in medium containing 2% raffinose and 0.2% sucrose, then galactose was added to 2% to induce protein expression. For fluorescence microscopy, cells were grown in SCD–Trp medium with casamino acids (a rich source of nutrients) and the same medium was used for imaging. The majority of pikostatin experiments were performed in rich (YP) medium because pikostatin is much less potent on cells grown in defined (SC) medium. Hygromycin B (Invirogen, 50mg/mL in PBS) was used at 300 µg/mL, where applicable, for plasmid selection. Higher concentrations of pikostatin (10x) were used in imaging medium used for fluorescence microscopy. For plates containing pikostatin or pyrazolopyrimidine-based inhibitors, drug stock was added to a single-plate-sized aliquot of medium to the desired final concentration and then the plate was poured. Pikostatin (ST016598, molecular formula C_{20}H_{25}N_{3}O_{2}, MW 339.431 g/mol) was purchased from TimTec LLC (Newark, DE) and prepared as a 10 mM stock solution in DMSO. 1-NM-PP1, 1-NA-PP1, and 2-NM-PP1 inhibitors were kindly donated by Kevan Shokat (UCSF).
Table 2.1. *Saccharomyces cerevisiae* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ2168</td>
<td>MATα gal2 leu2 trp1 ura3-52 prb1-1122 pep4-3 pre1-451</td>
<td>Jones, 1991</td>
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<tr>
<td>BY473</td>
<td>MATα/MATα his3Δ/his3Δ leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ LYS2/lys2Δ0</td>
<td>Research Genetics</td>
</tr>
<tr>
<td>JTY3350</td>
<td>BY4743 pik1Δ::KanMX4/PIK1</td>
<td>Strahl et al., 2005</td>
</tr>
<tr>
<td>JKY01</td>
<td>BY4743 stt4Δ::KanMX6/STT4</td>
<td>Research Genetics</td>
</tr>
<tr>
<td>JKY02</td>
<td>BY4743 mss4Δ::KanMX6/MSS4</td>
<td>Research Genetics</td>
</tr>
<tr>
<td>JKY03</td>
<td>BY4743 frq1Δ::KanMX6/FRQ1</td>
<td>Research Genetics</td>
</tr>
<tr>
<td>JKY04</td>
<td>BY4743 plc1Δ::KanMX6/PLC1</td>
<td>Research Genetics</td>
</tr>
<tr>
<td>JKY05</td>
<td>BY4743 KanMX::pGAL1-GST-PIK1/PIK1</td>
<td>This study</td>
</tr>
<tr>
<td>JKY06</td>
<td>BY4743 eGFP-PIK1/PIK1 (marker-less)</td>
<td>This study</td>
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<tr>
<td>JTY3650</td>
<td>BY4741 FRQ1-GFP::KanMX6</td>
<td>Strahl et al., 2005</td>
</tr>
<tr>
<td>YPH499</td>
<td>MATα ade2-10oc his3-Δ200 leu2Δ-1 lys2-801am trp1-Δ63 ura3-52</td>
<td>Sikorski and Hieter, 1989</td>
</tr>
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<td>JTY2940</td>
<td>YPH499 MATα PIK1::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
<tr>
<td>JTY2941</td>
<td>YPH499 MATα pik1-11::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
<tr>
<td>JTY2942</td>
<td>YPH499 MATα pik1-21::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
<tr>
<td>JTY2947</td>
<td>YPH499 MATα pik1-22::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
<tr>
<td>JTY2945</td>
<td>YPH499 MATα pik1-24::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
<tr>
<td>JTY2948</td>
<td>YPH499 MATα pik1-41::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
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<td>JTY2943</td>
<td>YPH499 MATα pik1-63::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
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<td>JTY2944</td>
<td>YPH499 MATα pik1-83::TRP1</td>
<td>Schnieders, 1996</td>
</tr>
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<td>JTY2308</td>
<td>YPH499 MATα plc1Δ2::LEU2</td>
<td>Flick and Thorner, 1993</td>
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<td>JTY3125</td>
<td>YPH499 MATα mss4Δ::HIS3MX6 [CEN LEU2 mss4-102Δs]</td>
<td>S. Emr / T. Strahl</td>
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<tr>
<td>JTY3108</td>
<td>YPH499 MATα stt4Δ::HIS3 [CEN LEU2 stt4-4Δs]</td>
<td>S. Emr / F. Roelants</td>
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<td>JKY07</td>
<td>YPH499 MATα nup42Δ::KanMX6</td>
<td>This study</td>
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JKY08A  YPH499  MATa  pik1-83::TRP1  nup42Δ::KanMX6  This study
JKY08B  YPH499  MATa  pik1-83::TRP1  nup42Δ::KanMX6  This study
JKY09  YPH499  MATa  plc1Δ2::LEU2  nup42Δ::KanMX6  This study
JKY10  YPH499  MATa  mss4Δ::HIS3MX6 [CEN LEU2 mss4-102ts] nup42Δ::KanMX6  This study
JKY11  YPH499  MATa  stt4Δ::HIS3 [CEN LEU2 stt4-4ts] nup42Δ::KanMX6  This study
RR01  YPH499  MATa  pik1-83::TRP1  ho::P_{PIK1}-GFP-pik1-CCaaX  R. Rajaii
RR02  YPH499  MATa  pik1-63::TRP1  ho::P_{PIK1}-GFP-pik1-CCaaX  R. Rajaii
W303-1A  MATa  ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100  Thomas and Rothstein, 1989
JKY12  W303-1A  MATa  nup42Δ::KanMX6  This study
JTY3158  W303-1B  MATα  plc1Δ::LEU2  Weirich et al., 2006
JKY13  W303  plc1Δ::LEU2  nup42Δ::KanMX6  This study
2.2. Plasmids and recombinant DNA methods. Plasmids used in this study are listed in Table 2.2. Plasmids were constructed and propagated in *E. coli* using standard recombinant DNA methods. pJK01 and pJK02 were generated by site-directed mutagenesis of pJT2517 (*P*<sub>PIK1</sub>-GFP-*PIK1*). The selectable marker on a set of plasmids used in pikostatin experiments was switched from *URA3* or *HIS3* to HygB<sup>+</sup> using the marker swap cassette amplified from pRS306H or pRS303H (Taxis and Knop, 2006) and co-transformed into live yeast together with the target plasmid. In this case, HygB was appended to the original plasmid name and the original source was listed in the plasmid table. Plasmids with the new HygB<sup>+</sup> marker were rescued in *E. coli*; marker replacement was confirmed by restriction endonuclease digest, and insert integrity was verified by nucleotide sequence analysis. pRR1 was constructed by ligating the *Bam*<sub>H</sub>/Sac<sub>I</sub> fragment of pJT2518 (*P*<sub>PIK1</sub>-GFP-*pik1*-CCaaX) into pJT3483 ((*HO<sup>R</sup>*-higG-*URA3*-hisG-*HO<sup>L</sup>)) (Voth *et al.*, 2001) between *HO<sup>R</sup>* and the adjacent hisG repeat.

**Table 2.2.** Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS315</td>
<td><em>CEN LEU2</em></td>
<td>Sikorski and Hieter, 1989</td>
</tr>
<tr>
<td>pRS316</td>
<td><em>CEN URA3</em></td>
<td>Sikorski and Hieter, 1989</td>
</tr>
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<td>pRSHygB</td>
<td><em>CEN HygB</em></td>
<td>This study</td>
</tr>
<tr>
<td>pJT720HygB</td>
<td>pRSHygB <em>P</em>&lt;sub&gt;GAL1&lt;/sub&gt;-FRQ1-myc</td>
<td>Hendricks <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>pJT721HygB</td>
<td>pRSHygB <em>P</em>&lt;sub&gt;FRQ1&lt;/sub&gt;-FRQ1-myc</td>
<td>Hendricks <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>pJT2517</td>
<td>pRS316 <em>P</em>&lt;sub&gt;PIK1&lt;/sub&gt;-GFP-*PIK1</td>
<td>Strahl <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>pJK01</td>
<td>pRS316 <em>P</em>&lt;sub&gt;PIK1&lt;/sub&gt;-GFP-*PIK1(V842A)</td>
<td>This study</td>
</tr>
<tr>
<td>pJK02</td>
<td>pRS316 <em>P</em>&lt;sub&gt;PIK1&lt;/sub&gt;-GFP-*PIK1(V842G)</td>
<td>This study</td>
</tr>
<tr>
<td>pJT2518</td>
<td>pRS316 <em>P</em>&lt;sub&gt;PIK1&lt;/sub&gt;-GFP-<em>pik1</em>-CCaaX</td>
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</tr>
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<td>pJT2509</td>
<td>pRS316 <em>P</em>&lt;sub&gt;GAL1&lt;/sub&gt;-GFP-*PIK1</td>
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<td>pJT2650</td>
<td>pRS316 <em>P</em>&lt;sub&gt;GAL1&lt;/sub&gt;-GFP-*pik1(D918A)</td>
<td>Strahl <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Construct</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------------------------------------</td>
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<td>pJT2541</td>
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<td>pJT2651</td>
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<td>pJT2625</td>
<td>pRS315 $P_{GAL1}$-GST-GFP</td>
<td>P. Pryciak (U. Mass. Med.)</td>
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<td>pJT1496</td>
<td>pRS316 $P_{PHO5}$-GFP-OSH2^{2xPH}</td>
<td>T. Levine (U. London)</td>
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<td>pJT2523</td>
<td>pRS316 $P_{GAL1}$-myc-$pik1$-CCaaX</td>
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<td>pJT3156HygB</td>
<td>pRSHygB $P_{PRC1}$-GFP-STT4</td>
<td>Audhya et al., 2002</td>
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<tr>
<td>pJT2517HygB</td>
<td>pRSHygB $P_{PIK1}$-GFP-PIK1</td>
<td>Strahl et al., 2005</td>
</tr>
<tr>
<td>pJT2518HygB</td>
<td>pRSHygB $P_{PIK1}$-GFP-$pik1$-CCaaX</td>
<td>Strahl et al., 2005</td>
</tr>
<tr>
<td>pJT2509HygB</td>
<td>pRSHygB $P_{GAL1}$-GFP-$pik1$-CCaaX</td>
<td>Strahl et al., 2005</td>
</tr>
<tr>
<td>pJT2650HygB</td>
<td>pRSHygB $P_{GAL1}$-GFP-$pik1(D918A)$</td>
<td>Strahl et al., 2005</td>
</tr>
<tr>
<td>pJT2625HygB</td>
<td>pRSHygB $P_{GAL1}$-GST-GFP</td>
<td>P. Pryciak (U. Mass. Med.)</td>
</tr>
<tr>
<td>pJT1496HygB</td>
<td>pRSHygB $P_{PHO5}$-GFP-OSH2^{2xPH}</td>
<td>T. Levine (U. London)</td>
</tr>
<tr>
<td>pYGFPGn</td>
<td>$loxp$-kanMX4-$loxp$-yeGFP</td>
<td>Prein et al., 2000</td>
</tr>
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<td>pSH47</td>
<td>$P_{GAL1}$-cre</td>
<td>Guldener et al., 1996</td>
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<tr>
<td>pJT3483</td>
<td>$HO^{R}$-hisG-URA3-hisG-$HO^{L}$</td>
<td>Voth et al., 2001</td>
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<tr>
<td>pRR1</td>
<td>$HO^{R}$-$pPIK1$-GFP-$pik1$-CCaaX-hisG-URA3-hisG-$HO^{L}$</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.3. Serial dilutions, growth curves, and viability assays. Yeast strains were cultured in the appropriate selective medium overnight, diluted to $A_{600\text{nm}} = 0.15$, and grown to mid-exponential phase ($A_{600\text{nm}} = 0.5-0.7$) for 4-6 h. For serial dilutions, cultures were adjusted to $A_{600\text{nm}} = 0.5$ and 5-fold dilutions were made in 96 well plates, before cells were spotted on dry, pre-warmed plates. For growth curves, cultures were adjusted to final $A_{600\text{nm}} = 0.1$ in 96-well plates with varying pikostatin concentrations arrayed in triplicate across the plate. Absorbance was read every 15 min for 36 h in an Infinite M1000 plate reader (TECAN, Durham, NC) with orbital shaking between time points to prevent cell settling. For IC$_{50}$ determination, cultures were diluted to $A_{600\text{nm}} = 0.1$ in glass tubes with pikostatin and absorbance was recorded at 6 h of treatment. For viability assays, equal numbers of cells were collected from pikostatin- and DMSO-treated cultures and serial dilutions were performed in a 96 well plate, before cells were spotted on pikostatin-free YPD plates.

2.4. Cycloheximide pulse-chase analysis for determining protein stability. Overnight cultures were diluted into YP raffinose/sucrose medium and grown to mid-exponential phase ($A_{600\text{nm}} = 0.15-0.3$), protein expression from $p\text{GAL1}$ was induced by adding 2% galactose for 4 h, followed by the addition of 10 $\mu$g/mL cycloheximide together with either 16 $\mu$M pikostatin or volume-matched DMSO. An equal number of cells (2 $A_{600\text{nm}}$ units) were collected at the indicated times for immunoblot analysis. Briefly, total protein was extracted by TCA precipitation, solubilized in SDS sample buffer (Laemmli, 1970), resolved by SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane. GST-Pik1 was detected using $\alpha$-GST antibody (rabbit polyclonal sc-459, Santa Cruz Biotechnology) and $\alpha$-Cdc3 antibody (rabbit polyclonal, generously provided by Michael McMurray, this lab) was used as a loading control. Both antibodies were used at 1:1,000 dilution.

2.5. GST- and GFP- pull-downs and immunoprecipitations. For GFP pull-downs, strain BJ2168 transformed with pJT2509HygB ($p\text{GAL1-GST-PIK1}$) was used. For GST pull-downs, strain JKY05 was used for GST-Pik1 (chromosomal) and BY4743 transformed with pJT2625HygB ($p\text{GAL1-GST-GFP}$) was used for the GST only control. For Frq1 immunoprecipitations, JKY05 ($p\text{GAL1-GST-PIK1/PIK1}$) was transformed with pJT720HygB ($p\text{GAL1-FRQ1-myc}$) and only the epitope-tagged Frq1 was detected by immunoblot analysis to avoid using the same antibodies for immunoprecipitation and detection. Cells were grown in YP raffinose/sucrose medium and GST-tagged protein expression from $p\text{GAL1}$ was induced as described above. Cultures were then treated with either pikostatin at concentrations indicated or volume-matched DMSO for 2 h, and cells were subsequently harvested and snap-frozen in liquid N$_{2}$. Whole cell lysates were prepared using the standard glass beads protocol (Burke et al., 2000) and 15 mg lysate was taken per pull-down (or immunoprecipitation) in a total volume of 0.8-1.0 mL lysis buffer: 20 mM Tris-HCl (pH 7.5), 125 mM potassium acetate, 0.5 mM EDTA, 0.5 mM EGTA, 1-2 mM DTT, 0.1% Tween-20, 12.5% glycerol, one crushed Complete protease inhibitor tablet (Roche).
Lysate was incubated either with glutathione-Sepharose™ 4B resin (GE Healthcare) for GST pull-downs or with polyclonal α-Frq1 anti-sera (Hendricks et al., 1999) at 1:100 dilution and Protein A/G agarose resin (Calbiochem, a subdivision of EMD Chemicals/Merck, Gibbstown, NJ) for immunoprecipitations, for 3-5 h. To capture GFP-Pik1p for initial in vitro assays, Chromotek GFP-Trap beads (IZB Martinsried, Germany) were used. Beads/resins were washed three times with ice-cold IP buffer and either boiled in SDS sample buffer (Laemmli, 1970) for immunoblot analysis as described above or used immediately in the in vitro lipid kinase assay described below. A soluble GST-GFP construct was used for the GST only control and pre-immune serum served as the control for Frq1 immunoprecipitations. Antibodies used for immunoblot analysis of Pik1 protein-protein interactions included α-GST antibody (rabbit polyclonal sc-459, Santa Cruz Biotechnology), rabbit polyclonal α-Pik1C sera #1261 and #1262, 1:100 dilution (Schnieders, 1996) and α-Frq1 sera #1137, 1:1,000 dilution (Hendricks et al., 1999), α-myc (9E10) antibody (mouse, purified in-house from tissue culture medium, 1:100 dilution). For loading control, rabbit polyclonal α-Pgk1 antibodies were used 1:10,000 dilution (Baum, Thorner, and Honig, 1978).

2.6. In vitro assay for Pik1 lipid kinase activity. The in vitro assay for Pik1 phosphatidylinositol 4-kinase activity was adapted from Knight et al., 2007. Briefly, Pik1-containing beads were resuspended in 25 mM Tris-HCl (pH 7.5) and mixed phosphatidylinositol/TritonX-100 micelles were added as substrate (final concentrations in the assay were 0.25 mM phosphatidylinositol (soy PtdIns, Avanti Polar Lipids), 0.25% TritonX-100, 75 mM Tris-HCl (pH 7.5), 10 mM MgCl₂). In some later assays, 1 mg/mL bovine serum albumin (BSA) was also used as a stabilizing agent and results obtained +/- BSA were comparable. The reaction mixture was incubated for 5 min at 34°C and aliquoted into a 96 well plate. Pikostatin was added to concentrations indicated and 10-15 min at room temperature were allowed for its potential binding to Pik1p. Reactions were initiated by the addition of 5 mM ATP containing 1 µCi [γ-32P]ATP per well. At the indicated time points (0, 5, 15, 30, 45, and 60 min), reactions were terminated by spotting 4 µL from each well onto a nitrocellulose membrane. Membranes were air-dried for 15-30 min, rinsed once with 1M NaCl containing 5% phosphoric acid solution and washed overnight in fresh 1M NaCl containing 5% phosphoric acid solution to remove excess [γ-32P]ATP. Dried membranes were exposed to a Phosphorimager™ screen for 12-16 h and imaged on a Typhoon™ Phosphorimager (Molecular Dynamics Division, Amersham Pharmacia Biotech, Inc.). Radioactive PtdIns4P signal was quantified using free Spot software from the Shokat lab (UCSF; http://shokatlab.ucsf.edu/SPOT.htm; Knight et al., 2007). Data were compiled in Excel and analyzed using GraphPad Prism statistical software. To calculate initial reaction rates, measurements within each replicate were normalized to the DMSO-treated control at 60 min post-ATP addition, then four replicates per time point were averaged and plotted as the amount of PtdIns4P product formed over time, except for samples treated with α-Pik1C antibody or pre-immune serum, where three independent replicates were used. Graphical data were
fitted with a linear regression curve in Graphpad Prism and the regression slope (product formed per unit time) for each condition was normalized to the DMSO-treated control (set to 100%) and reported as the initial rate. To approximate specific activity, initial rates were calculated as described above for four independent experiments using Pik1 pulled-down from both pikostatin- and DMSO-treated cells. Initial rates were then plotted against the amount of Pik1 pulled-down on the beads in each experiment as detected by immunoblot analysis. These data were fitted with a linear regression curve in Graphpad Prism and the regression slope (initial reaction rate over unit enzyme) was reported as specific activity in arbitrary units.

2.7. Bright Field and Fluorescence Microscopy. For observing the gross morphology of pikostatin-treated cells, cultures were spotted onto a glass coverslip, inverted onto a glass slide, and imaged directly on a model BH-2 microscope (Olympus America) at 100X magnification. Images were collected using a charge-coupled device camera (Olympus America) and processed in Magnafire SP imaging software (Optronics). For fluorescence microscopy, yeast strains to be imaged were cultured overnight in selective SC medium supplemented with casamino acids without tryptophan or adenine. For microscopy, cultures were diluted to A$_{600nm}$ = 0.05-0.1 and grown to mid-exponential phase (A$_{600nm}$ = 0.2-0.4). To achieve the same amount of growth inhibition as in rich medium, pikostatin had to be used in imaging medium at 50-100 µM, which is approximately 10 times higher than the concentration normally effective in YPD. The amount of DMSO was kept below 2% in all experiments. Cells were treated for the length of time indicated, attached to concanavalin A–coated coverslips, which were sealed to slides with vacuum grease (Dow Corning). All imaging studies were performed at ~25°C using a microscope (IX-81OMAC; Olympus) equipped with 100X/NA 1.4 objective and a charge-coupled device camera (Orca II; Hamamatsu) and processed in Metamorph imaging software (Molecular Devices). Image analysis was performed with ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/). For reversibility experiments, cells were kept in an open-air chamber, old medium was removed by gentle aspiration with a P1000 pipette and then fresh medium was added immediately and changes in GFP-tagged protein localization were observed.

2.8. In situ hybridization for mRNA export defect detection. To assess an mRNA export defect, cultures of relevant strains were grown in YPD to mid-exponential phase (A$_{600nm}$ = 0.15-0.25) at 25°C, split into 5 mL aliquots and either shifted to 37°C or put back at 25°C for 2 h. Formaldehyde (Fisher Chemical cat # F79-500) was added directly to each culture to 5% final concentration. Cells were first fixed for 15 min at the shift temperature and then all cultures were moved to 25°C and fixation continued for a total of 2 h. From this point on, solutions were prepared using distilled water pre-treated with in water that had been pre-treated with diethylpyrocarbonate (DEPC), as indicated, to inhibit RNase activity. Cells were pelleted, washed twice in cold buffer A (100 mM potassium phosphate (pH 6.5), 0.5 mM MgCl$_2$ in DEPC-treated water) and resuspended in buffer B (1.2 M sorbitol, 100
mM potassium phosphate (pH 6.5), 0.5 mM MgCl₂ in DEPC-treated water) to A₆₀₀nm = 2.0. Sometimes cells were stored at 4°C overnight in buffer B before further processing. To remove the cell wall, cells were incubated for 35 min at 37°C in the presence of 0.4 mg/mL Zymolyase 100T (Seikagaku Corp., Tokyo, Japan) in buffer B supplemented with 0.4% β-mercaptoethanol (Fisher Chemicals). Samples were then cooled on ice to prevent overdigestion during subsequent steps. Spheroplasts were gently pelleted in a tabletop microcentrifuge at medium speed and resuspended in 100 μL fresh ice-cold buffer B. The resulting spheroplast suspension was applied to poly-L-lysine- (SigmaAldrich, cat # P8920) coated 5 mm diameter wells on 12-well glass microscope slides (Electron Microscopy Sciences, Hatfield, PA, cat # 63425-05), using 20 μL spheroplast suspension per well. Spheroplasts were allowed to adhere for 10 min and then the wells were washed twice with 20 μL buffer A and allowed to air-dry at room temperature. Slides were dessicated in ice-cold methanol for 6 min, followed by ice-cold acetone for 30 sec, and allowed to air-dry in the ventilation hood for 1-2 h to remove trace methanol/acetone. Sometimes slides were stored at 4°C for several days before hybridization. All subsequent incubations were carried out inside a humid chamber at 37°C. For hybridization, wells were first equilibrated for 2 min in pre-hybridization buffer (75 mM sodium citrate (pH 7.0), 750 mM NaCl, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.01% Tween, 0.1 mg/mL ssDNA, 0.2 mg/mL E. coli tRNA in DEPC-treated water), aspirated, and incubated with fresh pre-hybridization buffer for 1 h. Pre-hyb buffer was then replaced with hyb buffer, which was pre-hyb buffer supplemented with digoxigenin-labeled oligo(dT)₅₀ probe, and hybridization was carried out for 12-18 h. Subsequently, slides were aspirated and washed in a jar stepwise in the following four solutions: 2xSSC (30 min at RT), 1xSSC (30 min at RT), 1xSSC (30 min at 37°C), and 0.5xSSC (30 min at RT). SSC refers to saline and sodium citrate solution and was made from a 20x stock (3 M NaCl, 0.3 M sodium citrate (pH 7.0) in DEPC-treated water). Afterwards, wells were incubated in 1xPBS supplemented with 1 mg/mL BSA and 0.1% NP-40 twice for 2 min at a time and then blocked in PBS/BSA for 2 h. Primary antibody (mouse α-digoxigenin, 1:50 dilution in PBS/BSA, gift of the Weis lab, UC Berkeley) was applied overnight, then wells were washed three times in 1xPBS and secondary antibody was applied for 1 h (FITC-conjugated donkey α-mouse, 1:100 dilution in PBS/BSA, Jackson Labs, gift of the Drubin lab, UC Berkeley). Finally, nuclei were visualized with DAPI and slides were mounted in Vectashield and imaged on the IX-81OMAC microscope (Olympus) equipped with 100X/NA 1.4 objective and a charge-coupled device camera (Orca II; Hamamatsu). Images were processed in Metamorph imaging software (Molecular Devices) and analyzed in CellProfiler. To calculate mRNA export defect score, the densities of the FITC signal were compared inside the nucleus, defined by the DAPI stain, versus over the area of the entire cell, defined by the diffuse FITC signal present in the majority of the cells that were examined. An export defect score greater than 1 indicates a higher density of mRNA inside the nucleus, indicative of a partial mRNA export defect. The higher the ratio above 1, the greater the mRNA export defect.
CHAPTER 3

Pikostatin is a novel small-molecule inhibitor of Pik1

3.1 Background

3.1.1 Rationale – the need for isoform-specific inhibitors. Functional studies of different phosphatidylinositol 4-kinase isoforms have been hindered by a lack of isoform-specific inhibitors, especially in mammalian cells where conditional alleles are difficult to create and knock-downs can be inefficient and time-consuming to generate. Even in yeast, available temperature-sensitive alleles are not always practical for dissecting certain isoform-specific processes, such as endocytosis defects in pik1<sup>ts</sup> mutants (Walch-Solimena and Novick, 1999; Audhya et al., 2000), because temperature shift can elicit additional physiological effects separate from inactivation of the lipid kinase per se, that may confound interpretation of the outcome observed. The following sections briefly describe my efforts and those of others to inhibit PtdIns kinases in an isoform-specific manner.

3.1.2 Analog-sensitive lipid kinase alleles – the Shokat approach. First attempts at isoform-specific inhibition of PtdIns kinases aimed to apply to lipid kinases a genetic engineering technique that revolutionized protein kinase research. The “bump-hole” method developed by Shokat and colleagues (Bishop et al., 2000) is based on creating an artificially expanded pocket in the active site of any protein kinase by mutating a key gatekeeper residue and thus making the active site accessible to synthetic ATP analogs that contain a bulky substituent (“bump”) that can occupy the enlarged pocket (“hole”) of the engineered kinase, but is too bulky to bind in the active site of the wild-type kinase (or any other normal protein kinase) (Bishop et al., 2000).

Since the time Shokat and colleagues demonstrated the success of this “bump-hole” approach in their study of v-Src (Liu et al., 1998a and 1998b; Bishop et al., 1998), analog-sensitive alleles of many protein kinases have been engineered successfully, including S. cerevisiae Fus3 and Cdc28 (Bishop et al., 2000), Cla4 (Weiss et al., 2000), Pho85 (Carroll et al., 2001), Ime2 (Benjamin et al., 2003), Kin28 and Srb10 (Liu et al., 2004), lpl1 (Kung et al., 2005), Hog1 (Westfall and Thorner, 2007), Kss1 (Patterson et al., 2010), Sch9 (yeast Akt/PKB ortholog) and all three catalytic subunits of yeast PKA (Yorimitsu et al., 2007), among many others.

Analog-sensitive protein kinase alleles have been used to study a variety of processes in fungi, including, but not limited to, mitotic and meiotic cell cycle progression (Bishop et al., 2000; Benjamin et al., 2003), stress response (Westfall and Thorner, 2007; Patterson et al., 2010), transcription (Liu et al., 2004; Lee et al., 2009), and telomere maintenance (Frank et al., 2006).

Because the catalytic domains of lipid kinases have a basic architecture similar to those of protein kinases, with structurally conserved residues in the N-
C-terminal lobes contacting the ATP nucleotide bound in the active site, engineering lipid kinase alleles seemed to be a logical next step. However, when the Shokat group applied the “bump-hole” method to Class I and III PtdIns 3-kinases, mutating the active site gatekeeper residue either abolished kinase activity altogether (Class I PI3KαIle848Ala/Gly) or resulted in mutant alleles that were no more sensitive to inhibition by known “bulky” ATP analogs than the corresponding wild-type kinase (Class III Vps34Ile670Ala) (Alaimo et al., 2005).

3.1.3 Analog-sensitive lipid kinase alleles – my approach. In an independent analysis of a published PtdIns 3-kinase structure (Walker et al., 2000), I identified Val842 in Pik1 as a putative gatekeeper residue (Figure 3.1). I therefore introduced Val842Ala and Val842Gly mutations into otherwise wild-type Pik1 and tested both the in vivo function and the sensitivity to “bulky” analogs of these candidate Pik1-AS mutants (Figure 3.2). Although both Pik1(V842A) and Pik1(V842G) rescued the growth of pik1-83ts cells at restrictive temperature, neither could be inhibited by the three different “bulky” pyrazolopyrimidine-based inhibitors we tested. This result is consistent with the conclusions of Alaimo and et al. (2005) that “a different set of residues in lipid kinases are likely responsible for controlling sensitivity to small molecule inhibitors”.

Figure 3.1. Val842 in Pik1 is a putative gatekeeper residue. Upper panel, the crystal structure of the mammalian p110γ PI3K ATP-binding site (PDB 1E8Z (A), Walker et al., 2000) is shown in the diagram (cyan ribbons), with the bound ATP nucleotide (stick model). The putative gatekeeper residues at the back of the ATP-binding pocket are highlighted in green. Note that the substituents added to the amino group at C6 or to N7 of the purine ring would, in principle, sterically clash with Ile737 or Val842. Lower panel, sequence alignment of p110γ with yeast Pik1. Ile737 in p110γ and Val842 in Pik1 (red box) are also indicated in the crystal structure (arrow). Ribbon diagram was generated in PyMol with the assistance of J.C. Patterson (this lab).

Figure 3.2. Candidate Pik1-AS alleles are functional, but not sensitive to analogs. Serial dilutions of the pik1-83fs strain transformed with pRS316 (EV), pJT2517 (PIK1), pJK01 (PIK1 V842A), or pJK02 (PIK1 V842A). Plates were incubated at 37°C for 3 dy before imaging and the two putative analog-sensitive Pik1 alleles were evaluated for their ability to complement pik1-83fs (JTY2944) at the restrictive temperature in the absence of any inhibitor (right-most panels) as well as in the presence of three different pyrazolopyrimidine-based inhibitors: 1-NM-PP1, 1-NA-PP1, and 2-NM-PP1 (inhibitor concentrations increase to the left). The inhibitor concentrations tested span the range of expected for biological activity.
Figure 3.1 Val842 in Pik1 is a putative gatekeeper residue.

![Image of protein structure showing Val842 and ATP binding site]

Figure 3.2 Candidate Pik1-AS alleles are functional, but not sensitive to analogs.

<table>
<thead>
<tr>
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<th>2-NM-PP1</th>
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<tr>
<td></td>
<td>10μM</td>
<td>25μM</td>
<td>20μM</td>
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<tr>
<td>EV</td>
<td></td>
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<td>EV</td>
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<td>PIK1</td>
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<td>PIK1(V842A)</td>
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<td>PIK1(V842G)</td>
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1-NM-PP1
3.1.4 Synthetic isoform-specific PtdIns 4-kinase inhibitors. Historically, Type I PtdIns 3-kinase inhibitors wortmannin and LY294002 have also been used to inhibit PtdIns 4-kinases, exhibiting selectivity both for yeast Stt4 (Cutler et al., 1997) and, to a lesser extent, for mammalian PI4KIIIα (Balla et al., 2008a). However, the major drawback of these inhibitors is that they inhibit PtdIns 3-kinase activity at a much lower concentration than is needed to inhibit PtdIns 4-kinase activity, with the notable exception of S. cerevisiae PtdIns 3-kinase Vps34p, which is resistant to wortmannin (see Table 3.1 for IC₅₀ values and references). One more PtdIns 4-kinase inhibitor specific for PI4KIIIα has been characterized, phenylarsine oxide (PAO), but its action depends on the presence of two active site cysteine residues that are poorly conserved in the PI4KIIIα protein family (Balla et al., 2008b). Only one inhibitor specific to PI4KIIIβ has been described: PIK93 (Balla et al., 2008b). However, a rotation student in the Thorner lab, Ms. Andrea Pezda, found that it was not an effective inhibitor of S. cerevisiae Pik1. Moreover, PIK93 also inhibits the major mammalian PtdIns 3-kinase isoform, PI3Kγ, in the same concentration range (Balla et al., 2008b). Thus, there is still a need for reliable isoform-specific PtdIns 4-kinase inhibitors.

Table 3.1. IC₅₀ Values for Phosphatidylinositol 4-kinase Inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>Pik1</th>
<th>PI4KIIIβ</th>
<th>Stt4</th>
<th>PI4KIIIα</th>
<th>Vps34</th>
<th>p110α</th>
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<tbody>
<tr>
<td>Wortmannin</td>
<td>resistant¹</td>
<td>0.05 µM²</td>
<td>1 nM¹</td>
<td>0.05 µM²</td>
<td>3 µM¹</td>
<td>4 nM⁵</td>
</tr>
<tr>
<td>LY294002</td>
<td>n.d.</td>
<td>100 µM²</td>
<td>n.d.</td>
<td>50 µM²</td>
<td>50 µM¹</td>
<td>1 µM⁵</td>
</tr>
<tr>
<td>PAO</td>
<td>n.d.</td>
<td>30 µM²</td>
<td>n.d.</td>
<td>1 µM²</td>
<td>n.d.</td>
<td>20 µM⁴</td>
</tr>
<tr>
<td>PIK93</td>
<td>resistant⁶</td>
<td>19 nM³</td>
<td>n.d.</td>
<td>1.9 µM³</td>
<td>n.d.</td>
<td>16 nM³</td>
</tr>
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</table>

¹Strahl and Thorner, 2007; ²Balla and Balla, 2006; ³Balla et al., 2008b; ⁴Han and Kohanski, 1997; ⁵Walker et al., 2000; ⁶Andrea Pezda (this lab), unpublished results.

3.1.5 Pikostatin is a novel and specific inhibitor of Pik1. This chapter characterizes a novel potential inhibitor of Pik1, the Type IIIβ phosphatidylinositol 4-kinase ortholog in the budding yeast S. cerevisiae (Figure 3.3). First, I show that the commercially available compound ST016598, dubbed optimistically "pikostatin", specifically inhibits the growth of cells with reduced dosage of PIK1 both on plates and in liquid media, without affecting Pik1 stability, in vivo protein interactions, or localization of fluorescently-tagged Pik1. Although ST016598 (pikostatin) is only a weak inhibitor of the lipid kinase activity of Pik1 in vitro, pikostatin treatment nevertheless results in a specific, rapid, and reversible depletion of the Pik1-
dependent Golgi body-specific pool of PtdIns4P, without affecting the separate pool of PtdIns4P created at the plasma membrane by Stt4. I also found that a subset of pik1ts alleles exhibit hypersensitivity to pikostatin even under permissive conditions. Finally, I discuss the implications of these findings for our understanding of Pik1 function and outline future research directions to determine the exact mode of action of this novel specific inhibitor of Type IIIβ phosphatidylinositol 4-kinase activity in S. cerevisiae.

Figure 3.3. The chemical structure of pikostatin. Both the schematic representation (left) and stick model (right) of the chemical structure of ST016598 (“pikostatin”, molecular formula C$_{20}$H$_{25}$N$_{2}$O$_{2}$, FW = 339.431 g/mol) is shown. Other proprietary names for this compound include ZINC00131423, CID4665860, and A0748/0034948 (Robert St Onge, personal communication). The compound used for this work was purchased from TimTec LLC (Newark, DE).

Figure 3.3 The chemical structure of ST016598 (pikostatin).
3.2 Results

3.2.1 A chemical genomic screen identifies ST016598 (pikostatin). Two basic approaches to finding direct molecular targets of chemical inhibitors exist: screening for resistance, caused either by mutations that render the target incompatible with the inhibitor or by an increase in the dosage of the target, or screening for sensitivity, caused by a decrease in target dosage, also known as haploinsufficiency.

Recently, genome-wide haploinsufficiency profiling (HIP) has been used in the budding yeast *S. cerevisiae* to identify potential protein targets of small-molecule inhibitors that are essential for growth (for reviews, see Hillenmeyer *et al.*, 2008, 2010), including new targets of previously characterized inhibitors and previously unknown targets of uncharacterized compounds.

In a HIP assay of ~1,200 diploid *S. cerevisiae* strains, where each strain was heterozygous for an essential gene, it was found that a commercially available compound, ST06598, potently and specifically inhibited growth of the *pik1Δ/PIK1* diploid in competitive culture format (Robert St Onge, Stanford Genome Center, personal communication). I set about to determine whether ST016598 (pikostatin) (Figure 3.3) is, in fact, a potentially useful inhibitor of the *S. cerevisiae* phosphatidylinositol 4-kinase Pik1.

3.2.2 Pikostatin inhibits growth of cells with low dosage of PIK1. To corroborate the results of the chemogenetic screen, I first tested the pikostatin sensitivity of various diploid strains, heterozygous for genes involved in lipid metabolism. Of all the strains tested, only the *pik1Δ/PIK1* diploid was sensitive to concentrations of pikostatin as low as 4 µM and it was the only heterozygous strain that failed to grow on 7.5 µM pikostatin (Figure 3.4).

In contrast, cells with low dosage of *STT4*, the other major essential yeast phosphatidylinositol 4-kinase, grew similar to the wild-type control at all pikostatin concentrations, indicating that the effect of pikostatin was specifically dependent on *PIK1* haploinsufficiency. Reduced dosage of the only yeast PtdIns4P 5-kinase, *MSS4*, or the yeast phospholipase C ortholog, *PLC1*, likewise had no effect on pikostatin sensitivity.

Similar to its effect in plate assays, pikostatin inhibited growth in liquid culture (Figure 3.5) with the experimentally determined IC$_{50}$ of $2.00 \pm 0.04$ µM (R=0.9934) for *pik1Δ/PIK1* cells versus $3.19 \pm 0.05$ µM (R=0.9899) for control *PIK1/PIK1* diploids. Moreover, cells exposed to pikostatin in rich YPD medium showed no specific cell-cycle arrest phenotype and were viable up to 6 h after exposure to the drug (Figure 3.6). Viability declined to <1% for both *pik1Δ/PIK1* and *PIK1/PIK1* cells upon exposures longer than 24 h (data not shown).
Figure 3.4. Pikostatin inhibits growth of cells with low dosage of PIK1. Serial dilutions of BY4743 and heterozygous strains JTY3350 (pik1Δ/PIK1), JKY01 (stt4Δ/STT4), JKY02 (mss4Δ/MSS4), JKY03 (frq1Δ/FRQ1), JKY04 (plc1Δ/PLC1) on YPD plates with pikostatin at the concentrations indicated are shown. Plates were incubated at 30°C for 3 dy before imaging. The heterozygous strains were obtained from Research Genetics, Inc. (except BY4743 and JTY3350) and their genotypes were verified by PCR.

Figure 3.4 Pikostatin inhibits growth of cells with low dosage of PIK1.

![Image of growth inhibition assays](image)

Figure 3.5. Pikostatin dose-response and IC<sub>50</sub> in liquid culture. Pikostatin dose-response curves pik1Δ/PIK1 (JTY3350) versus PIK1/PIK1 (BY4743) diploids grown in YPDex liquid culture in glass tubes are shown. Cells were inoculated in varying pikostatin concentrations at A<sub>600nm</sub> = 0.1 and optical density was measured after 6 h of pikostatin treatment. Data shown are the average of three independent replicates with (error bars, SEM). Non-linear regression analysis and IC<sub>50</sub> calculations were performed using GraphPad Prism software.

Figure 3.5 Pikostatin dose-response and IC<sub>50</sub> in liquid culture.
Figure 3.6. Pikostatin does not cause visible cell-cycle phenotypes or lead to loss of viability when exposure is less than 6 h. DIC images of pik1Δ/PIK1 (JTY3350) and PIK1/PIK1 (BY4743) diploids grown to log phase in YPDex liquid media and exposed to 7.5 µM pikostatin for the indicated times. Equal amounts of cells were collected at indicated time points and serially diluted onto YPD plates in the absence of pikostatin to assess viability. The experiment was repeated three times, but only one representative data set is shown.

**Figure 3.6 Pikostatin does not cause visible cell-cycle arrest or lead to loss of viability when exposure is less than 6 h.**

![DIC images of pik1Δ/PIK1 and PIK1/PIK1 diploids grown to log phase in YPDex liquid media and exposed to 7.5 µM pikostatin for the indicated times.](image)

<table>
<thead>
<tr>
<th>PIK1/PIK1</th>
<th>pik1Δ/PIK1</th>
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<tbody>
<tr>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>7.5 µM pikostatin</td>
<td>7.5 µM pikostatin</td>
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</table>

![Equal amounts of cells collected at indicated time points and serially diluted onto YPD plates in the absence of pikostatin to assess viability.](image)
3.2.3 Increasing dosage of PIK1 restores resistance to pikostatin. If Pik1 is the direct target of pikostatin, increasing the dosage of PIK1 should confer pikostatin resistance. Therefore, I examined the effect of adding back phosphatidylinositol 4-kinase activity to pik1Δ/PIK1 cells. As expected, PIK1 heterozygotes with an additional copy of the wild-type PIK1 gene (but not the non-functional pik1-CCaaX allele) expressed from a low-copy plasmid under the control of the native PIK1 promoter now grew similarly to wild-type cells on pikostatin-containing plates (Figure 3.7). In contrast, the expression of additional copies of STT4 failed to rescue pikostatin sensitivity of PIK1 heterozygotes, even when STT4 was mildly overexpressed from the constitutive PRC1 promoter.

Overexpressing PIK1 from the GAL1 promoter rescued the pikostatin sensitivity of pik1Δ/PIK1 cells on galactose medium, but not on dextrose medium, as expected (Figure 3.7). However, expression of PIK1 from the GAL1 promoter in PIK1 heterozygotes (Figure 3.7) or in wild-type diploid cells (data not shown) did not permit cells to grow at doses of pikostatin greater than 8 µM. In contrast, either decreasing (Figure 3.4) or increasing (Figure 3.8) dosage of FRQ1, the calcium-binding protein that helps Pik1 localize to Golgi membranes, had no effect on pikostatin sensitivity of pik1Δ/PIK1 diploids.

Growth in liquid medium in the presence of pikostatin also showed a clear dependence on PIK1 dosage. At intermediate pikostatin concentrations (5.0-7.0 µM), cells bearing only one copy of the PIK1 gene grew very poorly (if at all), cells with two copies of PIK1 showed a significant delay in growth, and cells with three copies of PIK1 grew similar to the DMSO-treated controls (Figure 3.9). Interestingly, the growth inhibition I observed in cells with lower copies of PIK1 was not caused by a change in doubling time once cells achieved exponential growth but was rather due to an increase in the lag time before the onset of growth (Figure 3.9).

Pikostatin sensitivity was specifically correlated to the dosage of the PIK1 gene in both plate and liquid growth assays, indicating that pikostatin somehow targets Pik1 function in vivo. I next examined how pikostatin may affect Pik1 stability, specific activity, protein-protein interactions, or localization.

Figure 3.7. Increasing dosage of PIK1 restores resistance to pikostatin. Serial dilutions of pik1Δ/PIK1 (JTY3350) cells, transformed with plasmids pRSHygB (EV), pJT3156HygB (pPRC1-GFP-STT4), pJT2517HygB (pPIK1-GFP-PIK1), pJT2518HygB (pPIK1-GFP-pik1-CCaaX), pJT2509HygB (pGAL1-GFP-PIK1), or pJT2650HygB (pGAL1-GFP-pik1 D918A) on YP plates with pikostatin at concentrations indicated are shown. The carbon source was galactose, except where otherwise indicated. One YPD plate is shown (right-most panel) to demonstrate that complementation by the pGAL1-GFP-PIK1 construct is abolished under repressive conditions. Plates were images after 5 dy at 30°C, except the YPD plate, which was imaged at 3 dy. The Pik1-CCaaX protein is constitutively tethered to membranes via the lipid modification motif from Ras2p; the D918A mutation renders Pik1 catalytically inactive.
Figure 3.7 Increasing dosage of PIK1 restores resistance to pikostatin.

*pik1Δ/PIK1* cells, transformed with:  
<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>3.75 μM pikostatin</th>
<th>7.5 μM pikostatin</th>
<th>galactose</th>
<th>dextrose</th>
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<tr>
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<tr>
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<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
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<td>pPIK1-GFP-PIK1</td>
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<td>pGAL1-GFP-PIK1</td>
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<td>pGAL1-GFP-pik1(D918A)</td>
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Figure 3.8. Increasing FRQ1 dosage does not restore resistance to pikostatin.  
Serial dilutions of the *pik1Δ/PIK1* (JTY3350) diploid transformed with plasmids pRSHygB (EV), pJT721HygB (*pFRQ1-FRQ1-myc*), pJT720HygB (*pGAL1-FRQ1-myc*), or pJT2517HygB (*pPIK1-GFP-PIK1*) on YPGal plates in the presence (right) and absence (left) of 7.5 μM pikostatin are shown. Plates were incubated at 30°C for 3 dy before imaging. HygromycinB was used at 300 μg/mL for plasmid selection.

Figure 3.8 Increasing FRQ1 dosage does not restore resistance to pikostatin.

*pik1Δ/PIK1* cells, transformed with:  
<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>7.5 μM pikostatin</th>
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<tr>
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<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
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<tr>
<td>pFRQ1-FRQ1-myc</td>
<td><img src="image21" alt="Image" /></td>
<td><img src="image22" alt="Image" /></td>
</tr>
<tr>
<td>pGAL1-FRQ1-myc</td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
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<tr>
<td>pPIK1-GFP-PIK1</td>
<td><img src="image25" alt="Image" /></td>
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Figure 3.9. Increasing *PIK1* dosage improves growth in liquid medium in the presence of pikostatin. Growth of diploid cells with varying dosage of the *PIK1* gene in 96-well plates, monitored in the TECAN microplate reader, is shown. Strains used were 1x*PIK1*: *pik1Δ/PIK1* (JTY3350) with pRSHygB; 2x*PIK1*: *PIK1/PIK1* (BY4743) with pRSHygB; 2.5x*PIK1*: *pik1Δ/PIK1* (JTY3350) with pJT2517HgB (*pPIK1-GFP-PIK1*); 3x*PIK1*: *PIK1/PIK1* (BY4743) with pJT2517HgB (*pPIK1-GFP-PIK1*). One of three representative curves is shown for each strain at each given growth condition. Absorbance was monitored at 600 nm at 15 min intervals over 36 h, with intermittent shaking to keep cells suspended and aerated.

**Figure 3.9 Increasing *PIK1* dosage improves growth in liquid media in the presence of pikostatin.**
3.2.4 **Pikostatin does not affect the stability of Pik1 in vivo.** Since Pik1 is not an abundant protein under standard growth conditions (1,600 molecules/cell; Ghaemmaghami et al., 2003), I first put one chromosomal copy of *PIK1* in a BY4742 diploid under the control of the inducible *GAL1* promoter, as well as introduced an N-terminal GST tag for ease of detection. GST-Pik1 expression was detectable by immunoblot analysis when cells were exposed to galactose (Figure 3.10).

Next, I performed pulse-chase analysis to assess the stability of GST-Pik1 in pikostatin-treated cells (Figure 3.10). First, GST-Pik1 expression was induced from the *GAL1* promoter, then protein synthesis was blocked by the addition of cycloheximide and the amount of GST-Pik1 remaining was followed over time. Over the tested time range of 24 h, GST-Pik1 was equally stable in cells treated with a lethal dose of pikostatin and in DMSO-treated control cells (Figure 3.10).

**Figure 3.10.** Pikostatin does not affect GST-Pik1 stability. Pulse-chase assay for GST-Pik1 stability in the absence (left panel) and presence (right panel) of 16 µM pikostatin. Strain JKY05 (pGAL1-GST-PIK1/PIK1) was grown to mid-exponential phase, then GST-Pik1 expression was induced by galactose addition for 4 h. After 4 h, cycloheximide (CHX) was added at 10 µg/mL to inhibit bulk protein synthesis and samples were taken at the indicated times. Equal numbers of cells were collected for each time point. Total protein was isolated by TCA precipitation and analyzed by SDS PAGE, followed by immunoblotting with α-GST antibodies. Cdc3 was used as a loading control.

3.2.5 **Pikostatin does not affect the specific activity of Pik1 in vitro.** I also assessed the effect of pikostatin on the specific activity of Pik1 *in vitro* (Figure 3.11). Briefly, GFP-Pik1 was immunoprecipitated from whole cell lysates and the Pik1-containing beads were washed and resuspended in the lipid kinase assay buffer. Then equal aliquots of the bead suspension were incubated with PtdIns-detergent micelles in the absence or presence of pikostatin, ensuring that equal amounts of Pik1 were present in each reaction. Reactions were initiated by the addition of ATP, and PtdIns4P formation was followed over time.
In this assay, pikostatin was a weak inhibitor of Pik1 phosphatidylinositol 4-kinase activity under all conditions tested (Figure 3.11). This lack of apparent efficacy might be due to the poor solubility of this compound. At the highest concentration tested (125 µM, 20x more than that necessary to fully inhibit cell growth, see Figure 3.5), pikostatin reduced the initial reaction rate by only 40%. In contrast, an antibody directed against the catalytic domain of Pik1 reduced initial rate by 80% (Figure 3.11), consistent with previous results (Schnieders, 1996).

Another possible explanation for the large discrepancy between the amount of pikostatin needed to inhibit growth in vivo and the amount of pikostatin needed to inhibit Pik1 lipid kinase activity in vitro is that pikostatin itself may be biologically inert and instead may need to be metabolized in cells and thereby converted to its biologically active form, even though pikostatin lacks obvious reactive groups (Figure 3.3). To address this possibility, I compared the specific activity of GST-Pik1 pulled down from lysates prepared either from cells treated with pikostatin or from DMSO-treated control cells.

I observed comparable levels of specific activity in both samples (Figure 3.12). Of course, the active inhibitor may have diffused away under these conditions. Although these data do not completely rule out pikostatin conversion by cellular machinery, they suggest that any modifications pikostatin may receive once it enters the cell do not result in very high-affinity binding of the derivative to Pik1.

**Figure 3.11. Pikostatin is a weak inhibitor of Pik1 PtdIns 4-kinase activity in vitro.** Initial reaction rates are shown for in vitro reactions using GFP-Pik1 (pJT2509 expressed in BJ2168) isolated on Chromotek beads from crude yeast lysate and incubated with mixed PtdIns-detergent micelles, with pikostatin added exogenously. The α-Pik1C antibody was used as a positive control, as it has been previously shown to inhibit Pik1 catalytic activity in vitro (J. W. Thorner, unpublished results). Results shown are the average of four independent experiments, except α-Pik1C and pre-immune composites, which are each the average of three independent experiments. GraphPad Prism software was used for linear regression analysis and initial rate calculations. Error bars reflect how accurately the linear regression fit the kinetic data.
Figure 3.12. Pikostatin treatment does not alter the specific activity of immunoprecipitated Pik1. *In vitro* specific activity of GST-Pik1 isolated on glutathione-agarose beads from crude yeast lysates, prepared from exponentially-growing JKY05 (pGAL1-GST-PIK1/PIK1) cells treated either with DMSO or with 16 µM pikostatin for 2 h, is shown. To obtain specific activity, initial rates were calculated from kinetic data from four separate experiments, plotted against the amount of bead-captured GST-Pik1 determined by immunoblot analysis, and fitted with a linear regression in GraphPad Prism software; the slope of the linear fit was taken as an approximation of specific activity, in arbitrary units.

Figure 3.12 Pikostatin treatment does not alter the specific activity of immunoprecipitated Pik1.
3.2.6 **Pikostatin does not alter normal Pik1 protein-protein interactions.** Three binding partners of Pik1 are known and together they regulate Pik1 partitioning between distinct cellular compartments. Frq1, a small Ca\textsuperscript{2+}-binding N-myristoylated protein, interacts with the N-terminus of Pik1 and assists in Pik1 localization to Golgi membranes (Hendricks et al., 1999; Ames et al., 2000; Strahl et al., 2005; reviewed in Strahl and Thorner, 2007).

The Pik1-Frq1 complex can also be found in the cytoplasm where it interacts with the two yeast 14-3-3 orthologs Bmh1 and Bmh2 via a consensus motif phosphorylated by an as yet unknown yeast protein kinase (Demmel et al., 2008a). The interactions between Pik1, Frq1, Bmh1, and Bmh2 are thought to regulate the distribution of Pik1 between the Golgi compartment and the cytoplasm and between the cytoplasm and the nucleoplasm.

To test whether pikostatin treatment disrupts the binding of Pik1 to Frq1, its major regulatory partner, I performed GST pull-downs from lysates prepared either from cells treated with pikostatin or from DMSO-treated control cells. Equivalent amounts of Frq1 co-purified with GST-Pik1, but not with the GST only control, in the absence and presence of pikostatin (Figure 3.13). Likewise, Frq1 immunoprecipitated from whole-cell lysates co-purified GST-Pik1, regardless of whether cells were naïve or treated with pikostatin (Figure 3.14).

I have not yet performed extensive analysis of how pikostatin may affect the interaction of Pik1 with Bmh1/2 but preliminary results indicated the electrophoretic mobility of Pik1 upon pikostatin treatment is unaltered (data not shown), suggesting that pikostatin does not change the phosphorylation status of Ser396, which has been shown by Demmel et al. (2008a) to result in a gel mobility shift of native Pik1 and to regulate Pik1 interaction with the two yeast 14-3-3 proteins, Bmh1 and Bmh2.
Figure 3.13. GST-Pik1p pulldowns co-purify Frq1p in the absence and presence of pikostatin. GST-Pik1 pull-downs on glutathione-agarose beads were performed in crude yeast lysates prepared from exponentially-growing cells (JKY05 (pGAL1-GST-PIK1/PIK1) or BY4743 transformed with pJT2625HygB (pGAL1-GST-GFP)) treated with pikostatin at the indicated concentrations for 2 h. Upper panel, control GST pull-downs using either GST-GFP (left) or GST-Pik1 (right), without pikostatin treatment. Lower panel, GST-Pik1 pull-downs from pikostatin-treated JKY05 cultures. Lanes marked “input” for lysate and “sup” for post-pull-down supernatant solution represent 5% of total protein used in each pull-down. A non-specific band appears below the Frq1 band in immunoblots using α-Frq1 polyclonal antibodies and is marked with an asterisk.
Figure 3.14. Frq1 immunoprecipitation co-purifies GST-Pik1 in the absence and presence of pikostatin. Frq1 was immunoprecipitated using polyclonal α-Frq1 antibodies from crude yeast lysates prepared from exponentially-growing cells (JKY05 (pGAL1-GST-PIK1/PIK1) transformed with pJT720HygB (pGAL1-FRQ1-myc)) treated with pikostatin at the indicated concentrations for 2 h. Upper panel, control Frq1 immunoprecipitations using either α-Frq1 antibodies (left) or pre-immune serum (right), without pikostatin treatment. Lower panel, Frq1 immunoprecipitations from pikostatin-treated cultures. Lanes marked “input” for lysate and “sup” for post-pull-down supernatant solution represent 5% of total protein used in each immunoprecipitation. Both myc-tagged and untagged Frq1 were present in crude lysate but only the myc-tagged species were visualized by immunoblot to avoid using the same antibodies for immunoprecipitation and detection. Frq1-myc is functionally equivalent to untagged Frq1 (Hendricks et al., 1999).
3.2.7 Pikostatin treatment results in a rapid, reversible depletion of the Golgi body-specific PtdIns4P pool without concomitant Pik1 relocalization. Finally, I examined the localization of Pik1 and related proteins in live cells treated with pikostatin. Normally, in exponentially growing cells, Pik1 localizes to Golgi membranes in distinct punctae that also contain the late Golgi marker Sec7 (Strahl et al., 2005; Gloor et al., 2010). When cells enter stationary phase and the need for constitutive secretion decreases, Pik1 leaves the Golgi and relocates to the cytoplasm, where it is maintained in a complex with Bmh1/2.

As expected, eGFP-Pik1, expressed in diploids under the control of the native PIK1 promoter, localized to Golgi punctae in exponentially-growing cells (data not shown). These punctae persisted upon pikostatin treatment and, in my hands, appeared indistinguishable from the punctae observed in DMSO-treated control diploids, for exposures to pikostatin up to 2 h (Figure 3.15). I observed a similar pattern for the localization of Frq1-GFP in haploid cells in response to pikostatin treatment (Figure 3.16), suggesting that the Pik1-Frq1 complex maintains its normal Golgi localization in the presence of pikostatin.

In addition to assessing Pik1 localization, I also visualized the product of Pik1 lipid kinase activity using a PtdIns4P-specific probe in control and pikostatin-treated cells. A dimer of the isolated pleckstrin homology (PH) domain of the yeast Osh2 protein has been shown to bind both the Pik1-dependent PtdIns4P pool at Golgi membranes and the Stt4-dependent PtdIns4P pool at the plasma membrane (Roy and Levine, 2004). At least one other group has also used the dual localization of the GFP-Osh2\textsuperscript{PHx2} reporter in yeast to monitor Pik1 and Stt4 activity \textit{in vivo} (Zhai et al., 2008). I therefore examined localization of this reporter in pikostatin-treated cells (Figure 3.17).

Interestingly, the reporter was lost from Golgi punctae immediately upon pikostatin addition, with a concomitant increase of fluorescent signal at the plasma membrane, indicating that pikostatin treatment resulted in a rapid depletion of the Golgi PtdIns4P pool specifically, while it did not affect lipid homeostasis at the plasma membrane. This effect was fully reversible, with the reporter returning to Golgi punctae within 30 min after cells were washed with fresh pikostatin-free medium (Figure 3.18).

**Figure 3.15.** eGFP-Pik1 localizes normally in pikostatin-treated cells. The localization of eGFP-Pik1 upon pikostatin treatment in JKY06 (eGFP-PIK1-PIK1) is shown. Cells were grown to mid-exponential phase and 75 µM pikostatin was added directly to the culture for 120 min before imaging. Cells were then immobilized on ConA-coated coverslips and imaged under an IX-81OMAC microscope at 100X magnification with an attached Hamamatsu camera.
Figure 3.15 eGFP-Pik1 localizes normally in pikostatin-treated cells.

Figure 3.16. Frq1-GFP localizes normally in pikostatin-treated cells. The localization of Frq1-GFP upon pikostatin treatment in JTY3650 (BY4741 FRQ1-GFP::KanMX6) is shown. Cells were grown to mid-exponential phase and 75 µM pikostatin was added directly to the culture for 120 min before imaging. Cells were then immobilized on ConA-coated coverslips and imaged under an IX-81OMAC microscope at 100X magnification with an attached Hamamatsu camera.
Figure 3.17. A PtdIns4P-specific probe mislocalizes in a time- and concentration-specific manner in pikostatin-treated cells. The localization of the GFP-OSH2<sub>2xPH</sub> reporter upon pikostatin treatment is shown in BY4743 transformed with pJT1496HygB (<i>pPHO5-GFP-OSH2<sub>2xPH</sub></i>). Cells expressing the reporter from a low-copy CEN plasmid were treated with pikostatin at specified concentrations for the indicated time. Cells were then immobilized on ConA-coated coverslips and imaged under an IX-81OMAC microscope at 100X magnification with an attached Hamamatsu camera.

**Figure 3.17** A PtdIns4P-binding probe mislocalizes in a time- and concentration-dependent manner in pikostatin-treated cells.
Figure 3.18. Golgi PtdIns4P-specific pool is rapidly and reversibly depleted upon exposure to pikostatin. The localization of the GFP-OSH2\(^{2xPH}\) reporter is shown before, during, and after pikostatin treatment in BY4743 transformed with pJT1496HygB (pPHO5-GFP-OSH2\(^{2xPH}\)). Cells expressing the reporter from a low-copy CEN plasmid were grown to mid-exponential phase and immobilized on a ConA-coated coverslip in an open air chamber. Media in the chamber was exchanged manually, using a P1000 pipetteman. The concentration of pikostatin used was 50 \(\mu\)M and drug-free media for the wash-out was volume-adjusted with DMSO.

![Figure 3.18 Golgi body PtdIns4P pool is rapidly and reversibly depleted upon exposure to pikostatin.](image)

### 3.2.8 Certain pik1\(^{ts}\) alleles are hypersensitive to pikostatin.

Since altering key residues in the active site of mammalian PtdIns 4-kinases can change sensitivity to a variety of inhibitors (Balla et al., 2008b), I turned to our collection of temperature-sensitive pik1 alleles (Schnieders, 1996), in hopes of gleaning insight into the structural determinants of pikostatin sensitivity. These alleles were created by error-prone PCR and together they represent a variety of mutations both in the catalytic domain and in the N-terminal regulatory region of Pik1.

To determine whether any of these mutations affect pikostatin sensitivity, I first observed the growth of seven haploid yeast strains containing different pik1\(^{ts}\) alleles on pikostatin plates at the permissive temperature (Figure 3.19). While all pik1\(^{ts}\) alleles we tested were more sensitive to pikostatin than the control PIK1 strain, three alleles were hypersensitive to pikostatin concentrations as low as 2 \(\mu\)M: pik1-11\(^{ts}\), pik1-21\(^{ts}\), pik1-24\(^{ts}\).

Increased pikostatin sensitivity did not correlate with the degree of temperature sensitivity conferred by these mutations (Figure 3.20). This observation suggests that drug sensitivity arises from specific structural changes conferred by
one or more of the mutated residues, rather than from the non-specific destabilization of the entire pik1-ts protein.

Interestingly, only one of the hypersensitive alleles, pik1-21-ts, contained mutations in the C-terminal catalytic domain of Pik1 (M830V and R1023G) (Figure 3.21). The other two hypersensitive alleles, pik1-11-ts and pik1-24-ts, both contained mutations in two different highly conserved residues in the N-terminal lipid kinase unique (LKU) motif (L40S in pik1-11-ts and E81G in pik1-24-ts) (Figure 3.21).

These findings are consistent with structural evidence (Strahl et al., 2007; Lim et al., 2011) that the LKU element in the N-terminal region of Pik1 must interact with the C-terminal catalytic domain to achieve full activation of Pik1 lipid kinase activity. Perhaps pikostatin acts by disrupting the LKU-catalytic domain association and mutations in conserved residues in the LKU motif that are present in certain pik1-ts alleles may additionally destabilize this association, thereby causing hypersensitivity to pikostatin.

Figure 3.19. Certain pik1-ts alleles are hypersensitive to pikostatin. Serial dilutions of JTY2940 (PIK1), JTY2941 (pik1-11-ts), JTY2942 (pik1-21-ts), JTY2947 (pik1-22-ts), JTY2945 (pik1-24-ts), JTY2948 (pik1-41-ts), JTY2943 (pik1-63-ts), and JTY2944 (pik1-83-ts) are shown on YPD plates with pikostatin at indicated concentrations. Plates were incubated at 25°C for 4 dy before imaging. All pik1-ts strains have been generated in this lab (Schnieders, 1996).

Figure 3.20. Pikostatin sensitivity does not correlate with temperature sensitivity. Serial dilutions of JTY2940 (PIK1), JTY2941 (pik1-11-ts), JTY2942 (pik1-21-ts), JTY2947 (pik1-22-ts), and JTY2945 (pik1-24-ts) are shown. Cells were spotted onto YPD plates with pikostatin at indicated concentrations and incubated at 25°C for 4 dy before imaging (upper panels for each strain). The same cells were also spotted onto an extra set of YPD plates and incubated at various temperatures. Growth at

39
25°C and 30°C is shown (lower panels for each strain) and all ts strains failed to grow at 37°C, as expected (not shown). All pik1<sup>ts</sup> strains have been generated in this lab (Schnieders, 1996).

**Figure 3.20** Pikostatin and temperature sensitivity do not correlate.

![Image showing different strains and conditions for pikostatin and temperature sensitivity](image)

**Figure 3.21.** Mutations present in hypersensitive pik1<sup>ts</sup> alleles. Schematic representation of pik1-11<sup>ts</sup>, pik1-21<sup>ts</sup>, and pik1-24<sup>ts</sup>. The LKU motif (yellow), Frq1-binding site (magenta), catalytic domain (green), and relevant mutations are marked. Mutations in conserved residues are indicated by an asterisk.
3.3 Discussion

I have characterized ST06598 (pikostatin), a novel putative inhibitor of Pik1, the PI4KIIIβ ortholog in the budding yeast S. cerevisiae. My data, including the sensitivity of PIK1, but not of STT4, heterozygotes to low micromolar concentrations of pikostatin and the ability of increased dosage of the PIK1, but not of the STT4, gene to rescue pikostatin sensitivity, demonstrate that pikostatin is indeed a specific inhibitor of Pik1 with an experimentally determined IC_{50} = 2 µM, consistent with the results obtained in an as yet unpublished chemogenomic screen conducted at the Stanford Genome Center (Robert St Onge, personal communication).

On the other hand, certain results cloud this simple picture. High-level expression of PIK1 from the GAL1 promoter did not confer a level of resistance greater than that of wild-type cells. Also, pikostatin was only a weak inhibitor of Pik1 catalytic activity in vitro. In view of these concerns, it is difficult to predict whether pikostatin will be a generally useful isoform-specific PI4KIII inhibitor in other organisms. However, Pik1 is not susceptible to either wortmannin or PIK93, which are both potent inhibitors of Type III PtdIns 4-kinases in mammals.

It is possible that pikostatin may inhibit Pik1 activity indirectly, either by regulating its access to its substrate PtdIns or to key positive regulators of its lipid kinase activity. Sec14, an essential yeast PtdIns transfer protein, has been proposed to deliver PtdIns to the cytoplasmic leaflet of Golgi membranes, where Pik1 can then convert it to PtdIns4P (for a review, see Strahl and Thorner, 2007; Bankaitis et al., 2010). It is still poorly understood how Sec14 activity is regulated, except that it can be bypassed by deletion of KES1, one of seven yeast orthologs of mammalian oxysterol binding proteins (OSBPs) and a Golgi PtdIns4P-binding effector (Li et al., 2002). I have not yet tested whether overproduction of SEC14 or loss of KES1 affect the degree of pikostatin sensitivity exhibited by pik1Δ/PIK1 cells or any of my hypersensitive pik1ts alleles.

Pikostatin may also provide insight into the activation of Pik1 lipid kinase activity once Pik1 is recruited to the Golgi body, a process that is not well understood. It has been shown that Frq1 not only assists in Pik1 membrane targeting (Hendricks et al., 1999; Huttner et al., 2003), but also enhances Pik1 lipid kinase activity by promoting the association of the N-terminal lipid kinase unique (LKU) domain of Pik1 with its C-terminal catalytic domain (Strahl et al., 2007; Lim et al., 2011).

Our microscopy and biochemical data show that at least the stability of the Pik1-Frq1 complex itself and its localization to Golgi membranes are preserved in cells treated with pikostatin. In contrast, the Golgi PtdIns4P pool is rapidly depleted during pikostatin treatment, suggesting that pikostatin could block LKU association with the catalytic domain, even when Frq1 is bound, and thereby inhibit its function (Figure 3.22).
Figure 3.22. Current working model for the mode of action of pikostatin, a novel, specific inhibitor of Pik1. The binding of Pik1 to Frq1 and its subsequent association with Golgi membranes is show as a schematic representation (adapted from Lim et al., 2011). For Pik1, the LKU motif (aa 23-98, gray), Frq1-binding site (aa 111-159, magenta), and the catalytic (kinase) domain (aa 642-916, orange) are indicated. For Frq1 in the absence of Ca\(^{2+}\), the N-terminal myristoyl group (red) is shown interacting with the internal hydrophobic cavity (yellow), flanked by a C-terminal \(\alpha\)-helix (\(\alpha_{10}\)). Upon Ca\(^{2+}\)-binding, both the myristoyl group and the C-terminal \(\alpha\)-helix are displaced and an additional hydrophobic patch becomes exposed in Frq1. Frq1-binding then forces a U-turn in the Pik1 polypeptide, bringing the N-terminal LKU motif in close proximity to the C-terminal catalytic (kinase) domain of Pik1. We propose that pikostatin may disrupt this LKU-catalytic association, even when Frq1 remains bound to Pik1.

Of course, other key Pik1-associated factors could be affected. One candidate under my consideration is Sec7, one of three GEFs for Arf1, a small Golgi body-localized GTPase involved in the formation of both clathrin- and COPI-coated vesicles (Springer et al., 1999; Roth, 1999; Jackson and Casanova, 2000). In has been recently shown that, when Sec7 function is compromised, Pik1 is much less efficient at producing PtdIns4P at the Golgi, although it is still able to localize normally to Golgi membranes (Gloor et al., 2010). Thus, Sec7 may directly or indirectly contribute to Pik1 activation in the Golgi compartment and pikostatin may directly or indirectly interfere with this function of Sec7.
In either case, pikostatin could potentially be a useful tool for future studies of the regulation of Pik1 function at the Golgi body. Overall, my partial characterization of the mode of action of pikostatin has opened new avenues to studying the function of Type IIIβ phosphatidylinositol 4-kinase regulation in yeast as well as other organisms.
CHAPTER 4

Efforts to address the role of Pik1 in the yeast nucleus

4.1 Roles for Pik1, Mss4, and Plc1 in nuclear PtdIns metabolism.

Ample claims have been made for the presence of PtdIns4,5P$_2$ inside the nucleus of mammalian cells (Cocco et al., 1987; York and Majerus, 1994; Boronenkov et al., 1998; Watt et al., 2002; Martelli et al., 2005; Irvine, 2003, 2006; Cocco et al., 2010). PtdIns4,5P$_2$ is likely found inside the yeast nucleus as well (Stefan et al., 2002; D. Böke, J. Maier, F. Roelants, and J. Thoerner, unpublished data). However, progress has been slow in identifying and characterizing all of the roles of phosphoinositides in the nucleus.

In animal cells, the enzymes that produce and break down nuclear phosphoinositides remain poorly characterized. Although there is evidence for nuclear entry of both phospholipase C$_{\delta1}$ (Yagisawa, et al., 2002; Okada et al., 2002) and phospholipase C$_{\beta1}$ (Manzoli et al., 1999; Martelli et al., 2000; Cocco et al., 2010), exactly how the substrate for these enzymes (PtdIns4,5P$_2$) is generated inside the nucleus remains unknown.

By contrast, in yeast, it has been demonstrated that the PtdIns 4-kinase Pik1 (Strahl et al., 2005), the sole yeast PtdIns4P 5-kinase Mss4 (Audhya and Emr, 2003), and the yeast PLC$_{\delta}$ ortholog Plc1 (Flick and Thorner, 1993; Stolz et al., 1998) all undergo nucleocytoplasmic shuttling, and thus are likely responsible for the synthesis and breakdown of nuclear PtdIns4,5P$_2$. Moreover, reportedly, one of the four yeast synaptojanin-like phosphatases, Inp52/Sjl2, has been shown to localize to the nucleus (Ooms et al., 2000; reviewed in Strahl et al., 2007).

Inp52/Sjl2 is purportedly involved in an astounding variety of nuclear processes, such as chromatin silencing, transcriptional activation, telomere maintenance, DNA replication, mitotic spindle assembly, and sister chromatid separation during meiosis, based on results obtained in two genome-wide screens for genetic interactions in yeast (Dixon et al., 2008; Fiedler et al., 2009). Interestingly, abnormal mitotic chromosome segregation also has been noted for a temperature-conditional allele of PLC1, plc1-1 (Payne and Fitzgerald-Hayes, 1993) and the loss of either PLC1 (Flick and Thorner, 1993), PIK1 or MSS4 (Rudge et al., 2004) function results in defects in meiosis and sporulation.

It is now clear that some functions of nuclear phosphoinositides are exerted via hydrolysis of PtdIns4,5P$_2$ and the generation of InsP$_3$ and its derived nuclear inositol-polyphosphates. InsP$_6$ is involved in controlling the function of mRNA export factors via formation of a Dpb5-Gle1-InsP$_6$ complex at the nuclear pore (Alcazar-Roman et al., 2006; Weirich et al., 2006; Montpetit et al., 2011). InsP$_7$ serves as a cofactor for the inhibition of the CDK-cyclin complex Pho85-Pho80 by the CDK inhibitor Pho81 (Lee et al., 2008).
Less clear is whether DAG, or PtdIns4P, and/or PtdIns4,5P$_2$ directly support any physiological processes in yeast via specific nuclear effectors that bind these molecules. Nonetheless, the observed ejection of Pik1 from the nucleus under conditions that cause secretory stress (Walch-Solimena and Novick, 1999) and its preferential accumulation in the nucleus upon the depletion of fermentable carbon sources (Demmel et al., 2008a), suggest that the cytoplasmic and nuclear phosphoinositide cycles may be regulated coordinately in response to intra- and extracellular cues.

To address whether Pik1 is responsible for generating the nuclear PtdIns4P that generates the PtdIns4,5P$_2$ that is hydrolyzed to produce InsP$_3$ that is converted to InsP$_6$ necessary for formation of the Dpb5-Gle1-InsP$_6$ complex, I have tested directly whether Pik1 plays a role in mRNA export. I also attempted to apply an unbiased genetic selection for dosage suppressors of the lack of nuclear Pik1 as a means to identify other potential phosphoinositide- or inositol-polyphosphate-binding effectors in the yeast nucleus.

4.2 Directed approach – the role of Pik1 in mRNA export via Dpb5-Gle1

4.2.1 Previous work – Dpb5-Gle1-InsP$_6$ function in the late steps of mRNA export. The export of messenger RNA from the nucleus is tightly coupled to transcription and mRNA processing steps for quality control and centers around the assembly of the messenger ribonucleoprotein (mRNP) export complex that includes the export receptor Mex67-Mtr2 in yeast (conserved as TAP-p15 in metazoans), which then interacts with the components of the nuclear pore complex (NPC) to shuttle the mature mRNP through the central NPC channel concomitant with its uncoating (reviewed in Hocine et al., 2010; Stewart, 2007, 2010; Kelly and Corbett, 2009; Iglesias and Stutz, 2008; Cole and Scarcelli, 2006; Weis, 2002).

Since the Mex67-Mtr2 receptor functions independently of the Ran gradient that controls the import and export of proteins (Weis, 2002; Mattaj and Müller, 2011), the directionality of mRNA transport is imposed by removing export factors as soon as the mature mRNP reaches the cytoplasm (Hodge et al., 1999; Strahm et al., 1999; Cole, 2000; Stewart, 2010).

Directional translocation through the NPC requires extensive remodeling of the mRNP as it exits the nucleus and, in yeast, is achieved through the action of the conserved DExD/H-box RNA helicase Dpb5/Rat8 (Snay-Hodge et al., 1998; Tseng et al., 1998), together with its activator Gle1/Rss1 (Murphy and Wente, 1996; Del Priore et al., 1996, Noble and Guthrie, 1996; Stutz et al., 1997).

Both Gle1 and Dpb5 are concentrated at the cytoplasmic face of the NPC by binding to the nucleoporins Nup42/Rip1 (Stutz et al., 1995; Murphy and Wente, 1996) and Nup159/Rat7 (Hodge et al., 1999; Schmitt et al., 1999), respectively. A co-stimulatory InsP$_6$ molecule is required for the optimal activation of Dpb5 RNA-remodeling activity by Gle1 in vitro and in vivo (York et al., 1999; Weirich et al., 2006; Alcazar-Roman et al., 2006).
Interestingly, InsP₆ production is dispensable under normal growth conditions (plc1Δ, ipk1Δ, ipk2Δ single mutants are viable), but becomes essential under stress conditions (Flick and Thorner, 1993) or when Dbp5-Gle1 function and/or localization is compromised (Miller et al., 2004; Weirich et al., 2006). In particular, double mutants defective both in InsP₆ biosynthesis and in Gle1 localization to the NPC (plc1Δ nup42Δ, ipk1Δ nup42Δ, ipk2Δ nup42Δ) show synthetic defects in growth and in poly(A)⁺ RNA export at elevated temperatures that can be rescued either by DBP5 overexpression or by the expression of DBP5L₃₂₇V or GLE1H₃₃₇R, two dominant alleles that partially bypass the requirement for InsP₆ in mRNA export (Weirich et al., 2006; Alcazar-Roman et al., 2006).

It seems reasonable to propose that the nuclear pools of Pik1 and Mss4 constitute a PtdIns4,5P₂ biosynthetic pathway inside the nucleus that produces substrate for nuclear Plc1, which, ultimately, generates the InsP₆ necessary for the maximal activation of Dbp5 RNA helicase activity (Figure 4.1). I therefore set out to test whether reducing Pik1 or Mss4 activity would lead to functional interactions with nup42Δ similar to those observed for plc1Δ, ipk1Δ, and ipk2Δ mutants.

**Figure 4.1. Proposed role of nuclear Pik1, Mss4, and Plc1 in the generation of IP₆ necessary for mRNA export.** Schematic representation of mRNA export through the nuclear pore via the action of the Dbp5-Gle1-InsP₆ complex is shown. The export-competent mRNP is depicted as a black ribbon and violet ovals represent RNA-binding proteins. Nucleoporins are shown in green, in no particular order, except for Nup42 and Nup159 (labeled), which are shown localizing to the cytoplasmic face of the NPC. The proposed complete InsP₆ biosynthetic pathway is shown to the right of the NPC, with enzymes depicted as colored ovals and different PtdIns species color-coded and labeled (except diacylglycerol, shown in red). Soluble InsPₓ species are represented by pink stars and labeled to show the degree to which they are phosphorylated.
Figure 4.1 Proposed role of nuclear Pik1p, Mss4p, and Plc1p in the generation of IP₆ necessary for mRNA export.
4.2.2 Construction and validation of double mutants in the sensitized nup42Δ background. To test directly how phosphatidylinositol kinases Pik1, Stt4, and Mss4 may contribute to the generation of PtdIns4,5P2 and ultimately the InsP6 necessary for mRNA export, I constructed several strains harboring previously characterized temperature-sensitive alleles of PIK1, STT4, and MSS4, in combination with the sensitizing nup42Δ mutation. For these initial studies, the pik1-83ts allele was selected from our sizeable collection of temperature-sensitive pik1ts alleles (Schnieders, 1996) because it causes the most rapid block to cell doubling upon shift to restrictive temperature and completely inhibits growth after 2 h at 37°C (Schnieders, 1996, p. 83).

I also constructed a plc1Δ nup42Δ double mutant in the YPH499 strain background for use as the positive control. Previous studies of this sort were in the W303 strain background (Weirich et al., 2006). I first tested my plc1Δ nup42Δ control strain for growth and mRNA export at elevated temperatures. The double mutant grew more poorly than either single mutant at 25°C and 30°C, and was inviable at 33°C and above (Figure 4.2). Furthermore, I observed an obvious mRNA export defect in these plc1Δ nup42Δ cells shifted to 37°C, whereas this defect was less pronounced in the plc1Δ single mutant and barely detectable in the nup42Δ single mutant (Figure 4.3), consistent with previous results (Weirich et al., 2006).

Figure 4.2. Double plc1Δ nup42Δ mutants show a growth defect at elevated temperatures independent of strain background. Serial dilutions of JTY3158 (W303 plc1Δ), JKY13 (W303 plc1Δ nup42Δ), JKY12 (W303 nup42Δ), JTY2308 (YPH499 plc1Δ), JKY09 (YPH499 plc1Δ nup42Δ), and JKY07 (YPH499 nup42Δ) onto YPD plates, incubated at the specified temperatures for 4 dy before imaging, are shown. The double mutant in each background was generated by crossing the two single mutants and then subjecting the resulting heterozygous diploid to tetrad dissection.

Figure 4.3. Double plc1Δ nup42Δ mutants show an mRNA export defect at 37°C in the YPH499 strain background. Cultures of JTY2308 (YPH499 plc1Δ), JKY09 (YPH499 plc1Δ nup42Δ), and JKY07 (YPH499 nup42Δ) were shifted to 37°C before fixation, immobilization, and permeabilization. Poly(A)+ mRNA localization was visualized by in situ hybridization with an oligo(dT)50 probe and is shown in green (upper panels). DNA was stained with DAPI (blue, lower panels). Fixed, immobilized, and stained samples were sealed in Vectashield and imaged at 100X magnification on the IX-81OMAC microscope. Arrows indicate nuclei with mRNA retained inside.
Figure 4.2 *plc1Δ nup42Δ* mutants show a growth defect at elevated temperatures independent of strain background.

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<tr>
<th>W303 background</th>
<th>YPH499 background</th>
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<td><em>plc1Δ nup42Δ</em></td>
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Figure 4.3 *plc1Δ nup42Δ* mutants show an mRNA export defect at 37°C in the YPH499 strain background.

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<thead>
<tr>
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<th><em>PLC1+ nup42Δ</em></th>
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<td>DNA</td>
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49
4.2.3 Pik1 and Mss4, but not Stt4, function is important for mRNA export. I next examined the growth and mRNA export phenotypes of the remaining double mutant strains. Contrary to my expectations, pik1-83ts nup42Δ, mss4-102ts nup42Δ, and stt4-4ts nup42Δ double mutants all grew equivalently to the respective pik1-83ts, mss4-102ts, and stt4-4ts single mutants, regardless of whether Nup42 was present or not (Figure 4.4).

I also examined localization of poly(A)+ RNA after a shift to 37°C in pik1-83ts, stt4-4ts, and mss4-102ts cells in the sensitized nup42Δ background, as compared to the nup42Δ mutant alone (Figure 4.5) and to plc1Δ nup42Δ cells (Figure 4.6). Upon gross observation, a modest fraction of pik1-83ts nup42Δ (Figure 4.7), as well as mss4-102ts nup42Δ cells (Figure 4.8), exhibited an appreciable enrichment of poly(A)+ RNA signal inside the nucleus. As judged in this fashion, about 15% of pik1-83ts nup42Δ and 17% of mss4-102ts nup42Δ cells examined showed a visible nuclear poly(A)+ RNA accumulation. In contrast, none of the stt4-4ts nup42Δ cells examined showed any visible mRNA export defect (Figure 4.9). Interestingly, I observed mRNA accumulation in the nuclei of a very small fraction (estimated at <5%) of nup42Δ cells (Figure 4.5), although this proportion of affected cells was much lower than observed in the pik1-83ts nup42Δ, mss4-102ts nup42Δ, and plc1Δ nup42Δ cultures.

To quantify these data in an unbiased manner, I developed an algorithm using the image processing software CellProfiler to calculate an mRNA export defect score for each cell, with a score greater than 1 indicating that an export defect is present. The average mRNA export defect scores calculated for three independent biological replicates for each double mutant strain are shown below the corresponding microscopy images (Figures 4.5-4.9).

Although the distributions of export defect scores were not entirely Gaussian, the large sample sizes of my data sets justified the use of the unpaired two-tailed Student’s t-test to compare the average mRNA export defect scores between different strains and conditions (Figure 4.10). My statistical analysis indicated that the modest mRNA export defect observed in pik1-83ts nup42Δ and mss4-102ts nup42Δ cells after a shift to 37°C was statistically significant (α= 0.05), compared to the same strains maintained at 25°C. The average mRNA export defect score calculated for the stt4-4ts nup42Δ strain was lower at 37°C compared to 25°C, but this difference was not statistically significant at a more stringent confidence level (α= 0.01). Interestingly, when analyzed in this way, the nup42Δ mutant alone exhibited a detectable mRNA export defect, which has not been observed before. I attribute this finding to a higher sensitivity of my image processing method for the detection of partial mRNA export defect that is invisible to the naked eye.

Together, these data suggest that the actions of Pik1 and Mss4, but not of Stt4, may be necessary to create the pool of PtdIns4,5P2 that serves as the precursor to InsP6 biosynthesis in vivo. However, further work is necessary to ensure that the mRNA export defect observed in pik1-83ts nup42Δ and mss4-102ts nup42Δ strains is not due to the presence of the nup42Δ mutation alone.
Figure 4.4. Mutants in phosphoinositide synthesis fail to show an enhanced growth defect in the sensitized nup42Δ background. Serial dilutions of JTY3125 (mss4-102ts), JKY10 (mss4-102ts nup42Δ), JTY3108 (stt4-4ts), JKY11 (stt4-4ts nup42Δ), JTY2944 (pik1-83ts), JKY08A (pik1-83ts nup42Δ spore A), JKY08B (pik1-83ts nup42Δ spore B), and JKY07 (nup42Δ) onto YPD plates, incubated at the specified temperatures for 4 dy before imaging, are shown. The double mutant in each background was generated by crossing the two single mutants and then subjecting the resulting heterozygous diploid to tetrad dissection.

Figure 4.4 Mutants in phosphoinositide synthesis fail to show an enhanced growth defect in the sensitized nup42Δ background.

![Image of yeast plates showing growth at different temperatures](image_url)

Figure 4.5. In situ analysis of mRNA export in the nup42Δ single mutant. Cultures of JKY07 (nup42Δ) were shifted to 37°C for 2 h before fixation, immobilization, and permeabilization. Poly(A)+ mRNA localization was visualized by in situ hybridization with an oligo(dT)$_{50}$ probe and is shown in the two upper panels. DNA was stained with DAPI (lower panels). Fixed, immobilized, and stained samples were sealed in Vectashield and imaged at 100X magnification on the IX-81OMAC microscope. Mean mRNA export defect score for each temperature is shown for two independent experiments as well as for the composite dataset. Error bars indicate SEM. Sample sizes were as follows (25°C /37°C): N$_{rep1}$= 314/106, N$_{rep2}$= 111/67, N$_{composite}$= 425/173.

Figure 4.6. In situ analysis of mRNA export in the plc1Δ nup42Δ double mutant. Cultures of JKY09 (YPH499 plc1Δ nup42Δ) were shifted to 37°C for 2 h before fixation, immobilization, and permeabilization. Poly(A)+ mRNA localization was visualized by in situ hybridization with an oligo(dT)$_{50}$ probe and is shown in the two upper panels. DNA was stained with DAPI (lower panels). Fixed, immobilized, and stained samples were sealed in Vectashield and imaged at 100X magnification on the IX-81OMAC microscope. Mean mRNA export defect score for each temperature
is shown for three independent experiments as well as for the composite dataset. Error bars indicate SEM. Sample sizes were as follows (25°C /37°C): N_{rep1}= 131/77, N_{rep2}= 223/185, N_{rep3}= 225/182, N_{composite}= 579/444.

**Figure 4.7. In situ analysis of mRNA export in the pik1-83<sup>ts</sup> nup42Δ double mutant.** Cultures of JKY08A (pik1-83<sup>ts</sup> nup42Δ spore A) and JKY08B (pik1-83<sup>ts</sup> nup42Δ spore B) were shifted to 37°C for 2 h before fixation, immobilization, and permeabilization. Poly(A)<sup>+</sup> mRNA localization was visualized by *in situ* hybridization with an oligo(dT)<sub>50</sub> probe and is shown in the two upper panels. DNA was stained with DAPI (lower panels). Fixed, immobilized, and stained samples were sealed in Vectashield and imaged at 100X magnification on the IX-81OMAC microscope. Mean mRNA export defect score for each temperature is shown for three independent experiments, each using two separate double mutant spores obtained from tetrad analysis (reps1-2 are from experiment 1, reps4-3 from exp 2, reps5-6 from exp3). Since the mRNA export defect phenotype was weak for both spores in experiment 1, two different composite means are shown on the right, with and without the suspect datasets (reps1-2). Error bars indicate SEM. Sample sizes were as follows (25°C /37°C): N_{rep1}= 440/24, N_{rep2}= 354/310, N_{rep3}= 184/302, N_{rep4}= 304/204, N_{rep5}= 184/305, N_{rep6}= 215/151, N_{compositeReps1-6}= 2,191/1,884, N_{compositeReps3-6}= 887/962.

**Figure 4.8. In situ analysis of mRNA export in the mss4-102<sup>ts</sup> nup42Δ double mutant.** Cultures of JKY10 (mss4-102<sup>ts</sup> nup42Δ) were shifted to 37°C for 2 h before fixation, immobilization, and permeabilization. Poly(A)<sup>+</sup> mRNA localization was visualized by *in situ* hybridization with an oligo(dT)<sub>50</sub> probe and is shown in the two upper panels. DNA was stained with DAPI (lower panels). Fixed, immobilized, and stained samples were sealed in Vectashield and imaged at 100X magnification on the IX-81OMAC microscope. Mean mRNA export defect score for each temperature is shown for three independent experiments as well as for the composite dataset. Error bars indicate SEM. Sample sizes were as follows (25°C /37°C): N_{rep1}= 440/24, N_{rep2}= 354/310, N_{rep3}= 132/207, N_{composite}= 926/541.

**Figure 4.9. In situ analysis of mRNA export in the stt4-4<sup>ts</sup> nup42Δ double mutant.** Cultures of JKY11 (stt4-4<sup>ts</sup> nup42Δ) were shifted to 37°C for 2 h before fixation, immobilization, and permeabilization. Poly(A)<sup>+</sup> mRNA localization was visualized by *in situ* hybridization with an oligo(dT)<sub>50</sub> probe and is shown in the two upper panels. DNA was stained with DAPI (lower panels). Fixed, immobilized, and stained samples were sealed in Vectashield and imaged at 100X magnification on the IX-81OMAC microscope. Mean mRNA export defect score for each temperature is shown for three independent experiments as well as for the composite dataset. Error bars indicate SEM. Sample sizes were as follows (25°C /37°C): N_{rep1}= 168/134, N_{rep2}= 284/233, N_{rep3}= 84/81, N_{composite}= 536/448.
Figure 4.5 *In situ* analysis of mRNA export in the *nup42Δ* single mutant.
Figure 4.6 *In situ* analysis of mRNA export in the *plc1Δ nup42Δ* double mutant.

25°C  37°C

poly(A)+ RNA

DNA

![Graph showing export defect scores for different conditions and replicates.](#)
Figure 4.7 In situ analysis of mRNA export in the \textit{pik1}^{ts} nup42Δ double mutant.

25°C  

poly(A)+ RNA

DNA

37°C

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure47}
\caption{In situ analysis of mRNA export in the \textit{pik1}^{ts} nup42Δ double mutant.}
\end{figure}
Figure 4.8 *In situ* analysis of mRNA export in the *mss4*<sup>ts</sup> *nup42Δ* double mutant.

![Images showing *in situ* analysis](image)

**poly(A)+ RNA**

25°C | 37°C
---|---

**DNA**

25°C | 37°C

![Graph showing export defect score](image)

Export defect score

rep1 | rep2 | rep3 | *mss4-102 nup42Δ* composite

| 25°C | 37°C |
Figure 4.9 *In situ* analysis of mRNA export in the *stt4*<sup>ts</sup> *nup42Δ* double mutant.

25°C  37°C

poly(A)<sup>+</sup> RNA

DNA

![Images of mRNA export at 25°C and 37°C](image)

![Bar chart showing export defect score](chart)

25°C  37°C
Figure 4.10. Summary and statistical analysis of *in situ* mRNA localization results. Mean mRNA export defect score for each temperature is shown for composite datasets representing three independent replicates, except for nup42Δ (two replicates) and pik1-83ts nup42Δ (six replicates). Error bars indicate SEM. Sample sizes were as follows (25°C /37°C): N_{nup42} = 425/173, N_{plc1nup42} = 579/444, N_{mss4nup42} = 926/541, N_{pik1nup42} = 2,191/1,884, N_{stt4nup42} = 536/448. The differences in mean mRNA export defect scores at the two temperatures were significant for all strains when compared using the unpaired two-tailed Student’s t-test with Welch’s correction. GraphPad Prism software was used for all calculations and statistical analyses.
4.3 Genetic selection for dosage suppressors of the lack of nuclear Pik1

4.3.1 Previous genetic screens for synthetic lethality with pik1<sup>ts</sup> alleles identified secretory genes. Several genetic screens for synthetic lethality have been performed with temperature-sensitive yeast pik1 alleles to identify potential components of the cellular processes in which Pik1 acts (Walch-Solimena and Novick, 1999; Nguyen et al., 2005; Sciorra et al., 2005; Demmel et al., 2008a and 2008b). These studies have confirmed the pivotal role of Pik1 in the secretory pathway (Hama et al., 1999) and identified several proteins that have been shown subsequently to be PtdIns4P-binding effectors: Sec2 (Mizuno-Yamasaki et al., 2010), Myo2 (Santiago-Tirado et al., 2011), and Drs2 (Muthusamy et al., 2009a, 2009b; Natarajan et al., 2004, 2009).

Although there is significant overlap in the genes identified in these screens, they cannot be considered comprehensive because of the limited number of strains and conditions tested. For example, these screens failed to identify known negative regulators, such as Kes1 and Bmh1/2. Kes1 is an important yeast PtdIns4P-binding protein that negatively regulates secretion, so that kes<sup>1</sup>Δ partially rescues growth of pik1-101<sup>ts</sup> cells at 37°C (Li et al., 2002; Fairn et al., 2007). Similarly, while combining a pik1-101<sup>ts</sup> allele either with bmh1Δ or with bmh2Δ has no effect, the overexpression of either BMH1 or BMH2 in the pik1-101<sup>ts</sup> background results in a strong growth defect, presumably due to the sequestration of Pik1 in the cytoplasm via its binding to Bmh1/2 (Demmel et al., 2008a).

On the other hand, the overexpression of positive regulators, such as FRQ1 (Hendricks et al., 1999), or effectors, such as SEC2 (Mizuno-Yamasaki et al., 2010) results in a partial suppression of pik1<sup>ts</sup> phenotypes. Interestingly, the results of the latest SGA screen (Demmel et al., 2008b) also include genes whose products localize to the nucleus and are involved in nuclear processes, such as transcription and meiotic recombination, as well as tRNA and ribosome biogenesis. However, I am unaware of any attempts to separate the function of Pik1 in these processes from its now well-characterized essential function in secretion.

4.3.2 Previous work with Pik1-CCaaX and Pik1(Δ10-192) suggests additional essential functions for Pik1 in the yeast nucleus. Previous work from this lab used two differentially-localized Pik1 constructs, Pik1-CCaaX and Pik1(Δ10-192), to demonstrate (Strahl et al., 2005) that localization of Pik1 both to the Golgi and to the nucleus is required for viability (Figure 4.11).

In the Pik1-CCaaX construct, the five native C-terminal residues, Gln-Gly-Ile-Tyr-Ser, have been altered to Cys-Cys-Ile-Ile-Ser, corresponding to the CCaaX box (C is cysteine, a is any aliphatic amino acid, and X is the carboxyl terminal residue), a conserved lipidation consensus motif that was first identified in the yeast plasma membrane protein Ras2 (Bhattacharya et al., 1995). CCaaX-containing proteins undergo a series of posttranslational modifications that promote association with membranes, including S-farnesylation, S-
palmitoylation, endoproteolysis, and carboxyl methylation (Hancock, 2003; Wright and Philips, 2006). This lab has previously shown that Pik1-CCaaX localizes predominantly to Golgi membranes and is excluded from the nucleoplasm, even when it is overexpressed (Strahl et al., 2005).

On the other hand, Pik1($\Delta$10-192) is a derivative of Pik1 with an internal N-terminal deletion that removes a putative nuclear export sequence (NES) as well as the binding site for Frq1, the Pik1-associated protein that directs Pik1 localization to the Golgi body (Hendricks et al., 1999; Strahl et al., 2007). In contrast to Pik1-CCaaX, Pik1($\Delta$10-192) is no longer membrane-localized and accumulates in the nucleus (Strahl et al., 2005).

Neither construct by itself can rescue the inviability of pik1$\Delta$ cells, but viability is maintained when Pik1-CCaaX and Pik1($\Delta$10-192) are simultaneously expressed in pik1$\Delta$ cells. Moreover, the catalytic activity of each construct is necessary for complementation, suggesting that PtdIns4P must be generated by Pik1 both at the Golgi compartment and inside the nucleus for normal cell function (Strahl et al., 2005).

**Figure 4.11** Pik1-CCaaX and Pik1($\Delta$10-192) are previously characterized Pik1 variants that localize differentially.
Figure 4.11. Pik1-CCaaX and Pik1(Δ10-192) are previously characterized Pik1p variants that localize differentially. Schematic representation of the Pik1-CCaaX and Pik1(Δ10-192) proteins, compared to wild-type Pik1 (left) is shown, along with fluorescence microscopy images showing the localization of the corresponding tagged constructs in vivo (right). Pik1-CCaaX is clearly excluded from the nucleus, while Pik1(Δ10-192) accumulates predominantly in the nucleus. Microscopy images were obtained by Strahl et al. (2005) and Pik1 schematics were kindly provided by J. Thorner.

4.3.3 Genetic selection for dosage suppressors – rationale and design. First, given the stringent requirement for PtdIns4P in secretion, I reasoned that globally decreasing Pik1 activity via a crippled pik1ts allele would perturb secretory function without affecting essential nuclear processes that may have a lower requirement for Pik1 activity. Therefore, I designed a genetic background where Pik1p activity could be selectively ablated in the nucleus while being maintained at the Golgi cisternae (Figure 4.12).

In collaboration with an undergraduate student Ms. Roxanne M. Rajaii, I inserted a copy of GFP-pik1-CCaaX under the control of the native PIK1 promoter at the HO locus of a haploid strain (HO-pPIK1-GFP-pik1-CCaaX-HO) where the native PIK1 locus has been replaced with either the pik1-63ts or the pik1-83ts allele (Schnieders, 1996). Upon shift to restrictive temperature, the ts mutant protein would be inactivated and Pik1 function would be supplied at the Golgi body through the action of Pik1-CCaaX, but Pik1 function would be absent in the nuclear compartment, resulting in cell death.

Second, I reasoned that the overexpression of positive regulators or effectors of nuclear Pik1 might rescue the lethality associated with the lack of Pik1 activity in the nucleus, in the same way that the overexpression of FRQ1 and SEC2 can rescue the lack of Pik1 activity at the Golgi body (Hendricks et al., 1999, Mizuno-Yamasaki et al., 2010). Toward this end, I could transform the pik1ts HO-pPIK1-GFP-pik1-CCaaX-HO strain (tester strain) with a yeast genomic library on a high-copy (2 μM) vector and select for plasmid-borne suppressors that are able to restore growth at the non-permissive temperature (Figure 4.12).

Figure 4.12. Design for a genetic selection for dosage suppressors for the lack of nuclear Pik1 activity. The phosphoinositide biosynthesis pathway initiated by Pik1 is shown on the left, to illustrate that in the absence of Pik1, overexpressing downstream effectors may compensate for decreased levels of pathway intermediates. The workflow for the genetic selection for these dosage suppressors is shown on the right. In the tester strain, the integrated Pik1-CCaaX allele will provide the only active form of Pik1 at the restrictive temperature and should supply PtdIns4P at the Golgi body, but be unable to supply PtdIns4P inside the nucleus, allowing for the selection of suppressors at 37°C.
4.3.4 \textit{Pik1(A10-192) was not sufficient to rescue the inviability of the tester strain under non-permissive conditions.} Both \textit{pik1-63ts} and \textit{pik1-83ts}-based tester strains yielded the expected growth phenotypes at the permissive (30°C) and restrictive temperatures (37°C) (Figure 4.13). Pik1-CCaaX, which can supply PtdIns4P only at the Golgi, is the only catalytically active form of Pik1 in these strains at the restrictive temperature, and, therefore, the tester strains did not grow at 37°C, although they grew normally at 30°C, as expected (Figure 4.13).
Serial dilutions of the parental pik1<sup>ts</sup> strains (JTY2944 (pik1-83<sup>ts</sup>) and JTY2943 (pik1-63<sup>ts</sup>); upper panels) as well as of the tester strains resulting from pPIK1-GFP-pik1-CCaaX insertion at the HO locus (RR01 (pik1-83<sup>ts</sup> ho::pPIK1-GFP-pik1-CCaaX) and RR02 (pik1-63<sup>ts</sup> ho::pPIK1-GFP-pik1-CCaaX); lower panels) are shown at permissive (left) and restrictive (right) temperatures. Both tester strains are unable to grow at 37°C, as expected, because Pik1-CCaaX cannot supply the nuclear function of Pik1 and is therefore unable to complement temperature-sensitive growth.

Next, I sought to rescue the lethal phenotype of the pik1-83<sup>ts</sup> HO-pPIK1-GFP-pik1-CCaaX-HO tester strain at 37°C by introducing "catalytically active" Pik1(Δ10-192), because such a rescue had been demonstrated in prior work from this lab (Strahl et al., 2005). However, despite numerous attempts to obtain this expected phenotype, I was unable to observe complementation of the tester strain by Pik1(Δ10-192) at 37°C, even when Pik1(Δ10-192) expression was driven to a high level by the inducible GAL1 promoter (Figure 4.14).

Figure 4.14. Introducing Pik1(Δ10-192) fails to rescue the temperature-sensitive growth of the pik1-83<sup>ts</sup> tester strain. Upper panels, the expression of myc-Pik1(Δ10-192) driven by the GAL1 promoter is detectable 5 h after galactose addition in the pik1-83<sup>ts</sup> tester strain (RR01) transformed with pJT2541 (pGAL1-myc-pik1(Δ10-192)). A prominent band corresponding to a breakdown product appears directly below full length myc-Pik1(Δ10-192) and is marked with an asterisk. Pgk1 levels were monitored for loading control. Lower panels, serial dilutions of the pik1-83<sup>ts</sup> tester strain (RR01) transformed with pRS315 (EV), pJT2541 (pGAL1-myc-pik1(Δ10-192)), or pJT2651 (pGAL1-myc-pik1(Δ10-192 D918A)) are also shown on both dextrose and galactose media. Two separate overnight cultures were used for each plasmid to produce duplicate dilutions.
I speculated that this might be due to inadequate expression of Pik1-CCaaX in the tester strain. Indeed our α-GFP antibody could not detect the low level of GFP-Pik1-CCaaX protein expressed from the PIK1 promoter at the HO locus in whole-cell lysates (data not shown). Work is currently in progress to determine whether GFP-Pik1-CCaaX can be detected in preparations of Frq1 immunoprecipitated from lysates that were prepared from the tester strain.

Next, I tested whether additional Pik1-CCaaX overexpressed from a plasmid would rescue the inviability of the tester strain at 37°C, in conjunction with plasmid-borne overexpressed Pik1(Δ10-192) (Figure 4.15). Although the expression of both myc-Pik1-CCaaX and myc-Pik1(Δ10-192) could be detected by immunoblot upon galactose induction in liquid medium, I observed no growth of the tester strain transformed with both plasmids on galactose-containing plates incubated at 37°C (Figure 4.15).
Figure 4.15. Pik1(Δ10-192) and Pik1-CCaaX together fail to rescue the temperature-sensitive growth of the pik1-83<sup>ts</sup> tester strain. *Upper panels,* the expression of myc-Pik1-CCaaX and myc-Pik1(Δ10-192) driven by the GAL1 promoter is detectable 4.5 h and 6 h after galactose addition in the pik1-83<sup>ts</sup> tester strain (RR01) transformed with pJT2541 (pGAL1-myc-pik1(Δ10-192)) or pJT2523 (pGAL1-myc-pik1-CCaaX). A prominent band corresponding to a breakdown product appears directly below full length myc-Pik1(Δ10-192) and is marked with an asterisk. Pgk1 levels were monitored for loading control. *Lower panels,* serial dilutions of the pik1-63<sup>ts</sup> (RR02) and pik1-83<sup>ts</sup> (RR01) tester strains transformed with different combinations of pRS315 (EV LEU2), pRS316 (EV URA3), pJT2541 (Δ10-192), and pJT2523 (CCaaX) are also shown on both dextrose and galactose media. No growth was observed on galactose medium at either temperature.

Finally, I checked whether overexpressing Pik1(Δ10-192) was toxic in our strain background (YPH499). However, wild-type diploids transformed with pGAL1-myc-pik1(Δ10-192) grew similarly to the empty-vector control under both inducing (galactose) and repressing (dextrose) conditions (Figure 4.16).
Figure 4.16. Pik1(Δ10-192) overexpression is not toxic. Serial dilutions of BY4743 transformed with pRS315 (EV), pJT2541 (myc-pik1(Δ10-192)), pJT2651 (myc-pik1(Δ10-192 D918A)), or pJT3219 (GFP-PIK1) are shown on SCDex –Leu (left) and SCGal –Leu (right) media. Plates were incubated at 30°C for 3 dy before imaging. Expression from all plasmids was driven by the inducible GAL1 promoter; the D918A mutation renders Pik1 catalytically inactive (Strahl et al., 2005).

Thus, although I was able to reproduce the lethal phenotype of cells lacking nuclear Pik1 activity, I was unsuccessful in reproducing the complementation in trans that was previously observed with Pik1-CCaaX and Pik1(10-192) (Strahl et al., 2005), even when both constructs were overexpressed from separate plasmids.
One goal of my dissertation research was to attempt to dissect the poorly characterized role of Pik1 in the nucleus, which is separate from its well-defined role in the Golgi compartment where it creates a pool of PtdIns4P necessary for protein and lipid transport along the secretory pathway. I focused my efforts in two areas: creating new tools for studying the nuclear compartment-specific function of Pik1 by trying to develop ways to specifically, quickly, and reversibly inhibit Pik1 catalytic activity (Chapter 3) and using both a directed and an unbiased approach in an attempt to determine what the function(s) of Pik1 in the yeast nucleus may be (Chapter 4).

5.1. Pikostatin (ST016598). Since isoform-specific inhibition had been achieved for many protein kinases by engineering the kinase active site to accept “bulky” ATP analogs, I first attempted to construct an analog-sensitive Pik1 allele by using structural and sequence similarity of the PtdIns 4-kinase catalytic domain to the catalytic domain of well-characterized PtdIns4,5P2 3-kinases (PI3Ks). However, the failure of our Pik1 gatekeeper mutants to respond to ATP analogs, together with similar results for PtdIns 3-kinase gatekeeper mutants obtained by Alaimo and colleagues (Alaimo et al., 2005), indicate that structural conservation between protein and lipid kinases is not precise enough to make the “bump-hole” technology directly applicable to the lipid kinase active site.

Therefore, I focused my efforts on exploring the mode of action for a novel apparent Pik1 inhibitor, pikostatin (ST016598). My results showing PIK1 haploinsufficiency upon pikostatin challenge and rescue of pikostatin sensitivity dependent on PIK1 dosage indicate that pikostatin specifically inhibits Pik1 function in live cells. Moreover, in vivo microscopy studies of PtdIns4P distribution in pikostatin-treated cells suggest that pikostatin acts to reduce Pik1 activity at the Golgi body, thus depleting the pool of PtdIns4P in the Golgi compartment that is necessary for secretion. Additional studies tracking the progress of standard secretory cargoes, such as chitin synthase Chs3 and invertase Suc2, along the secretory pathway will be needed to confirm that pikostatin treatment results in a general secretion block.

It would also be interesting to see whether elevating Golgi body PtdIns4P levels via overexpression of SEC14 or deletion of KES1 would result in pikostatin resistance. Since PIK1 is epistatic to SEC14, I would expect to see resistance when the negative regulator KES1 is deleted, but not when SEC14 is overexpressed. Conversely, either ablating the function of Sec14 via a sec14ts allele or enhancing the function of Kes1 via overexpression should result in increased pikostatin sensitivity. In fact, a selection for dosage suppressors that confer resistance to intermediate doses of pikostatin (~7.5 µM) when expressed in the sensitive pik1Δ/PIK1 diploid may help identify additional positive regulators of Pik1 activity in the Golgi compartment as well as Golgi body-localized PtdIns4P-binding effectors.
5.2 Nuclear Pik1 Perspectives: mRNA export. Building on previous work demonstrating that Pik1 activity was indispensable both at the Golgi body and in the nucleus (Strahl et al., 2005), I also attempted to determine what the function of nuclear Pik1 may be, using both a directed and an unbiased approach. My studies of mRNA export in cells where Pik1, Stt4, Mss4, or Plc1 function were ablated in the presence of a sensitizing nup42Δ mutation indicate that both Pik1 and Mss4 may be important for mRNA export, but Stt4 is most likely dispensable, as stt4-4ts nup42Δ cells shifted to the restrictive temperature failed to exhibit an increased accumulation of mRNA in the nucleus, compared to permissive conditions, in clear contrast to pik1-83ts nup42Δ, mss4-102ts nup42Δ and plc1Δ nup42Δ double mutants.

The observed mRNA export defect in cells lacking Pik1 and Mss4 activity was much less pronounced than that previously detected in cells lacking the PLC1 gene, probably because, after Pik1 or Mss4 shut-off, the hydrolysis of residual PtdIns4,5P2 by Plc1 was sufficient to generate adequate levels of InsP3 and InsP6 in the majority of cells during the 2 h incubation at 37°C.

Additional experiments are necessary to confirm that the mild mRNA export defect observed in pik1-83ts nup42Δ cells is due to a decrease in intracellular InsP6 and a concomitant decrease in Dbp5p ATPase activity. My model predicts that no mRNA export defect would be seen in pik1-83ts nup42Δ cells carrying DBP5L327V or GLE1H337R, dominant alleles that bypass the requirement for InsP6 in mRNA export (Weirich et al., 2006).

I have not resolved the question of whether local production of InsP6 inside the nucleus is necessary for optimal Dbp5 activity. I predict that the nuclear Pik1(Δ10-192) construct, but not the cytoplasmic Pik1-CCaaX, should rescue the mRNA export defect of pik1-83ts nup42Δ cells when the proteins are expressed at a near-native level.

It is not clear why I was unable to reproduce the trans-complementation previously observed (Strahl et al., 2005) between Pik1-CCaaX and Pik1(Δ10-192) alleles. Experiments are in progress to determine whether Pik1-CCaaX is expressed at low levels, as expected, in my pik1ts HO-pPIK1-GFP-pik1-CCaaX-HO strains. I would like to test whether Pik1-CCaaX alone could provide a sufficient amount of Golgi PtdIns4P to support normal secretory function.

First, I plan to assess the ability of Pik1-CCaaX to rescue the viability of sec14ts and frq1ts cells at restrictive temperature. These mutants are specifically defective in secretion and this lab has previously shown that their inability to grow at the restrictive temperature can be suppressed by the overexpression of PIK1 (Hama et al., 1999; Hendricks et al., 1999). If Pik1-CCaaX can rescue the temperature-sensitive growth of sec14ts and frq1ts cells when Pik1-CCaaX is overexpressed from the inducible GAL1 promoter, but not when it is expressed at low levels from the native PIK1 promoter, such a result would explain the lack of rescue that I observed when I attempted to complement the ts growth of my tester strains by introducing the Pik1(Δ10-192) construct.
Second, if Pik1-CCaaX indeed specifically complements the essential function of Pik1 at the Golgi, then I would predict that the localization of a PtdIns4P-binding probe to the Golgi compartment should be completely restored *in frq1-t15* mutants at restrictive temperature when Pik1-CCaaX is expressed in these cells.

The genetic selection for dosage suppressors of the lack of nuclear Pik1 that I have designed does not directly address the question of whether nuclear PtdIns4P and/or PtdIns4,5P₂ specifically (versus soluble inositol polyphosphates or DAG) play a role in the nucleus, other than as precursors to InsP₆, which is necessary for mRNA export. This question could be addressed by using *plc1Δ* cells, which are viable at 30°C on rich medium with glucose as the carbon source, and then establishing conditions to selectively deplete nuclear phosphoinositides by inhibiting only the nuclear function of Pik1 either by a compartment-specific drug, by using differentially-localized Pik1 alleles (Strahl *et al.*, 2005), or by targeting a phosphoinositide phosphatase to the nucleus, and then examining the consequences. A screen could then be conducted in the *plc1Δ* background under one of the above conditions to identify specifically only PtdIns4P- and PtdIns4,5P₂-binding nuclear effectors.

### 5.3 Nuclear Pik1 Perspectives: mRNA processing

Pik1, Mss4, and Plc1 may also participate in other aspects of nuclear biology, such as mRNA 5’-end capping, splicing, 3’-end processing, and polyadenylation that are all necessary for assembling an export-competent mRNP (Fasken and Corbett, 2009). In mammalian cells, pre-mRNA processing factors are enriched in nuclear speckles (Spector, 1993; Lamond and Spector, 2003) which also contain PtdIns4,5P₂ (Boronenkov *et al.*, 1998) as well as the enzymes necessary for its production and break-down (Boronenkov *et al.*, 1998; Martelli *et al.*, 2005).

In particular, phosphoinositides appear to be important for the activity of at least two mammalian nuclear speckle-resident proteins, Aly/Ref (Okada *et al.*, 2008) and Star-PAP (Mellman *et al.*, 2008), involved in splicing and polyadenylation, respectively. Aly/Ref (Zhou *et al.*, 2000) is the mammalian ortholog of yeast Yra1 (Portman *et al.*, 1997; Strasser and Hurt, 2000), an mRNA export adaptor that is loaded onto nascent pre-mRNA by splicing machinery. Aly/Ref is recruited to nuclear speckles via binding to nuclear PtdIns4,5P₂ and/or PtdIns3,4,5P₃ and is subsequently phosphorylated and activated by nuclear Akt (Okada *et al.*, 2008). Aly/Ref phosphoinositide-binding mutants no longer localize to nuclear speckles, resulting in bulk poly(A)⁺ mRNA export defect and growth arrest (Okada *et al.*, 2008).

Similarly, mammalian Star-PAP, a speckle-resident poly(A) polymerase necessary for processing mRNAs induced in response to oxidative stress, is activated by nuclear PtdIns4,5P₂ *in vitro* and *in vivo* (Mellman *et al.*, 2008). Depleting Star-PAP or its binding partner PIPKⅰα, a nuclear PtdIns4P 5-kinase homologous to yeast Mss4, inhibits 3’-end cleavage and processing of *HO-1* mRNA encoding heme oxygenase (Mellman *et al.*, 2008). While PIPKⅰα is highly similar to the sole yeast PtdIns4P 5-kinase Mss4, there is no known Star-PAP homolog in *S. cerevisiae* or
any other fungi, so it is unlikely that this mechanism for phosphoinositide-sensitive polyadenylation in response to oxidative stress operates in yeast.

However, two non-canonical poly(A) polymerases in S. cerevisiae, Pap2/Trf4 and Trf5, are part of a complex that localizes to the nucleolus and is involved in RNA surveillance and quality control (TRAMP complex) (Anderson and Wang, 2009; Houseley and Tollervey, 2006). Interestingly, a PtdIns4,5P₂-binding probe (GFP-PLC₅₉Phx²) has previously been observed to co-localize with the Nsr1 nucleolar marker (D. Böke, J. Maier, F. Roelants, J. Thorner, this lab, unpublished results), but, so far, no effort has been made to determine whether PtdIns4,5P₂ in the nucleolus might affect the activity of the TRAMP complex or any other nuclear protein.

5.4 Nuclear Pik1 Perspectives: chromatin remodeling and transcription. The remaining known mammalian phosphoinositide-binding effectors all influence chromatin structure (Yu et al., 1998; Zhao et al., 1998; Gozani et al., 2003).

Of special interest is the PtdIns5P-binding protein ING2 (Inhibitor Of Growth) that modulates the activity of histone acetylases and deacetylases in response to p38-mediated stress response signaling (Gozani et al., 2003; Jones et al., 2006). In particular, p38 MAPK phosphorylates and inhibits a nuclear PtdIns5P 4-kinase PIPKIβ, which works together with a Type I PtdIns 4-phosphatase to regulate PtdIns5P levels inside the nucleus. Under stress conditions, PIPKIβ activity is inhibited, resulting in PtdIns5P accumulation and the recruitment of ING2 to chromatin (Gozani et al., 2003; Jones et al., 2006).

Three ING1 homologs are known in S. cerevisiae: Yng1, Yng2 and Pho23 (Loewith et al., 2000). Pho23 associates with Rpd3, one of two major yeast histone deacetylases (HDAC) (Rundlett et al., 1996), and is necessary for Rpd3 HDAC activity in vivo (Loewith et al., 2001). Similarly, Yng1 and Yng2 function as part of histone acetyltransferase (HAT) complexes, NuA3 and NuA4, respectively (Loewith et al., 2000; Howe et al., 2002; Nourani et al., 2003). Moreover, Yng1 has been shown to promote NuA3 HAT activity in vivo, in a way that influences transcription of a specific subset of yeast genes (Taverna et al., 2006).

The recruitment of Yng1 to chromatin depends both on its ability to bind trimethylated lysine 4 of histone 3 (H3K4me3) via its plant-homeodomain (PHD) finger (Martin et al., 2006) as well as on critical determinants outside of the PHD finger (Chruscicki et al., 2010). It would be interesting to see how phosphoinositides may influence the nuclear localization of the yeast ING proteins (Pho23, Yng1, and Yng2) and/or their ability to bind chromatin, thereby regulating the access of various histone-modifying complexes to their nucleosome substrates.

PtdIns5P also activates the nuclear ubiquitin ligase Cul3-SPOP in a p38-dependent manner in mammalian cells (Bunce et al., 2008). Interestingly, two HECT-domain (Homologous to the E6-AP Carboxyl Terminus; Huibregtse et al., 1995) ubiquitin ligases, Rsp5 (Rodriguez et al., 2003; Neumann et al., 2003) and Tom1 (Utsugi et al., 1995; Duncan et al., 2000), localize to the nucleus and have
been implicated in mRNA export in *S. cerevisiae*. It would be interesting to see whether their activities may be regulated by nuclear phosphoinositides.

### 5.5 Nuclear Pik1 Perspectives: nuclear architecture and organization

Phosphoinositides may also participate in the transcriptional activation or silencing of genes tethered at the nuclear periphery. The eukaryotic nucleus is highly organized spatially, with telomeres and other silent chromatin domains clustering at the nuclear periphery, but excluded from the immediate vicinity of nuclear pore complexes (NPCs) where actively transcribed genes are concentrated, coupling transcription and mRNA export (Taddei *et al.* 2010). This type of organization suggests that distinct subdomains exist at the nuclear periphery, some for silencing and some for active transcription, and phosphoinositides in the inner nuclear membrane are perfectly poised to regulate gene expression in these subdomains.

Interestingly, an acidic protein Esc1 mediates Sir4-dependent relocation of telomeres to the nuclear periphery during G1 phase (Andrulis *et al.*, 2002; Taddei *et al.*, 2004), raising the possibility that phosphoinositides could form a barrier around NPCs, repelling Esc1 and associated silent chromatin. Interestingly, the protein involved in S phase telomere tethering to the nuclear envelope, Mps3 (Schober *et al.*, 2009), also has an acidic domain at its N-terminus, which is exposed to the nucleoplasm. The mechanism by which actively transcribed genes are recruited to NPCs remains obscure and the docking factors at the NPC necessary for gene recruitment are not known. Theoretically, phosphoinositides and/or phosphoinositide-binding effectors may be key contributors in this process.

### 5.6 Nuclear Pik1 Perspectives: a role in sporulation

The role of nuclear Pik1 in meiosis and sporulation remains unexplored, in spite of the fact that *pik1*<sup>ts</sup>, *ms4*<sup>ts</sup>, and *plc1*Δ (Flick and Thorner, 1993; Rudge *et al.*, 2004) mutants are all defective in meiosis and sporulation. Moreover, both Sec14, a PtdIns/PtdCho transfer protein, and Spo14, a PtdIns4,5P<sub>2</sub>-dependent phospholipase D, are also required for sporulation, indicating a central role for phosphoinositides in this process (Rudge *et al.*, 2004). Two interesting questions remain regarding this role.

First, it is not clear whether nuclear localization of Pik1 and Mss4 is necessary for normal sporulation. This possibility could be tested using the Psr-Mss4 fusion protein (Miller *et al.*, 2004), which was able to support viability, but purportedly was excluded from the nucleus, and therefore should not be able to support nuclear PtdIns4,5P<sub>2</sub> production necessary for normal sporulation.

Second, Flick and Thorner noted that *plc1*Δ / *plc1*Δ diploids fail to sporulate (Flick and Thorner, 1993), indicating that not only PtdIns4,5P<sub>2</sub> but also inositol polyphosphates produced upon the hydrolysis of PtdIns4,5P<sub>2</sub> by Plc1 are necessary for sporulation. It would be interesting to explore which specific inositol polyphosphate species is required and what protein targets they affect.

Finally, it appears that HDAC activity, specifically that of Rpd3, and not that of Hda1 (Rundlett *et al.*, 1996), is required for sporulation. Sporulation is completely
absent in \textit{rpd3\Delta / rpd3\Delta} diploids (Dora \textit{et al.}, 1999), as opposed to homozygous \textit{hda1\Delta} mutants (Briza \textit{et al.}, 2002), which sporulate, but produce spores with abnormal spore membranes. In view of reports that connect Pho23, one of the three ING1 orthologs in yeast (Loewith \textit{et al.}, 2000), to Rpd3 HDAC activity (Loewith \textit{et al.}, 2001), it is conceivable that nuclear phosphoinositides may regulate Pho23 localization and/or activity during sporulation, thereby recruiting Rpd3 to a specific subset of genes, which must be transcriptionally repressed, in order for sporulation to occur.
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