DEFINING THE ESSENTIAL AND ENDOCYTIC FUNCTIONS OF PAN1 IN *SACCHAROMYCES CEREVISIAE*

by
Mary Katherine Bradford

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ABSTRACT

Endocytosis is the process by which cells internalize extracellular and transmembrane cargo from their environment. The process requires dozens of proteins to be recruited in a specific order to the site of endocytosis on the plasma membrane. These proteins bind the cargo to be internalized, form a coat on the membrane, deform the membrane using the force of actin polymerization to create the nascent vesicle, and finally pinch off the vesicle to allow for the cargo to be trafficked within the cell. Clathrin-mediated endocytosis (CME) is the most studied endocytic mechanism; both the order of events and the proteins involved are well conserved in eukaryotic organisms. Due to quick reproduction times and ease of genetic manipulations, *Saccharomyces cerevisiae* is an ideal model system to gain insight into the precise mechanisms that are required to coordinate CME.

The timeline of CME is separated into four steps based on the proteins that are recruited during that stage: early coat, late coat, actin polymerization, and scission/uncoating. Although much is known about what proteins are recruited and when, there are still open questions concerning how each stage transitions to the next. The scaffold protein Intersectin/Pan1 may function as a coordinator between endocytic stages due to its multiple interactions with several proteins in the early, late, and actin polymerization stages. Intersectin and Pan1 are essential for life, making research of their function technically difficult. In this dissertation, a degron allele of Pan1 (Pan1-AID) was constructed to acutely deplete cells of Pan1 protein and observe the resulting phenotypes *in vivo*. Using Pan1-AID, it was discovered that Pan1 is critical for strengthening interactions during three transitions in CME: early coat to late coat, actin regulators to

actin machinery, and coat/actin machinery to the plasma membrane. In addition, the regions and domains that are critical for Pan1's endocytic and essential functions have been defined. Portions of Pan1 were found that can support viability, but not endocytosis, suggesting that these functions can be separated and Pan1, like Intersectin, may have additional roles in the cell.

Thesis Committee Members

Beverly Wendland (Thesis Advisor)

Kyle Cunningham (Thesis Reader)

Rejji Kuruvilla

Steve Farber

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CHAPTER 1: INTRODUCTION

Discovery of endocytosis

Eukaryotic cells are surrounded by a lipid bilayer called a plasma membrane; this thin, flexible, dynamic membrane is what separates the extracellular from the intracellular environment of the cell. The surface of the plasma membrane must protect the cell from environmental stressors, relay informational signals, and selectively choose what extracellular matter to allow passage into the cell and when. The simple act of cargo internalization by the plasma membrane has evolved into an incredibly diverse, highly regulated, complex set of pathways collectively called endocytosis. Endocytosis is critical for several cellular homeostatic functions including nutrient uptake, signal transduction, regulation of membrane composition, cell adhesion and migration, and cell polarity. Pathogens, such as bacteria and viruses, have evolved ways hijack a host cell's endocytic machinery in order to induce their own internalization into the cytoplasm. Through careful observation, researchers have learned from pathogens how to use endogenous endocytic routes to induce the proper uptake and trafficking of therapeutics to specific organelles. Over the past 80 years, the various endocytic mechanisms have been meticulously characterized, yet there are still many remaining open questions.

Membrane internalization was first visualized in the 1930s from movies of phagocytic cells, but it wasn't until the development of electron microscopy that endocytosis was truly described in the 1960s by Thomas Roth and Keith Porter (Roth & Porter, 1964). Their careful observations of electron micrographs of yolk uptake by mosquito oocytes found that vesicles containing yolk were created at curved, dense regions of the plasma membrane. These regions deformed into vesicles that were surrounded by a "bristle coat" which fell off after vesicle scission, but before the

internalized vesicles fused together. Just a decade later, biochemical studies led to the discovery that this bristle coat was made of a protein called clathrin (Pearse *et al.,* 1975; Crowther *et al.,* 1976). Around the same time, electron microscopy was again used to discovered altered trafficking pathways in a disease model (Goldstein and Brown, 1973; Brown and Goldstein, 1974; Goldstein *et al.,*1979). In this work, Michael Brown and Joseph Goldstein set out to characterize the cellular phenotypes of the Familial Hypercholesterolemia (FH) disorder. They discovered that Low Density Lipoprotein (LDL) binds to a specific cell surface receptor for its uptake and it is this receptor that is mutated in FH. These findings not only led to therapeutics for FH, but it was the first description of receptor-mediated endocytosis; Goldstein and Brown were awarded the Nobel Prize in 1985 for their discoveries.

Between the 1980s and 1990s, much was learned about the components of the endocytic vesicles, but the next wave of discoveries came with the development of GFP-labeling and live-cell imaging. In 1999, clathrin-mediated endocytosis (CME) was visualized for the first time in real-time by tagging clathrin with GFP (Gaidarov *et al.,* 1999). Live-cell imaging revolutionized the endocytic field and much has been learned since about the highly regulated order of events that are critical for proper internalization by the plasma membrane.

Endocytosis and Human Disease

Like all necessary basic cellular functions, disruption of endocytosis causes disease. Misregulated endocytic trafficking pathways are common phenotypes found in various cancers and neurodegenerative disorders (reviewed in Zhang, 2009 and Mosesson

et al, 2008). Additionally, some endocytic proteins have been implicated in specific diseases, such as Parkinson's Disease, Huntington's Disease, Alzheimer's Disease, and Downs Syndrome. Thus, it is imperative to understand the complexities of CME in order to find suitable therapeutic targets.

The main characteristic of cancer cells is their ability to evade death and proliferate freely. These actions require that cells alter the regulation of signaling pathways important for growth, death, migration, and adhesion. One method some cancers use to regulate these pathways is via altering the trafficking of proteins involved (Mosesson *et al,* 2008). Defective endocytic trafficking is a newly characterized hallmark of malignant cancer cells. Many cancers have been found to have aberrantly expressed endocytic proteins, including clathrin heavy chain, cortactin, Eps15, ITSN1, and HIP1/HIP1/R among many others (Ross *et* al., 1998; Bridge *et* al., 2001; Argani *et al.,* 2003; Rao *et al.,* 2003; So *et al.,* 2003; Buday *et al*., 2007; Gu *et al*., 2015). By controlling endocytosis, cancer cells can reduce down regulation of growth signaling by delaying the internalization of the cell surface receptors. Additionally, cancer cells can upregulate migration and control adhesion via endocytosis. Further characterization of how cancer controls and regulates endocytosis to promote its metastasis is essential for finding therapeutic targets.

Neurons depend on proper synaptic vesicle (SV) trafficking in order to relay signals; a key hallmark of neurodegenerative diseases is dysfunction of SV trafficking due to misregulation of exo- and endocytosis. Three neurodegenerative disorders: Alzheimer's Disease (AD), Parkinson's Disease (PD), and Huntington's Disease (HD) are characterized by accumulation and aggregation of misfolded protein fragments; the

cleavage and processing of these fragments depends on the endocytic trafficking. Although the exact mechanism of pathogenesis is still murky, it is thought that the protein aggregates alter endocytic dynamics leading to alterations of SV recycling and, eventually, neurodegeneration.

In PD, the accumulation of α-synuclein (α-syn) in dopaminergic neurons leads to neurodegeneration; as a result, PD patients experience resting tremors, hypokinesia, rigidity, and postural instability. The α-syn aggregates in intracellular inclusions called Lewy Bodies. One common mutation found in PD patients is in the *LRRK2* gene, a protein that has been implicated in the regulation of SV cycle and can interact with some endocytic proteins (reviewed in Esposito *et al.,* 2011). It is thought that these LRRK2 mutant proteins may contribute to the altered SV dynamics found in PD neurons.

HD is an autosomal dominant neurological disease, which manifests as involuntary movement, cognitive impairment, and emotional disturbance. The disease is caused by mutation of the Huntingtin (Htt) protein that expands an N-terminal polyglutamine stretch leading to altered biochemical and biophysical properties (reviewed in Qin and Gu, 2004). The N-terminus of mutated Htt is cleaved and processed though the endosomal-lysosomal system and these fragments localize to intracellular inclusions and aggregates (Kegel *et al*., 2000). These inclusions and aggregates are thought to contribute to misregulation of endocytosis and neurodegeneration. Additionally, HIP1, Huntingtin Interacting Partner 1, is directly involved in CME as a coat/adaptor protein and binds to wild-type, but not mutated, Htt (Kalchman *et al.*, 1997; Wanker *et al.*, 1997; Engqvist-Goldstein *et al*., 1999; Metzler *et al.,* 2001; Waelter *et al.,* 2001).

AD is a progressive neurodegenerative disease that is the leading cause of dementia. Two hallmarks of AD are the accumulation of extracellular amyloid-β (Aβ) plaques composed of a fragment of the amyloid-precursor protein (APP) and intracellular neurofibrillary tangles of the tau protein. However, increasing evidence supports that prior to the formation of tangles, extracellular and intracellular soluble amyloid-β oligomers (oAβ) are the toxic species of Aβ which are responsible for synaptic dysfunction and neuronal death in AD, although the initial mechanisms leading to dysfunction are unclear (Minano-Molina *et al.,* 2011; Benilova *et al.*, 2012). Misregulation of endocytosis is a common phenotype seen in the brains of AD patients and mouse models of AD. Further, several endocytic proteins, including PICALM (Yap1802), Intersectin, Sla1, and Inp52 have been implicated in AD (Cataldo *et al*., 2000; Dunckley *et al.,* 2006; Wilmot *et al.,* 2008; Harold *et al*., 2009; Thomas *et al*., 2011; Treusch *et al.,* 2011). Recent work has shown a role for CME in Aβ-induced growth cone collapse, which leads to neurodegeneration and memory impairment; inhibition of CME can reverse these effects *in vivo* (Kuboyama *et al.,* 2015).

Endocytosis and trafficking of misfolded proteins seem intimately linked in these disorders, but the exact mechanism leading to disease and neuronal dysfunction is unknown. Further research of this endocytic misregulation is crucial for understanding the pathogenesis of these disorders.

Clathrin-mediated endocytosis

Although cells have evolved several mechanisms for endocytosis, the most well conserved and well studied is Clathrin-Mediated Endocytosis (CME). As the name

implies, CME is differentiated from other modes of endocytosis by the presence of clathrin—a protein that forms a cage-like structure around the nascent vesicle. CME requires the coordination of over 60 proteins that arrive to plasma membrane at the endocytic site or "patch" in a specific order to recognize the cargo that is to be internalized, induce membrane curvature to form the vesicle, and bind together to form a coat around the nascent vesicle. The process begins with clathrin, adaptor proteins, and cargo converging at the site; adaptor proteins are so called because they link the clathrin coat to the cargo and the membrane. Next, several other coat proteins are recruited, many of which bind and/or regulate actin polymerization. Once the coat and cargo are stable, actin, which binds the coat proteins, is polymerized and the force created is used to pull the membrane into the cell. Scission factors pinch off the vesicle at the neck near the plasma membrane and the now completed vesicle is propelled into the cytosol. Various events mediate the un-coating of the vesicle. Finally, the vesicle delivers its cargo within the cell and the endocytic proteins are recycled back to the membrane for another round of endocytosis.

Conservation of CME from yeast to humans

All eukaryotic cells use CME as one of many methods for internalization of the plasma membrane and cargo. *Saccharomyces cerevisiae* is an ideal model system for genetic and molecular studies of CME as both the order of events, as well as the proteins involved, are well conserved from yeast to humans. Only three main differences have been found between the yeast and mammalian CME: the adaptor proteins, necessity of actin polymerization, and scission mechanisms.

CME starts with the recruitment of clathrin, cargo, early-arriving proteins (Eps15/Ede1), and adaptors to the site of endocytosis on the plasma membrane. In mammals, the heterotrimeric adaptor complex AP-2 is the "core" adaptor protein and is crucial for most CME events as it binds numerous cargo proteins (reviewed in Sorkin, 2004). However, AP-2, while present in yeast, is not essential for CME, does not bind clathrin, and only has one known cargo: K28 killer toxin (Huang *et al.,* 1999; Yeung *et al.,* 1999; Carroll *et al.,* 2009). Instead, yeast use four adaptor proteins that are partially redundant: Yap1801/2 (CALM/AP180s) and Ent1/2 (Epsins) (Maldondo-Baez *et al.,* 2008; Burston *et al.,* 2009). As there are hundreds of various cargos that need to be internalized either specifically or in bulk, it is necessary for cells to have both specific and non-specific adaptor proteins. Both yeast and mammals have accessory adaptor proteins, including FCHO1/2/Syp1, HIP1R/Sla2, and Sla1/Cin85 (Wesp *et al.,* 1997; Yang *et al.,* 1999; Henry *et al.,* 2002; Howard *et al.,* 2002, Piao *et al.,* 2007, Reider *et al.,* 2009).

The Arp2/3 complex is at the center of actin polymerization in both yeast and mammalian CME. The proteins that activate the Arp2/3 complex are conserved: N-WASP/Las17, Syndapin/Bzz1, Intersectin/Pan1, and the Type 1 Myosins/Myo3/5. While the actin polymerization process is very similar in yeast and mammals, it is the necessity of this step that differs: it is essential for all CME events in yeast, but not in mammals. If actin polymerization is disrupted in yeast cells either by mutating actin regulatory proteins or the addition of the actin depolymerizing drug Latrunculin A, then CME is either greatly reduced or arrested (Kaksonen et al 2003, 2005; Newpher and Lemmon, 2006). It is thought that the inward force of actin polymerization is necessary for the

membrane to overcome the outward force of turgor pressure created by the cell wall; removal of the cell wall to relieve this pressure results in an increase in CME (Aghamohammadzadeh and Ayscough, 2009; Basu *et al.,* 2014). In mammalian cells, actin polymerization is only necessary when internalizing a large cargo or in regions of increased pressure, such as between tight contacts of cells (Saffarian *et al.,* 2009; Collins *et al.,* 2011). Thus, while the mechanisms of the actin polymerization step are very well conserved, the necessity varies depending on species and circumstances.

The final difference between yeast and mammals is in the mechanism of fusion of the opposing membranes to close the vesicle and pinch it off from the plasma membrane. Both yeast and mammals use amphiphysins (yeast: Rvs161/167) that contain BAR domains to bring the opposing membranes together (Wigge *et al.,* 1997; Lombardi and Riezman, 2001). In mammals, dynamin uses GTP hydrolysis to pinch off the membrane (Sever *et al.,* 2000). However, the contribution of the yeast dynamin homologue, Vps1, to CME is not certain. It is thought that amphiphysins and the force of actin polymerization may be enough to pinch off the vesicle, but growing evidence hints that Vps1 may coordinate with Rvs167 in this step (Nothwehr *et al.,* 1995; Yu and Cai, 2004; Nannapaneni *et al.,* 2010; Smaczynska-de Rooij *et al.,* 2010).

Although there are some differences between yeast and mammalian CME events, they are similar enough that much can be learned from doing experiments in yeast and applying this knowledge to mammalian research.

Regulation of Endocytosis—Remaining Questions

Although much has been learned in the past six decades about the components and order of events of CME, there are still several remaining questions regarding the exact mechanisms of regulation at each step of endocytosis. CME can be broken down into four stages based on the arrival of certain protein modules: early coat, late coat, actin polymerization, and scission/uncoating. We know many of the proteins that arrive during each stage and how they bind each other, but why do they arrive at that time? The first stage, the early coat, has a highly variable lifetime at the endocytic patch, but the other three stages have a more predictable lifetime. It is hypothesized that there is some checkpoint that occurs before the wave of recruitment of the final three coats--what protein interactions are important for the sequential recruitment? What protein(s) signal that the patch is ready to mature? Does the amount or identity of the cargo play a role in the transition? N-WASP/Las17, the strongest actin nucleator in CME, is recruited with the late coat yet remains inhibited until some unknown signal indicates that the membrane is ready to be invaginated. During this invagination, why do some proteins remain at the plasma membrane, while others internalize at the tip of the expanding vesicle? What interactions are important for maintaining the coat structure during the actin polymerization?

We hypothesize that there are one or more key coordinators that act as scaffolds throughout CME to help transition between stages. These scaffold proteins would need to have several binding regions for many protein interactions. Flexibility would also be necessary in order to properly transition and change interactions during each stage. Two proteins stand out as possible candidates for these coordinator roles: Eps15/Ede1 and

Intersectin/Pan1. Recently, Ede1 was proven to be critical for the initiation of CME and formation of the early coat (Boeke *et al.,* 2014). And while its presence is important for the late coat, it is unable to properly regulate all of those interactions. This dissertation provides evidence that Pan1 is the key coordinator for the late coat. Thus, Eps15/Ede1 and Intersectin/Pan1 may function together to initiate and form the early coat, recruit the late coat, and provide overall structural organization to the entire CME clathrin coat.

Intersectin

The mammalian protein Intersectin (ITSN) is so named because it has roles in both endocytosis and cellular signaling and thus is thought to act at the intersection between these pathways. ITSN was first discovered in a screen for Src homology 3 (SH3) containing proteins in *Xenopus laevis* oocytes; upon further characterization it was found to contain 5 SH3 domains and 2 Eps15-homology (EH) domains, both of which are commonly found in endocytic machinery (Yamabhai *et al.*, 1998). Based on this analysis, ITSN was proposed to be a CME component. Later work found that ITSN is involved in several cellular signal transduction pathways independent of its endocytic scaffolding function (reviewed in Tysba *et al.,* 2011). In addition, ITSN has been implicated in cancer and several neurological diseases, including Alzheimer's Disease, Downs Syndrome, and Huntington's Disease (Scappini *et al.,* 2007; Hunter *et al.*, 2011).

ITSN is a multi-domain scaffold protein that contains two N-terminal EH domains, a central coiled-coil region, five SH3 domains, and a variably expressed actin-regulatory region (Figure 1.1) (Guipponi *et al.,* 1998; Yamabhai *et al.*, 1998). These regions allow ITSN to localize to clathrin-coated vesicles and interact with numerous

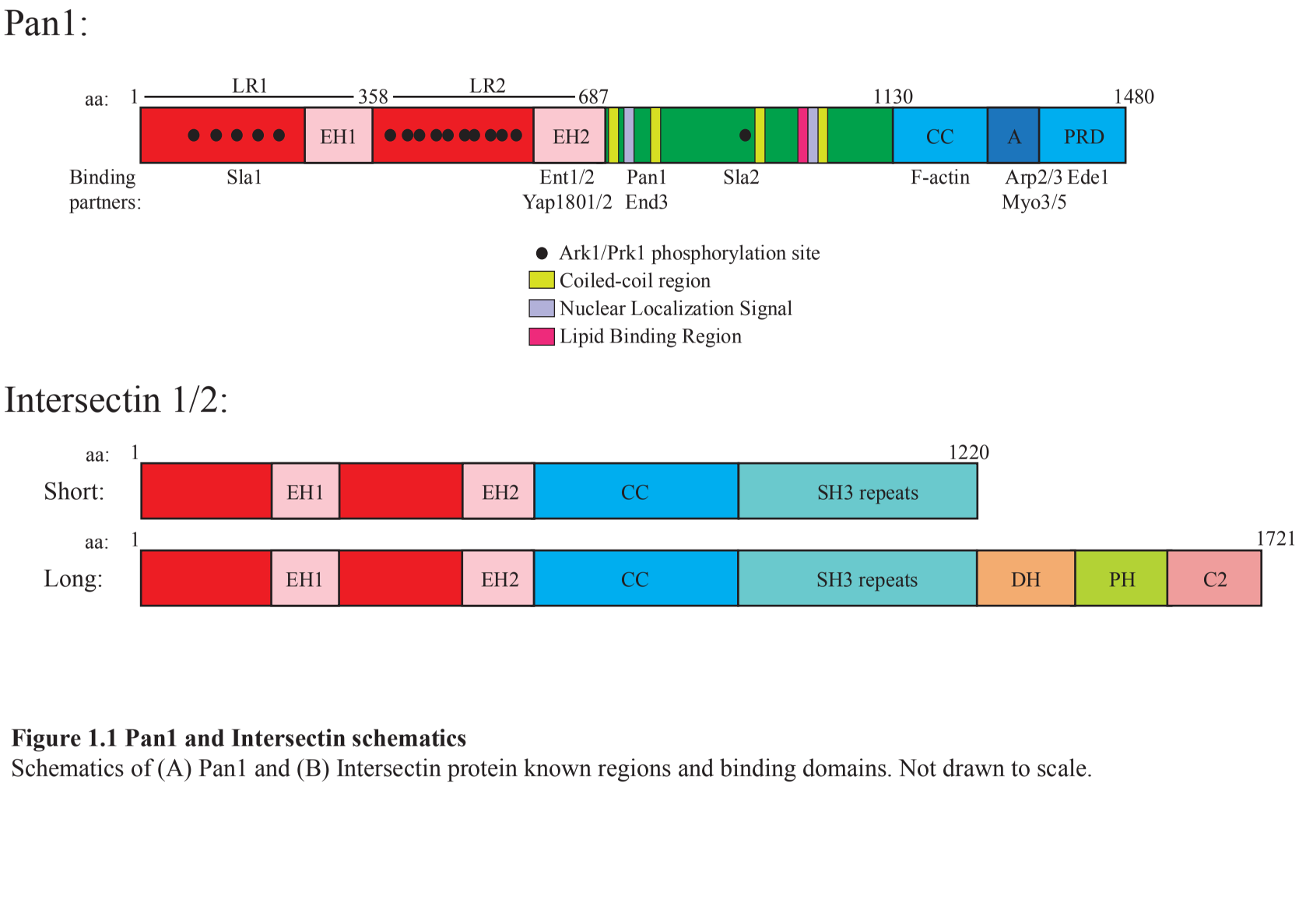


Figure 1.1 Pan1 and Intersectin schematics
Schematics of (A) Pan1 and (B) Intersectin protein known regions and binding domains. Not drawn to scale.

other endocytic proteins (Adams *et al.,* 2000). Through its EH domains, ITSN binds Asn-Pro-Phe (NPF) tripeptide motif containing proteins, such as the adaptor proteins Epsin1/2; the coiled-coil region binds another coat protein Eps15 (Yamabhai *et al.,* 1998; Sengar *et al.,* 1999). The five SH3 domains in ITSN, which bind proline-rich sequences, provide the most broad functional binding regions as they interact with proteins involved in signaling, cytoskeletal organization, and membrane trafficking (Li *et al.,* 2005, Tsyba *et al.,* 2011). Although there are several putative serine/threonine phosphorylation sites throughout ITSN, the function of these sites has yet to be investigated (Ballif *et al.,* 2004; Tweedie-Cullen *et al.,* 2009). Finally, ITSN has three C-terminal regions that are present in the long isoforms: Dbl homology domain (DH), Pleckstrin homology domain (PH), and C2 domain (Guipponi *et al.,* 1998; Sengar *et al.,* 1999). ITSN activates Cdc42 through coordination of the DH and PH domains and Cdc42 then stimulates WASp to promote the activation of Arp2/3 (Hussain *et al.*, 2001). Both the PH and the C2 domain can bind ITSN to lipid membranes (Rizo and Sudhof, 1998).

ITSN homologues are found in several eukaryotic organisms, including *Drosophila melanogaster* (Dap-160), *Xenopus laevis* (X-ITSN), *Caenorhabditis elegans* (ITSN-1), *Cryptococcus neoformans* (Cin1), and *Saccharomyces cerevisiae* (Pan1) (Roos and Kelly, 1998; Yamabhai *et al.,* 1998; Okamoto *et al.,* 1999; Sengar *et al.,* 1999; Rose *et al.,* 2007). ITSN is only essential in some cell types and organisms due to its redundancy with other ITSN genes; mammals have two ITSN genes: ITSN1 and ITSN2, both of which have a long and a short isoform. While ITSN2 is ubiquitously expressed in all tissues, ITSN1-long is primarily found in neurons (Guipponi *et al.,* 1998; Pucharcos *et al.,* 1999, 2000, 2001). A double knockout of ITSN1/2 is lethal and 1 out of every 8

ITSN1 knockouts dies shortly after birth. Interestingly, the viable pups have no gross phenotypic differences besides a reduced rate of synaptic vesicle endocytosis (Yu *et al.,* 2008). ITSN is a scaffolding protein that can form oligomers and is therefore sensitive to changes in concentration; thus both overexpression and reducing ITSN levels beyond the optimal concentration can cause similar effects. Overexpression and silencing of ITSN in cells leads to a disruption of endocytosis and silencing ITSN leads to an increase in early endosome size (Sengar *et al*., 1999; Pucharcos *et al.,* 2000; Martin *et al.,* 2006; Thomas *et al.,* 2009).

The numerous binding regions of ITSN allow it to serve as a scaffolding protein not only in endocytosis, but also in several signaling pathways (reviewed in Tysba *et al.,* 2011). In its endocytic role, ITSN is critical for receptor tyrosine kinase (RTK) signaling because CME is important for down regulation of activated RTKs by removal of the receptor from the cell surface. However, ITSN can also act independently of endocytosis in stimulation of the Elk-1 transcription factor (Mohney *et al.,* 2003; Patel *et al.,* 2013). Through a yet uncharacterized pathway, ITSN's EH domains can activate JNK-dependent signaling cascades (Adams *et al.,* 2000; Mohney *et al.,* 2003). Finally, in *D. melanogaster*, ITSN has been shown to directly interact with and activate aPKC and PAR6, which are part of a cell polarity complex important for asymmetric cell divisions (Nishimura *et al.,* 2007; Chabu *et al.,* 2008; Gonczy, 2008). Although this function hasn't been found in mammalian cells yet, ITSN can interact with Numb, which is a PAR complex activator (Nishimura *et al.,* 2006).

ITSN is a large, complex protein, yet the acute details of all of its functions are difficult to study due to redundancies and its essential nature. Pan1 shares many of the

same binding regions, interacting partners, and functions, and is therefore an ideal model system for a more careful study of this family of scaffolding proteins.

Implications of Intersectin in Human Disease

ITSN1 is a key component of endocytosis in neurons, thus any mutations in ITSN could lead to misregulation of SV recycling, a common phenotype of neurological disorders. Two hallmarks of AD, endocytic misregulation and an increase in early endosome size, resemble phenotypes seen in overexpression and silencing of ITSN. (Cataldo *et al*, 1997, 2000; Pucharcos *et al*., 1999; Predescu *et al.,* 2003; Koh *et al*., 2004; Marie *et al*., 2004; Yu *et al.,* 2008; Treusch *et al*., 2011). Although there is currently no direct link between ITSN1 and AD, ITSN1 transcript and protein levels are altered in AD patients (Dunckley *et al.,* 2006; Wilmot *et al*., 2008). In humans, the ITSN1 gene is on chromosome 21 and is trisomied in Downs Syndrome patients, leading to increased protein expression (Pucharcos *et al.,* 1999). All DS patients develop early-onset AD-like neuropathology; therefore, proteins expressed from genes located on the triplicated region of chromosome 21, such as ITSN, may be causal to AD and DS. Neurons in DS patients, like AD, have large early endosomal compartments, which is characteristic of ITSN1 overexpression (Pucharcos *et al*., 1999; Cataldo *et al*., 2000, 2004; Predescu *et al*., 2003; Yu *et al*., 2008).

Independent of its role in endocytosis, ITSN1 is involved in several signaling pathways. Through activation of the JNK-MAPK pathway, ITSN1-S has been implicated in increasing the aggregation of mutant Htt protein in HD (Mohney *et al.,* 2003; Scappini *et al.,* 2007). Thus, ITSN could be a potential therapeutic target for HD, AD, and DS.

The short isoform of ITSN1 is overexpressed in glioblastoma cells and there is evidence that ITSN1-S is essential for glioblastoma cell proliferation through its role it the RAF/MEK/ERK signaling cascade (Gu *et al.,* 2015). The precise roles of ITSN are difficult to study because there are two ITSN genes in mammals that both express numerous isoforms. The double knockout of ITSN1/2 is lethal, and the redundancy of the proteins makes experiments with single knockouts difficult to interpret. Research on more simple organisms, such as yeast, can be done to characterize the role of the ITSN family proteins.

Pan1

In 1992, Pan1 was erroneously identified as a poly(A) ribonuclease binding protein; this was corrected in 1996, the same year that Pan1 was found in two separate genetic screens to be important for actin cytoskeletal organization and endocytosis (Sachs and Deardorff, 1992; Boeck *et al.,* 1996; Tang and Cai, 1996, Wendland *et al.,* 1996). Over the last two decades, Pan1's role in CME has been defined by its abundant binding interactions to other endocytic proteins and its localization dynamics at the endocytic patch. The precise details of Pan1's mechanistic functions have yet to be described due to the fact that Pan1 is essential, making deletion experiments impossible.

The two genetic screens that identified Pan1 used temperature sensitivity (*ts*) to find candidate protein alleles. The Cai lab screened for mutants that were synthetically lethal with a *CDC28* mutation (Tang and Cai, 1996). The *pan1-4* allele was found and described to be required for normal actin cytoskeletal organization; this disorganization let to abnormal bud growth and defective cytokinesis. The Emr lab screen found mutants

that had inefficient uptake of the lipophilic dye, FM4-64 (Wendland *et al.,* 1996). This screen found the *pan1-20* allele, which in addition to its endocytic defects also displayed abnormal actin phenotypes, including loss of polarity of cortical actin patches and thicker actin bundles.

Pan1 binds several other proteins in the early, late, and actin polymerization stages of CME. At its N-terminus, Pan1 has two EH domains that are 72% similar, but only the second can bind to the adaptor proteins Ent1/2 and Yap1801/2 via their NPF motifs (Salcini *et al.*, 1997; Paoluzi *et al.*, 1998; Wendland and Emr, 1998; Wendland *et al.*, 1999; Maldonado-Baez *et al.*, 2008). The C-terminus of Pan1 has been implicated in binding to the early arriving protein, Ede1 via its coiled-coil region (S. Barker and B. Wendland, unpublished observations). Through a yet unspecified domain in the first of Pan1's N-terminal long repeat (LR) regions, Pan1 interacts with Sla2 (Toshima *et al.*, 2005; 2007). Pan1 has the strongest interactions with proteins in the late coat. Pan1 forms a complex with Sla1 and End3, which bind its N-terminus and central region, respectively (Tang *et al.*, 1997; 2000; Whitworth *et al.*, 2014). While the Pan1-End3 interaction is stable at the patch and in the cytosol, Ark1/Prk1 phosphorylation of both Sla1 and Pan1 leads to their dissociation at the uncoating step of CME (Tang *et al.*, 2000; Zeng *et al.*, 2001; Toshima *et al.*, 2005; 2007, Boeke *et al.*, 2014). Both proteins are dephosphorylated by Glc7 to allow their participation in the next round of CME (Zeng *et al.*, 2007). Pan1 can also bind itself and form homo-dimers and homo-oligomers via its central region (Miliaras and Wendland, 2004; Pierce *et al.*, 2013). Finally, Pan1 can both bind F-actin via a WH2-like motif, activate Arp2/3 through its acidic domain, and bind Myo3/5 in the proline-rich domain (PRD) (Duncan *et al.*, 2001; Barker *et al.*, 2007,

Toshima *et al.,* 2005). There are two putative regions of Pan1 that may be involved in an undiscovered Pan1 function: nuclear localization signals (NLS) and a Hog1-dependent stress-induced phosphorylation site (Kaminska *et al.,* 2005, Reiter *et al.,* 2012). Further research is required in order to confirm their validity and discover if/how these domains function in endocytosis or Pan1's essential role.

Like ITSN, Pan1 is a large protein with several diverse binding regions and domains; it contains two N-terminal EH domains, a central coiled-coil region, and a C-terminal actin regulatory region (Figure 1.1). Although Pan1 does not contain the five C-terminal SRC homology-3 (SH3) domains like ITSN, it does form a complex with Sla1, which contains three SH3 domains (Tang *et al,* 2000). Both Pan1 and ITSN interact with early arriving proteins, including adaptors and Eps15/Ede1 through conserved regions (Salcini *et al.*, 1997; Paoluzi *et al.*, 1998; Wendland and Emr, 1998; Yamabhai *et al.,* 1998; Sengar *et al.,* 1999; Wendland *et al.*, 1999; Koh *et al.,* 2007; Maldonado-Baez *et al.*, 2008; Pechstein *et al.,* 2010; S. Barker and B. Wendland, unpublished observations). They both interact with various CME coat proteins and can promote actin polymerization either directly or indirectly. ITSN has important signaling functions in mammalian cells, which leads us to believe that Pan1 may also be playing other, perhaps similar, signaling roles in *S. cerevisiae*. Much is still unknown about the mechanistic roles of ITSN in CME; the ease and genetic tractability of using yeast to study Pan1's essential roles may lead to new findings that can be applied to ITSN.

Methods for studying essential proteins

While yeast is a fantastic model system for uncovering specific details regarding the function of a majority of proteins, it does have limitations. The majority of yeast proteins are not critical for viability, allowing for simple gene deletion experiments for observation of the resulting phenotypes. However, this method does not work for the over 1000 essential yeast genes, which represent 18% of all open reading frames in *S. cerevisiae* (Nash *et al.*, 2007). There are numerous methods for mutating genes or conditionally inactivating essential proteins, each of which have advantages and caveats. Thus, it is important to use a combination of these methods to get a more complete picture of a protein's cellular function.

One of the most-widely used methods to study a protein is to generate temperature sensitive (*ts*) mutant alleles in which all or some of the functions of a protein are lost upon the switch to a higher or lower temperature. These mutations typically disrupt the folding of the protein leading to altered binding dynamics, localization, activity, or it targets the protein for degradation. These alleles can help clarify some of the protein's role, but often the mutation does not disrupt all of the protein's functions. Additionally, the insult of the temperature shift activates cellular stress pathways, which can complicate observations.

Another popular method is to alter the transcription levels of a gene using promoters that respond to changes in the levels of galactose (*GAL1*), methionine (*MET25*), or tetracycline (TetON/OFF) in the media (Johnston and Davis, 1984; Mountain *et al.,* 1991; Belli *et al*., 1998). While the *GAL1* and *MET25* promoters have the advantage of being natural yeast promoters, their activation requires a change in

cellular metabolism and their regulation is often not perfect. Tetracycline promoters avoid this metabolism switch, but do not avoid the biggest disadvantage of conditional promoters—long half-lives of proteins. Even if a gene is not being actively transcribed and translated, there is still previously made protein in the cell, which may take hours or days to degrade. This remaining protein causes a phenotypic lag, which makes acute observations due to the absence of a protein impossible.

Another way to study a protein is to alter the protein's translation levels via the DAmP method: Decreased Abundance by mRNA Perturbation (Schuldiner *et al.*, 2005). Hypomorphic alleles are generated by insertion of an antibiotic resistance cassette into the 3' untranslated region (UTR) of the target gene, thus destabilizing the corresponding transcript leading to a decrease in mRNA and protein levels. This method allows a protein to remain functional, but the decreased levels will alter the efficiency of the pathways in which it is involved, giving insight into the protein's role. Obviously, the main caveat of this method is that the some of the protein is still expressed and functional.

The final methods used to discover a protein's function focus on conditional expression of the target protein, instead of altering its encoding gene. If a protein is known to function in a particular location, a tag can be added to the protein that will, under the nonpermissive conditions, relocalize and "anchor" the protein to another location; for example, a nuclear protein can be anchored to the cytoplasm (Chen *et al.,* 1995; Belshaw *et al*., 1996; Haruki *et al*., 2008). This method uses the natural dimerization of mTOR proteins that occurs in the presence of rapamycin. Rapamycin is toxic to yeast and thus yeast cells must be made rapamycin-resistant in order for these

"anchor away" experiments to be successful. However, the addition of rapamycin can have off-target effects on the cells that can confuscate results.

Lastly, the cells' endogenous ubiquitin-mediated degradation system can be used to conditionally degrade the target protein. In these degron alleles, a degron tag is added to the target protein and a corresponding E3 ligase is expressed in the cells. Upon the switch to nonpermissive conditions, the degron tag is recognized by the E3 ligase, leading to polyubiquitination and subsequent proteasome proteolysis of the target protein. Two of these degron tags, the DHFR and TIPI tags, depend on the N-end rule, which states that the *in vivo* half-life of a protein depends on the identity of the N-terminal amino acid (Bachmair *et al*., 1986; Varshavsky, 1996; Sanchez-Diaz *et al*., 2004; Taxis *et al*., 2009). In both of these degron tags, the N-terminal amino acid is unstable in the nonpermissive conditions and is recognized by the E3 ubiquitin ligase Ubr1. While both of these methods are successful in recognition and degradation of their target proteins, the nonpermissive conditions are not ideal—often a carbon source or temperature shift is required for expression of Ubr1 and exposure of the unstable N-terminal degron residue. Additionally, Ubr1 is endogenous to the cells and its overexpression can lead to the degradation of non-target proteins. To avoid these caveats, the Auxin Inducible Degron (AID) method was developed using a plant based degradation system to avoid endogenous targets (Nishimura *et al*., 2009). In the AID method, the target protein is tagged with a small plant protein, the "AID" tag and the plant E3 ligase TIR1 is constitutively expressed in the cells. TIR1 only recognizes the AID tag upon the addition of the plant hormone auxin to the media. Because TIR1, AID, and auxin are all plant

specific they should not have any endogenous targets in yeast and should not cause additional stress on the cells.

Pan1 is an essential protein and much has been learned about its functions through the use of *ts* mutant alleles. Attempts have been made to conditionally control *PAN1*, however, *PAN1* contains many methionines and internal start sites that allow truncated versions of Pan1 lacking any 5' UTR sequences to be expressed constitutively (Tang and Cai, 1996; K. Whitworth and B. Wendland, unpublished observations). Temperature and metabolism shifts are not ideal for experiments on endocytosis as these conditions perturb endocytic dynamics. This dissertation describes the development of the Pan1-AID degron allele and its use to elucidate the mechanisms of Pan1's role in endocytosis. The Pan1-AID allele is also used to carefully annotate which regions and domains of Pan1 are critical for each of its known functions.

CHAPTER 2: CREATION OF A PAN1 DEGRON ALLELE

Some of the work presented in this chapter is published at Molecular Biology of the Cell (Bradford *et al.,* 2015).

ABSTRACT

Much of the previous work on Pan1 used conditional mutants found through genetic screens (temperature sensitive (*ts*) alleles) or with engineered point mutations or truncations. However, findings from using the *ts* alleles are confounded by the effects of the nonpermissive conditions, which can also adversely affect endocytosis and all of these allele experiments are chronic and could be subject to compensatory changes. Additionally, it is not known if these alleles completely remove all Pan1 functions or just some of them. Attempts to make a conditionally expressed Pan1 allele were unsuccessful to due putative alternative translational start sites within Pan1 that made these alleles "leaky" by expressing truncated protein even in the nonpermissive conditions (Tang and Cai, 1996; K. Whitworth and B. Wendland, unpublished observations). For these reasons, it was necessary to create a degron allele of Pan1 that acutely and specifically degrades Pan1 protein *in vivo*. We used an Auxin-Inducible Degron (AID) to deplete cells of Pan1 protein *in vivo* (Nishimura *et al.*, 2009). Upon addition of auxin to the Pan1-AID cells, Pan1 is quickly degraded, endocytosis is arrested after one hour, and the cells start to die after 4-5 hours. These early time-points were used to assess the acute phenotypes of Pan1 depletion on living cells and to characterize how, and why, cells died in the absence of Pan1.

INTRODUCTION

Work with *pan1* mutant alleles suggests roles for Pan1 in endocytosis, actin cytoskeletal organization, polarized cell growth, and mitochondrial protein sorting (Zoladek *et al.*, 1995; Tang and Cai, 1996; Wendland *et al.*, 1996; Bidlingmaier *et al.*, 2001; Kamińska *et al.*, 2005). However, its precise mechanistic role(s) in these pathways is unknown, nor is it known which of these, if any, corresponds to Pan1's essential function(s). Although Pan1 has been characterized as a clathrin-mediated endocytic (CME) protein, CME is not an essential process, thus begging the question of what is Pan1's essential function if not in CME? Pan1 binds early and late endocytic factors, thus we hypothesized that its deletion would cause misregulation of recruitment of endocytic proteins to and lifetimes at the patch. In addition, although Pan1 is itself not a strong regulator of actin polymerization, it does bind several proteins involved in actin polymerization (both promotion and inhibition); therefore, it is predicted that Pan1 deletion could cause aberrant actin structure formation (Duncan *et al.*, 2001; Toshima *et al.*, 2005; Barker *et al.*, 2007). The exact mechanisms of Pan1's function was difficult to discern due to its essential nature.

Much of the previous work on Pan1 has been done using temperature sensitive (*ts*) conditional mutants; however, findings using *ts* alleles are confounded by the effects of the nonpermissive conditions, which can adversely affect endocytosis and elicit a stress response. Further, these allele experiments are chronic and could be subject to compensatory changes. Finally, it is not known if these alleles remove some or all Pan1 functions. Conditional expression of Pan1 has been unsuccessful due to putative alternative translational start sites and "leaky" expression of truncated protein even in the

nonpermissive conditions (Tang and Cai, 1996). Thus it was necessary to create a degron allele of Pan1 that acutely and specifically degrades Pan1 protein *in vivo*. An initial Pan1-degron, *pan1-td*, relied on both an increase in temperature and a change in carbon source to initiate degradation of Pan1 (K. Whitworth and B. Wendland, unpublished observations). This degron allele was not ideal due to the non-permissive conditions having off target effects on the phenotypes observed (discussed in Appendix I). Thus, a different degron tag that eliminated these stress-inducing conditions was required for studying Pan1.

We have used the methods of Nishimura *et al.* (2009) to produce a degron-fusion allele of *PAN1* (Pan1-AID) in order to efficiently and specifically degrade Pan1 upon addition of the plant hormone auxin. The AID method uses the plant specific auxin-inducible degron system in which certain proteins are recognized for degradation in the presence of auxin. In plants, the highly conserved E3 ligase SCF complex binds to various F-box proteins, which interact with specific substrates only in the presence of auxin to promote the substrate's degradation. In the Pan1-AID strain, the plant F-box protein TIR1 is constitutively expressed with the ADH1 promoter and *PAN1* is C-terminally fused with the *Arabidopsis thaliana* TIR1 target protein IAA17 (AID tag). In the absence of auxin, TIR1 binds the SCF complex, but does not interact with the AID tag. Upon addition of auxin, TIR1 binds auxin, which promotes the interaction between TIR1 and the AID-tag. This interaction recruits an E2 ubiquitin conjugating enzyme, which promotes the ubiquitination and subsequent degradation of Pan1-AID (Figure 2.1A). Expression of TIR1 does not alter growth of yeast; furthermore, TIR1 does not have any endogenous yeast targets because it is a plant protein. Thus, this system allows

for specific of degradation of only proteins with an AID tag. Pan1-AID is viable in the absence of auxin, allowing for propagation of the strain. Under the nonpermissive conditions, the cells are inviable and endocytosis is arrested. The Pan1-AID allele thus provides a novel, effective way to study the functions of Pan1 using acute perturbations with minimal changes in growth conditions.

MATERIALS AND METHODS

Media and Growth Conditions

Yeast cells were grown in rich (yeast extract-peptone) or synthetic (yeast nitrogenous base with amino acids selection of plasmid maintenance) medium with 2% dextrose. For experiments using auxin in liquid cultures, 3-Indoleacetic acid (Sigma I2886) was added to synthetic media to a final concentration of 500μm. For experiments using auxin on solid media, 1-Naphthaleneacetic acid (Sigma N0640) was added to synthetic media to a final concentration of 1mM. For cell-death and growth experiments, sorbitol was added at a final concentration of 1M, rapamycin was added at a final concentration of 0.2μg/ml and cycloheximide at a final concentration of 0.1μM.

Strains and Plasmids

The yeast strains and plasmids used in this study are listed in Tables 2.1 and 2.2, respectively. Strains were constructed using PCR-based genomic integration as described previously (Longtine *et al*., 1998; Goldstein and McCusker, 1999; Nishimura *et al.,* 2009). DNA manipulations for plasmid construction were preformed using standard techniques, employing either T4 DNA polymerase-mediated ligations in *Escherichia coli* or homologous recombination with overlapping DNA fragments followed by plasmid rescue in *S. cerevisiae*. All restrictions enzymes were purchased from New England Biolabs (Ispwich, MA).

TABLE 2.1 Yeast strains used in this chapter

Strain	Genotype	Source
W303	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2*	Laboratory Strain
BY25598	*MATa ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ADH1-OsTIR1-9myc::URA3*	Yeast Genetic Resource Center
BWY5393	MATα *ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA*	This Study
BWY5394	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ADH1-OsTIR1-9myc::URA3 ade2::ADE2 HisΔ::URA Pan1-AID::G418*	This Study
BWY6051	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU Mup1-pHl::HPH*	This Study
BWY6052	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Mup1-pHl::HPH*	This Study
BWY6053	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEUMup1-pHl::HPH*	This Study

TABLE 2.2 Plasmids used in this chapter

Plasmid	Details	Description	Source
pRS414	CEN, TRP1	Empty Vector (EV)	Laboratory plasmid
pBW626	pRS414::PAN1 (CEN, TRP1)	pPan1.414	Sachs et al., 1992

Protein Expression

TCA Precipitation**:**

Mid to late-log phase cells -/+ Auxin at indicated time points were harvested and resuspended in 1ml of 10% trichloroacetic acid (TCA) with 1mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, a protease inhibitor), incubated on ice for 20 min, and precipitates were centrifuged at 14,000 rpm for 10 min at 4°C. Precipitates were washed twice with cold acetone, resuspended by sonication, and air-dried. Protein samples were processed for SDS-PAGE separation by adding 2x protein sample buffer with 1mM AEBSF and solubilized by bead disruption.

SDS-PAGE and Immunoblotting:

Proteins were separated on polyacrylamide mini gels (7.5%) at 27mA in SDS running buffer (3mM SDS, 25mM Tris base, 192mm glycine) and then transferred onto nitrocellulose membranes at 80V for 90 min in cold transfer buffer (20% methanol, 0.0375% SDS, 48mM Tris base, 30mM glycine). The membranes were blocked in 5% milk in TBST (10mM Tris, pH 7.5, 0.25M NaCl, 0.025% Tween-20). Blots were incubated in the specified primary antibody overnight at the following concentrations: Rabbit-anti-Pan1 was a gift from D.G. Drubin and was used at 1:10,000; Rabbit-anti-Act1 was a gift from D. Pruyne and was used at 1:5,000; Rabbit-anti-End3 was a gift from the H. Riesman lab and was used at 1:2,000; and Rabbit-anti-Sla1 was a gift from L. Hicke and was used at 1:2,000. The blots were washed 3 times in TBST, incubated with secondary antibodies conjugated to HRP (Pierce, Rockford, IL) diluted 1:2,000 in milk solution for 45 min. Blots were washed again 3 times in TBST, and then developed with chemiluminescent substrate (Solution 1:2.5mM Luminol, 400uM paracoumaric acid, 100mM Tris-HCl, pH 8.5. Solution 2: 5.4mM H_2O_2, 100mM Tris-HCl, pH 8.5) for 2 min at room temperature. Chemiluminescence images were acquired using a FluorChem M FM0455 imager.

Growth Assays

Serial Dilutions: Log phase cells were diluted to an OD600nm of 0.25, from which four 1:5 serial dilutions were plated on YNB-tryptophan minus or plus 1mm NAA and grown for three days at 30°C.

Liquid growth: Log phase cells were diluted to an OD600nm of 0.15 in synthetic media minus or plus 500μm IAA in a 24-well clear plate. Absorbance at 600nm was measured every 30 m over 16 hours in a plate reader.

Propidium Iodide Staining

Aliquots of cells from each culture were taken at the indicated times after incubation in minus or plus 500μm IAA at 30°C and labeled with 1μg/ml propidium iodide (PI). 5,000 cells were immediately analyzed on a BD FACS-Calibur (Becton Dickinson & Co., Mountain View, CA) flow cytometry system to quantify the population of cells with fluorescent propidium iodide staining. For DNA content assays, cells were fixed in 70% ethanol overnight and washed in PBS prior to PI staining. Gates were used to count the percent of cells with 1N and 2N DNA content.

FM4-64 staining

FM4-64 images were collected using a Zeiss Axiovert 135TV inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a Sensicam QE CCD camera (Cooke, Romulus, MI), Zeiss 100x 1.4 NA Plan-Apochromat objective, motorized filter wheels, fluorescein isothiocynate (FITC) and Texas Red filter sets (Semrock, Rochester, NY), and Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO). Images were captured with 500ms exposure, identical binning, intensification and illumination intensity. Log phase cells were incubated in YNB-trp media minus or plus 500μM IAA for 30 min at 30°C. For FM4-64 labeling, cell aliquots were transferred to an eppendorf tube with 10ug/ml FM4-64 (Molecular Probes, Eugene, OR) and incubated for 15 min at

30°C. The cells were washed twice with fresh media to remove excess FM4-64, resuspended in 100ul fresh media, and then put on ice until visualization at the microscope. Cells were spotted onto uncoated glass slides and covered with a coverslip.

Fluorescence microscopy

Mup1-pHl:

Mup1-pHlourin images were collected using a Zeiss Axiovert 135TV inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a Sensicam QE CCD camera (Cooke, Romulus, MI), Zeiss 100x 1.4 NA Plan-Apochromat objective, motorized filter wheels, fluorescein isothiocynate (FITC) and Texas Red filter sets (Semrock, Rochester, NY), and Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO). Images were captured with 500ms exposure, identical binning, intensification and illumination intensity. For quantification of fluorescence intensity, 16-bit image files were analyzed in Image J v1.41n. Background subtraction was performed before measurement of integrated density, and values were corrected for cell size.

To perform kinetic analysis of endocytosis, cells expressing Mup1-pHlourin were grown overnight in synthetic YNB media lacking methionine (YNB-met). Cells were then diluted to a density of 0.35 OD/ml in YNB-met, and were grown to a density of 0.6 OD/ml. Cells were then seeded onto concanavalin A-coated 8-well glass-bottomed chamber slides (LabTek, Scotts Valley, CA) containing YNB-Met and were allowed to settle before imaging. Cells were/were not treated with 500μM IAA for 30 min, then methionine was added at a concentration of 20μg/ml and images were captured after 30

min or one hr. During image acquisition, cells were maintained at a constant temperature of 30°C. Experiments with Pan1 plasmids were performed in YNB-trp-met media.

Electron Microscopy

Fixation: Log phase cells were incubated in indicated conditions and were fixed overnight at 4°C in 10% gluteraldehyde, 0.1 M NaCacod pH 7.4, 5mM CaCl2, 5 mM MgCl2, 2.5% sucrose. Samples were washed in subsequent wash steps with: 100mM cacodylate, TDES, and then 0.1M phoscitrate/1M sorbitol. To the phoscitrate/sorbitol step, B-glucuronidase and 10mg/ml zymolase were added and cells were agitated for one hour. Cells were washed with 0.1M cacodylate/5mM CaCl2/1M sorbitol and stored at 4°C. Cells were emedded in 2% agarose, cut into pieces and fixed in 1% OsO4/1% potassium ferrocyanide in 0.1M cacodylate/5mM CaCl2, pH 6.8 at room temperature for 30 m.

Osmium-Thiocarbohydrazide-Osmium staining: Blocks were washed in subsequent steps with: 1% thiocarbohydrazise, ddH2O, 1% OsO4/1% potassium ferrocynide in cacodylate buffer pH 6.8, ddH2O, and then let sit in Kellenberger's uranyl acetate overnight. Blocks were dehydrated through a graded series of ethanol on ice, then transferred to 1:1 ethanol/propylene oxide, then 100% propylene oxide, then 2:1 propylene oxide/ Epon resin overnight. Blocks transferred to beam capsules in fresh Epon resin overnight. Samples were sectioned and placed on grids. Electron microscopy imaging was done with a Phillips 420 Transmission Electron Microscope.

Image/Statistical Analysis

Statistical significance between populations was determined by one-way ANOVA followed by Tukey's Multiple Comparison post hoc analysis.

RESULTS

Pan1-AID is an efficient tool for depleting Pan1 protein in cells

To investigate the function(s) of Pan1, we used the Auxin-Inducible Degron (AID) system to acutely deplete cells of Pan1 protein *in vivo* (Nishimura *et al.*, 2009). The Pan1-AID degron cells were created by endogenously C-terminally tagging Pan1 with the AID sequence and integrating the OsTIR1 gene with the constitutive ADH1 promoter (Figure 2.1A). OsTIR1 is a plant F-box protein that specifically binds and promotes the degradation of the AID tagged target protein only in the presence of auxin. For controls, we used a wildtype (WT) strain and a strain containing only OsTIR1 (TIR1) with empty vector (EV) plasmids, both of which grew similarly in the absence and presence of auxin (Figure 2.1B). In the absence of auxin, Pan1-AID cells grew to WT levels and Pan1 protein was stable; upon addition of auxin, growth was arrested and 84% of Pan1 protein was degraded within 15 min (Figure 2.1, B-D). Expressing full-length Pan1 on a plasmid in Pan1-AID cells restored growth in the presence of auxin (Figure 2.1, B and C). Depletion of Pan1 was specific and did not affect the stability of its binding partners Sla1 and End3; however, there was a gel mobility shift for Sla1 upon the addition of auxin (Figure 2.1D). This gel mobility shift of Sla1 was not seen in extracts from TIR1 cells treated with auxin, suggesting that the shift was due to the loss of Pan1, consistent with a predicted hyperphosphorylation phenotype (Figure 2.1E) (Zeng *et al.*, 2001; 2007). It has been proposed that auxin (specifically, indoleacetic acid (IAA)) can induce filamentous growth, adhesion, and/or growth arrest in WT cells; however, we did not observe any of these phenotypes in our experiments (Prusty *et al.*, 2004).

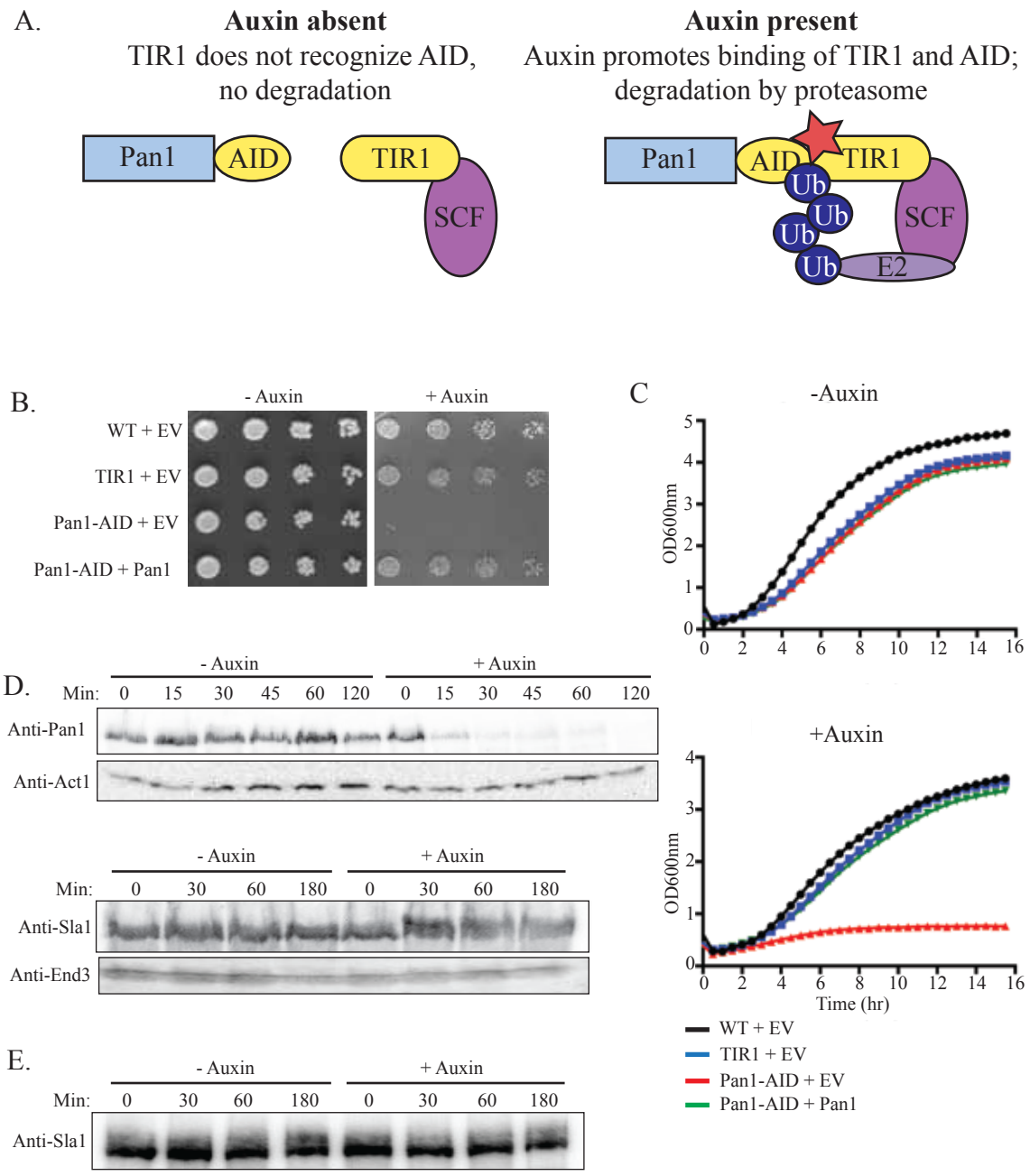


Figure 2.1. Pan1-AID is an efficient tool for depleting Pan1 protein in cells.
(A) Schematic of Auxin-Inducible Degron system for Pan1. (B) Serial dilutions of cells of indicated genotype were grown on synthetic media ± auxin for 3 d at 30°C. EV = empty vector. (C) Log phase cells were incubated in synthetic media ± auxin and the absorption at 600OD was measured every 30 min over 16hrs. (D-E) Immunoblots detecting Pan1, Sla1, and End3 in Pan1-AID (D) or TIR1 (E) cell lysates grown for indicated time ± auxin. Act1 serves as a loading control in D.

Degradation of Pan1 results in death of cells

To determine if the degradation of Pan1 led to either growth arrest or cell death, cells were grown the absence or presence of auxin and dead cells were stained with propidium iodide and quantified by flow cytometry. After 24 hours in the presence of auxin, Pan1-AID cells with EV reached almost 100% death, while death of the control strains was at similar levels to those with no auxin present (Figure 2.2A). To relate cell death to reduced Pan1 protein levels, cell death was analyzed over time. We found that Pan1-AID cells reach about 50% death after four hours in the presence of auxin, with over 75% of cells still alive between zero and two hours (Figure 2.2B). These early time points were used to assess the acute phenotypes of Pan1 depletion on living cells. The Pan1-AID death phenotype (Figure 2.2, A and B) was restored to WT levels with expression of full-length Pan1 from a plasmid.

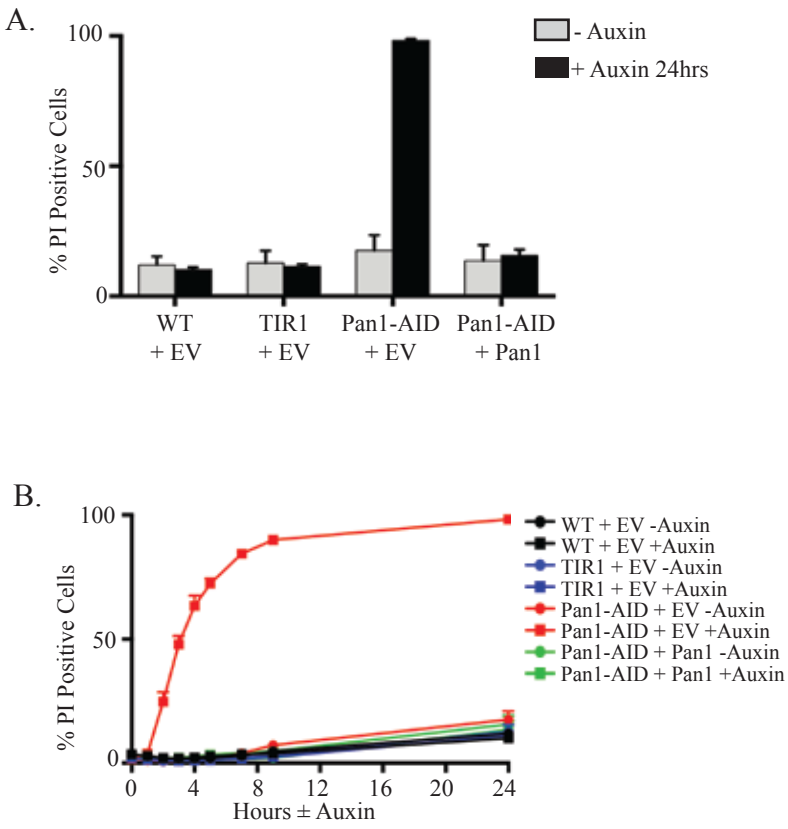


Figure 2.2. Pan1 is required for viability
(A) Percentage of cells that were positively stained with propidium iodide (PI) after grown for 24 h ± auxin at 30°C was quantified by flow cytometry. Graph represents the average of three replicate experiments (Mean ± SD). (B) Strains were grown ± auxin and samples were collected at indicated times, stained with PI, and quantified by flow cytometry. Graph represents the average of three replicate experiments (Mean ± SD).

Endocytosis is arrested in the absence of Pan1

Pan1's most well characterized role in the cell is as an endocytic coat protein. Previous studies have shown that endocytosis is aberrant in *pan1* mutant cells; thus, we hypothesized that endocytosis would be significantly affected in Pan1-AID cells upon depletion of Pan1 by the addition of auxin. We monitored bulk endocytosis using the lipophilic dye FM4-64 (Figure 2.3A). Cells were incubated in the absence and presence of auxin for 30 min and then stained with FM4-64 in similar auxin conditions for 15 min. In the control strains, WT and TIR1 plus EV, in the absence and presence of auxin and in Pan1-AID cells in the absence of auxin, the cells efficiently internalized the dye, which was trafficked throughout the cell, highlighting internal organelles such as endosomes and vacuoles. In contrast, FM4-64 was trapped at the plasma membrane in the Pan1-AID with EV cells plus auxin, indicating that bulk endocytosis had been arrested upon depletion of Pan1. This endocytic arrest was rescued by expressing full-length Pan1 from a plasmid.

To observe receptor-mediated CME, we used chimeras of the methionine permease Mup1 with pHlourin, a pH-sensitive variant of GFP (Miesenböck *et al.*, 1998). In the absence of methionine in the growth medium, Mup1 is localized to the plasma membrane; upon addition of methionine, Mup1 is quickly endocytosed through CME and targeted to the vacuole lumen. The pHlourin signal is quenched in the acidic lumen of the late endosome/multivesicular bodies (MVB) and vacuoles. Thus, endocytic efficiency can be quantified by measuring the amount of fluorescence remaining after the addition of methionine (Prosser *et al.*, 2010). In the absence of auxin, WT, TIR1, and Pan1-AID cells internalized Mup1-pHl to similar levels after the addition of methionine (Figure 2.3,

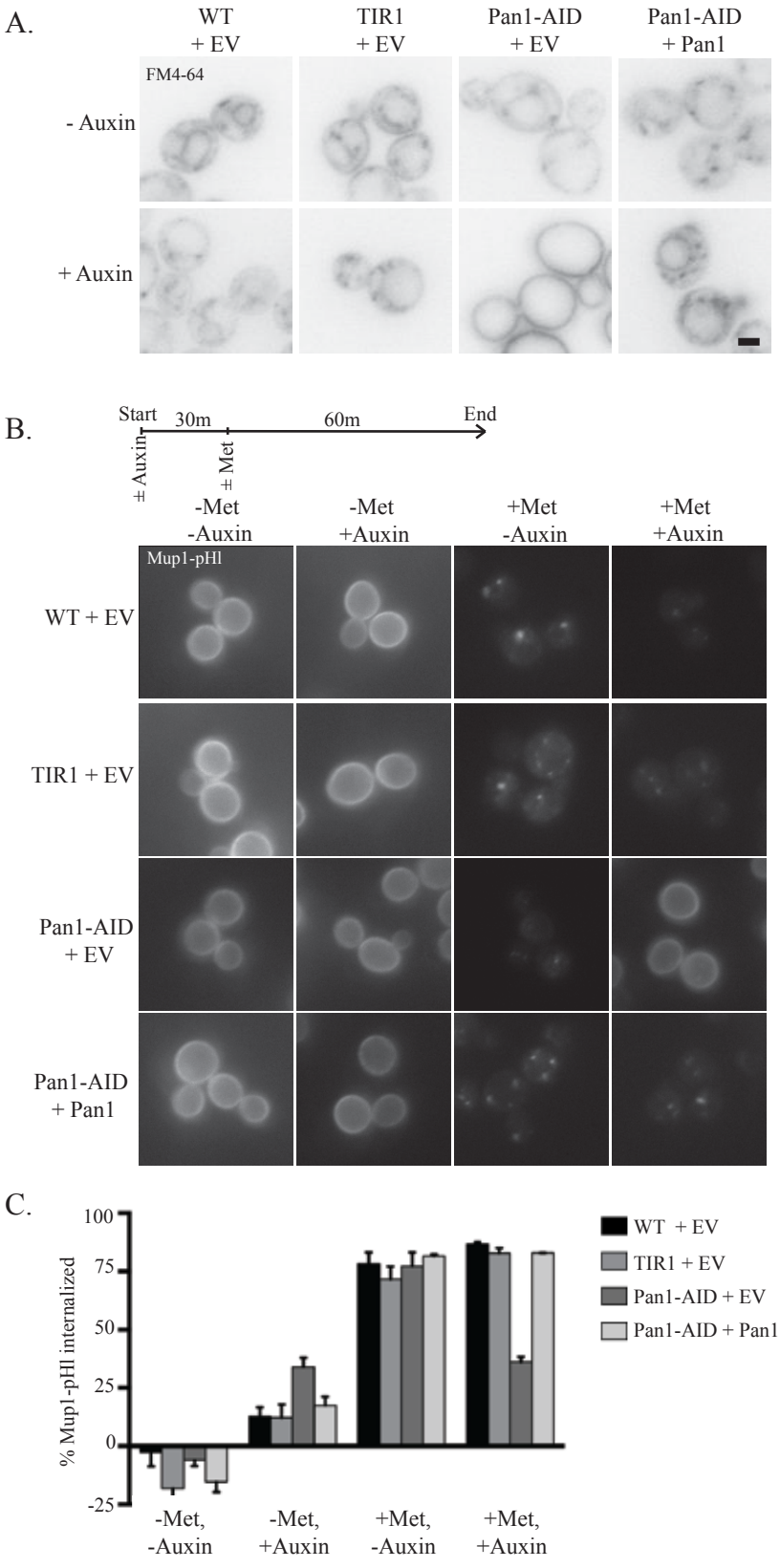

A.
WT + EV
TIR1 + EV
Pan1-AID + EV
Pan1-AID + Pan1
FM4-64
- Auxin
+ Auxin
B.
Start
30m
60m
End
± Auxin
± Met
-Met -Auxin
-Met +Auxin
+Met -Auxin
+Met +Auxin
Mup1-pHl
WT + EV
TIR1 + EV
Pan1-AID + EV
Pan1-AID + Pan1
C.
% Mup1-pHl internalized
100
75
50
25
0
-25
-Met, -Auxin
-Met, +Auxin
+Met, -Auxin
+Met, +Auxin
WT + EV
TIR1 + EV
Pan1-AID + EV
Pan1-AID + Pan1

Figure 2.3. Bulk and receptor mediated endocytosis is arrested in Pan1-AID in the presence of auxin.
(A) Representative images of log-phase cultures grown in ± auxin for 30 min before labeling with FM4-64 for 15 min. (B) Representative images of log-phase cultures ± pre-treatment with auxin for 30 min followed by addition of methionine for 1 h. Schematic of Mup-pHl experimental timeline. (C) Quantification of Mup1-pHl fluorescence internalized after 30 min ± auxin pre-treatment and 1 h ± methionine. Percent Mup1-pHl internalized was calculated by measuring the amount of fluorescence at the end of the assay compared to the amount at the beginning. Graph represents average of three replicate experiments (Mean ± SD). Scale bar, 2μm.

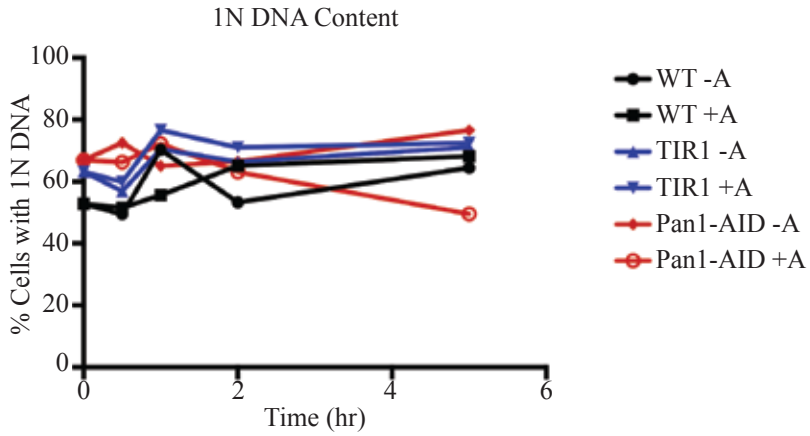
B and C). The addition of auxin did not affect Mup1-pHl internalization for the control cells. However, when Pan1-AID cells were pre-treated with auxin for 30 min prior to the addition of methionine, Mup1 remained at the plasma membrane, again confirming that the absence of Pan1 causes an endocytic arrest. This arrest in endocytosis was fully rescued by expressing full length Pan1 from a plasmid in the Pan1-AID cells.

In the absence of both auxin and methionine, all strains exhibited brighter fluorescence at the plasma membrane over the time course. We hypothesize this is due to the continued production of Mup1-pHl that is trafficked to the plasma membrane. The addition of auxin alone appears to cause a decrease in Mup1-pHl fluorescence at the plasma membrane. The decrease in fluorescence does not appear to be due to endocytosis as there are no internal Mup1-pHl structures visible as is seen in cells that are competent for endocytosis. One possibility is auxin-dependent changes in cytosolic pH; however, the reason for this decrease in fluorescence is currently unknown.

Depletion of Pan1 does not cause a cell cycle arrest

The depletion of an essential protein can often lead to an arrest in cell cycle prior to death of the cells. In order to assess if Pan1-AID cells were arresting in a particular stage of the cell cycle prior to death, the DNA content of the cells at various time points after the addition of auxin was measured by staining DNA with propidium iodide. There was no observable difference between the amounts of cells with 1N or 2N DNA for WT, TIR1, and Pan1-AID cells in early time points (Figure 2.4, A and B). At the five-hour time point, a majority (75%) of Pan1-AID cells plus auxin were dead, which confounds the results at this time point. The absence of an arrest was confirmed by observing the cells under DIC where there was no obvious arrest of Pan1-AID cells in a particular stage of the cell cycle. Therefore, these data suggest that Pan1-AID cells are not arrested prior to cell death.

A.



B.

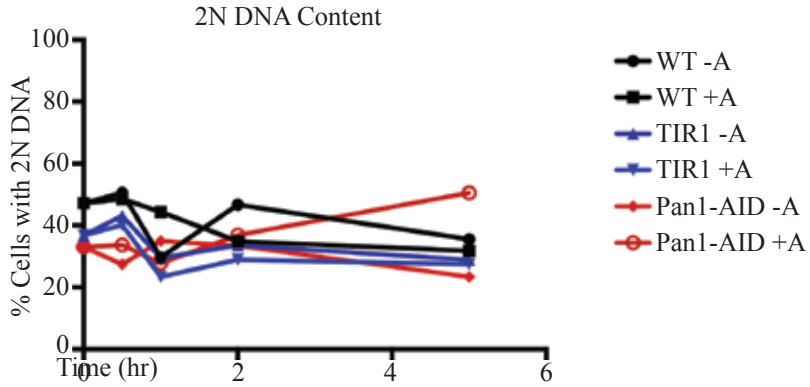


Figure 2.4 Pan1-AID cells do not arrest prior to cell death
(A and B) Strains were grown ± auxin and samples were collected at indicated times, fixed with ethanol overnight, stained with PI, and DNA content was quantified by flow cytometry: (A) percent of cells with 1N DNA content. (B) percent of cells with 2N DNA content.

Auxin-induced degradation of Pan1 is reversible

One of the benefits of the auxin-inducible degron system is the ability to wash-out the auxin to stop further degradation of the target protein and allow the cell to restore levels of newly synthesized protein. To test this in Pan1-AID cells, auxin was added to the cells for 2 hours, then cells were washed and the media was replaced with fresh media not containing auxin. Within one hour, expression of Pan1 protein was observed and cells began to grow (Figure 2.5, A and B). This confirms that, at least out to two hours, Pan1-AID cells can recover from the loss of Pan1.

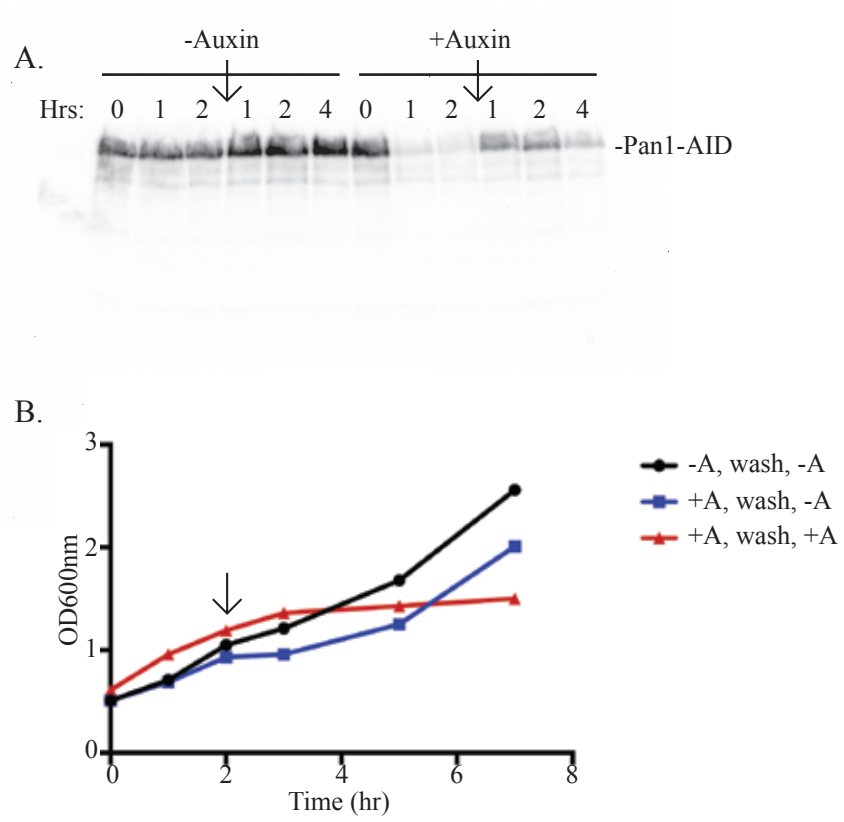


Figure 2.5 Pan1 degradation and Pan1-AID cell growth are reversible after a 2 hour auxin incubation.
(A) Immunoblot detecting Pan1 in Pan1-AID cell lysates from cells incubated in ± auxin for 2 hours, washed, and incubated in fresh media without auxin. (B) Pan1-AID cells were grown in ± auxin and the OD600nm was measured at indicated times. Arrow indicates time at which wash step occured.

Sorbitol does not restore defects of Pan1 depletion

Endocytosis is a mechanism used for plasma membrane and cell wall homeostasis. Some endocytic mutants result in weak cell walls that burst due to osmotic pressure; addition of sorbitol to the media can help support these weak cells walls. Alternatively, sorbitol helps other endocytic mutants by reducing the turgor pressure that makes actin polymerization necessary. However, sorbitol was not able to restore growth or viability in the absence of Pan1 (Figure 2.6, A-C). While the cell wall may be weakened due to loss of Pan1, the addition of sorbitol is not able to inhibit the death pathway induced in the absence of Pan1.

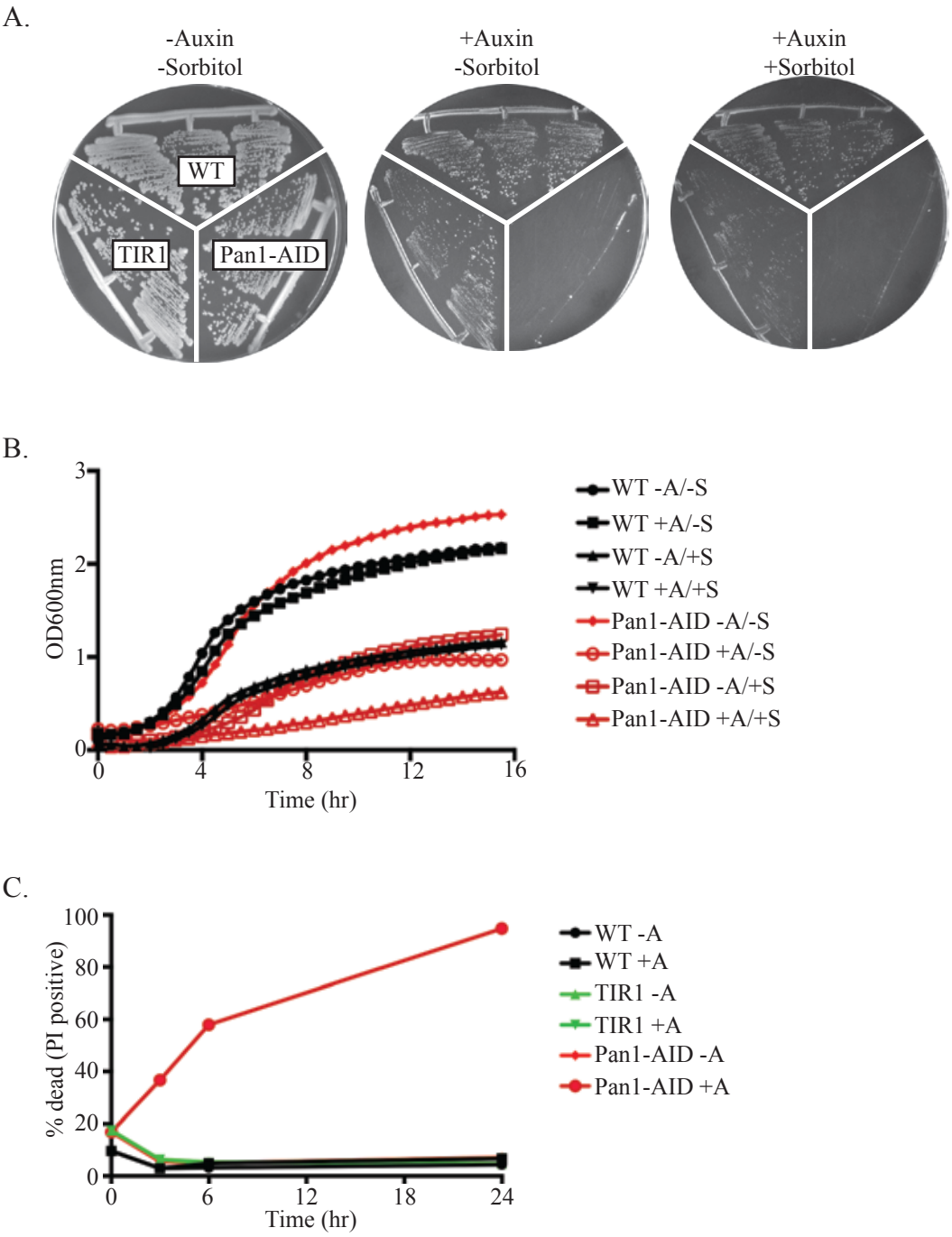


Figure 2.6 Providing osmotic support to Pan1-AID cells in the presence of auxin does not restore growth or viability.
(A) Serial dilutions of cells of indicated genotype were grown on synthetic media ± auxin, ± 1M sorbitol for 3 d at 30°C. (B) Log phase cells were incubated in synthetic media ± auxin, ± 1M sorbitol and the absorption at 600OD was measured every 30 min over 16hrs. (C) Strains were grown ± auxin +1M sorbitol and samples were collected at indicated times, stained with PI, and quantified by flow cytometry.

Cell death inhibitors do not rescue Pan1-AID cells

Although Pan1's most well known function is in CME, CME itself is not an essential process, which begs the question of what is Pan1's essential function? Determining how cells die in the absence of Pan1 may uncover what Pan1 is doing to maintain viability. Two well-known cell death inhibitors, rapamycin and cycloheximide, have been shown to inhibit cell death by either blocking the TOR pathway or inhibiting protein translation, respectively (Brown *et al*., 1994; Mattson and Furukawa, 1997; K. Cunningham, unpublished observations). However, neither drug was able to inhibit death of Pan1-AID cells in the presence of auxin, indicating that neither the TOR pathway nor protein translation is required for Pan1-AID cell death (Figure 2.7, A and B). Interestingly, cycloheximide did delay the death of cells by about 4 hours; this could mean that protein translation is required for the primary manner of death, but not secondary causes (Figure 2.7B).

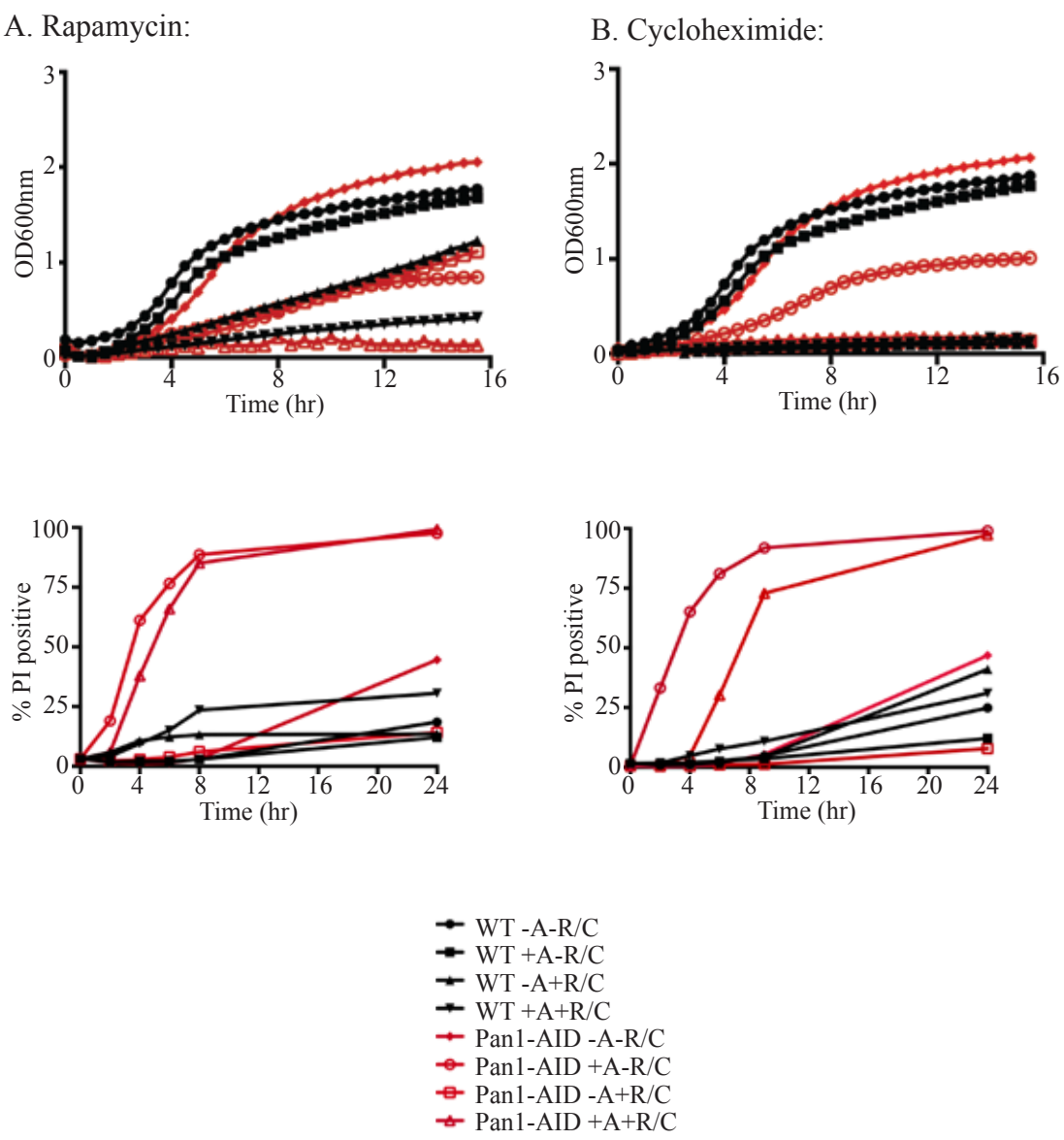
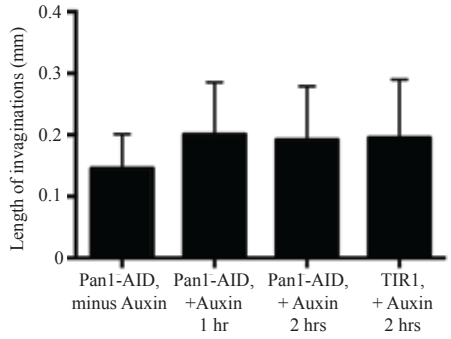


Figure 2.7. Death due to degradation of Pan1 is not dependent on the TOR pathway or protein translation.
Log phase cells were incubated in synthetic media ± auxin, ± rapamycin (A) or ± cycloheximide (B) and the absorption at 600OD was measured every 30 min over 16hrs (top) or samples were collected at indicated times, stained with PI, and quantified by flow cytometry (bottom).

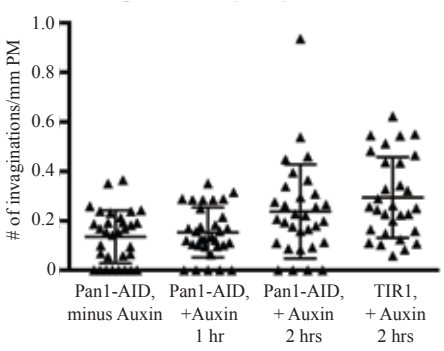
Pan1-AID cells do not have any obvious differences of plasma membrane invaginations

To observe any gross phenotypes due to loss of Pan1 or addition of auxin, electron microscopy was performed on Pan1-AID cells after 0, 1 and 2 hours in the presence of auxin and compared to TIR1 cells plus auxin for 2 hours as a control. The number and length of plasma membrane invaginations increased with longer times in auxin (Figure 2.8, A and B). However, whether or not these invaginations correspond to CME sites, eisosomes, or some other type of invaginated structure is unknown. It has been observed that eisosomes may become exaggerated in endocytic mutants (Buser and Drubin, 2013). In Pan1-AID samples that were incubated for two hours in auxin, 10% of cells observed appeared very sick and/or dead (Figure 2.8C) which correlates with the death rate calculated in Figure 2.2B. All cells in the other samples had no obvious differences, suggesting that the addition of auxin alone was not deleterious to cells (Figure 2.8D).

A.

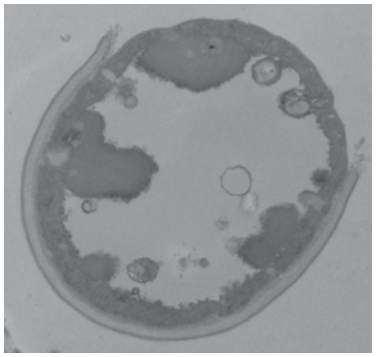


B.



C.

Pan1-AID + auxin 2hr



D.

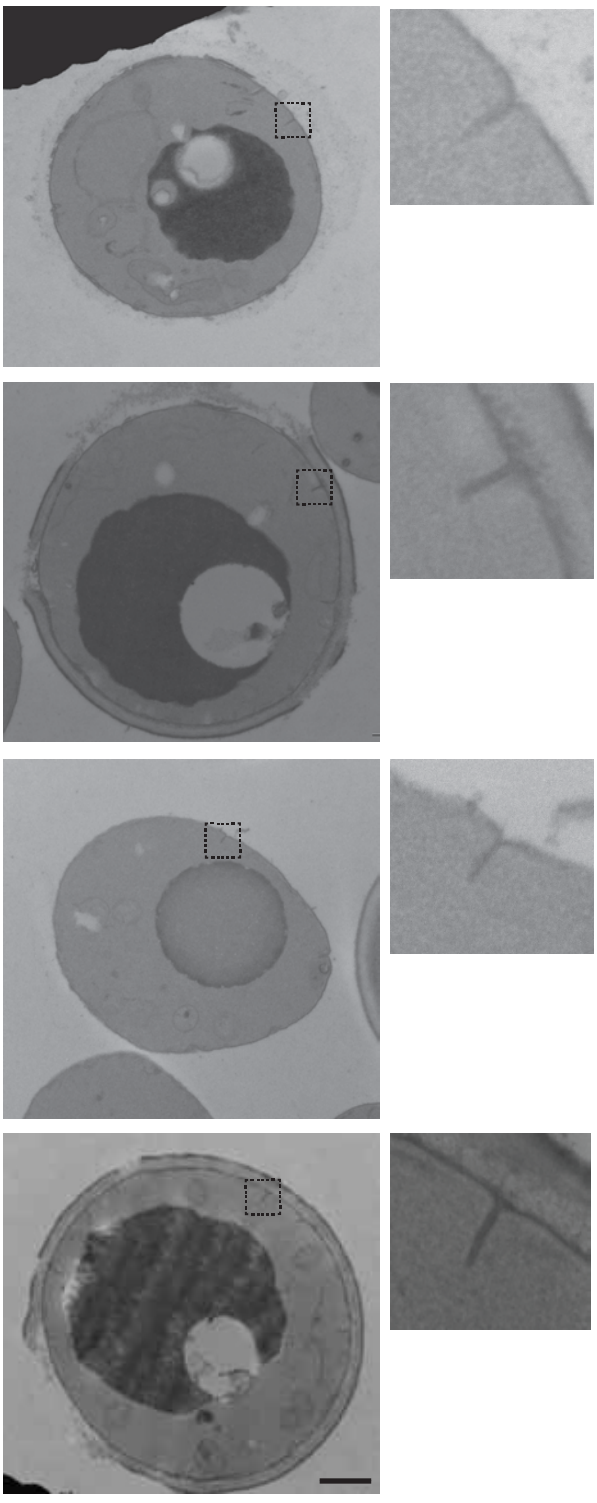


Figure 2.8. Gross morphologies of Pan1-AID and TIR1 cells incubated in auxin. (A and B) Quantification of the length (A) of invaginations and the number of invaginations (B) per micron plasma membrane (PM) for each sample. N > 65 invaginations, N > 30 cells. Images were collected and quantified blindly. Mean ±SD (C) Electron micrograph of a very sick/dead cell only seen in Pan1-AID plus auxin for two hours (10% of cells in that sample). (D) Electron micrographs of representative cells and invaginations from each sample. Boxed in area is magnified in image to the right of each cell. Scale bar, 1 μm.

DISCUSSION

Although much had been discovered about Pan1 using *ts* mutants and conditional expression of *PAN1*, these methods had caveats that left much unknown about Pan1's specific mechanistic functions. The creation of Pan1-AID has allowed for a much more in-depth study of not only Pan1's endocytic role, but also its essential function(s) and the regions of Pan1 that are critical in both of these roles.

In Pan1-AID cells, a majority of Pan1 protein is depleted within one hour after the addition of auxin, yet most cells remain viable; it requires four hours for over 50% of the cells to die (Figure 2.1D and 2.2B). How are Pan1-AID cells dying? CME is arrested after just one hour in the absence of Pan1 and although Pan1's major role is in CME, CME is not essential and probably not the cause of cell death (Payne *et al.*, 1988; Chu *et al.*, 1996; Madania *et al.*, 1999; Kaksonen *et al.*, 2005; Newpher and Lemmon, 2006; Prosser *et al.*, 2011)

The death of Pan1-AID cells was characterized in the hopes that discovery of how and why cells die in the absence of Pan1 could reveal Pan1's essential function. Pan1-AID cells do not arrest in a particular cell cycle stage prior to death and if auxin is washed out of the media after 2 hours, the cells will survive the brief period with minimal Pan1 levels (Figures 2.4 and 2.5). Thus, the death is not due to cell cycle disruption. Sorbitol can rescue several endocytic mutants by providing osmotic support to the cell wall or to reduce the necessity of actin polymerization; however, sorbitol did not restore growth or inhibit death of Pan1-AID cells in the absence of Pan1 (Figure 2.6). While this does not eliminate the possibility that loss of Pan1 results in cell wall or actin polymerization defects, these defects are not the primary cause of death.

Some cell death pathways can be reversed using drugs to inhibit or delay the death pathways. The TOR pathway is one of the main cell death pathways and it can be inhibited with rapamycin. This drug does not inhibit Pan1-AID cell death, confirming that it is a TOR-independent death pathway (Figure 2.7, A and B). Cycloheximide, which inhibits protein translation, has been suggested to inhibit programmed cell death by down regulating the expression of "death genes" (Mattson and Furukawa, 1997). While cycloheximide did not inhibit Pan1-AID cell death, it did delay the death by about 4 hours (Figure 2.7, C and D). Thus, protein translation may be important for the initial death pathway induced in the absence of Pan1, but not the entire, or secondary, death pathway.

Finally, death can be assessed by gross observation of the morphology of dead and dying cells. Electron micrographs of Pan1-AID cells after two hours in auxin showed that most cells looked similar to control cells (Figure 2.8D). The number and length of invaginations were similar between strains and auxin time points; however, whether or not these invaginations represent CME or other events has yet to be characterized (Figure 2.8, A and B). A small percentage of cells did have large, ruptured vacuoles that filled the majority of the cell; these cells were most likely dead, or dying (Figure 2.8, C). Observing Pan1-AID cells at more time points after auxin addition should reveal more details about the mechanism of death.

Pan1-AID is a powerful tool that was used to acutely deplete cells of the essential protein Pan1. Using this tool, we confirmed that Pan1 is necessary for growth, viability, and endocytosis. Further work to uncover how Pan1 functions in each of these roles is necessary.

CHAPTER 3: CHARACTERIZATION OF PAN1'S ENDOCYTIC ROLE

Some of the work presented in this chapter is published at Molecular Biology of the Cell (Bradford *et al.,* 2015).

ABSTRACT

Endocytosis is a well-conserved process by which cells invaginate small portions of the plasma membrane to create vesicles containing extracellular and transmembrane cargo proteins. Dozens of proteins and hundreds of specific binding interactions are needed to coordinate and regulate these events. There are several transitions during the process for which the exact mechanism of regulation is unknown: early coat maturation to late coat, coordination of actin regulators to actin machinery, and connection between the coat, membrane, and actin. Pan1 is thought to be a scaffolding protein due to its interactions with numerous proteins that act throughout the endocytic process and is therefore a prime candidate for regulation of these transition points. Previous research has characterized many Pan1 binding interactions, but due to Pan1's essential nature, the exact mechanisms of Pan1's function in endocytosis have been difficult to define. Using the Pan1-AID degron strain (explained in Chapter 2), we characterized the how endocytosis is affected in the absence of Pan1. The loss of Pan1 caused an arrest in CME due to a delay in endocytic progression, aberrant interactions between actin machinery and actin regulators, and weakened connections between the coat, membrane, and actin.

INTRODUCTION

Over 50 different proteins are involved in the initiation, maturation, and scission of endocytic events at the endocytic sites, or patches, and most of these proteins and their functions are well conserved between yeast and mammals. The formation of the clathrin coat, which consists of clathrin, adaptor proteins, coat proteins, and actin-regulatory complexes, begins with the recruitment of early coat proteins, such as Ede1 and Syp1, and adaptor proteins to the plasma membrane. The adaptors Yap1801/2 and Ent1/2 bind to cargo, late coat proteins, and clathrin, and connect the newly forming coat to the plasma membrane. Additional coat components, including Sla2, Sla1, End3, and Pan1 associate at the endocytic site and recruit proteins for the final stages of endocytosis. Actin and actin regulatory proteins, such as Las17, Myo3/5, Arp2/3, and Sac6, are recruited to the site to invaginate the membrane and form the vesicle. Finally, the scission proteins Rvs161/167 complete the process by pinching off the newly formed vesicle from the plasma membrane, allowing the vesicle to be propelled into the cell through the force produced by actin polymerization. Through phosphorylation and dephosphorylation of both proteins and lipids, the clathrin coat dissociates from the vesicle and the proteins are recycled though the cytosol and back to the membrane to participate in further rounds of CME.

The yeast protein Pan1 is most well known as a CME coat protein that can interact with adaptors, early and late coat proteins, and actin regulatory proteins, thus Pan1 is presumed to act as a central scaffold that could link the early and late stages of endocytosis (Sachs and Deardorff, 1992; Wendland *et al.*, 1996). Pan1 is a homolog of the mammalian protein Intersectin, which is also thought to be a CME scaffold as it also

binds several proteins involved throughout the endocytic process (Yamabhai *et al.*, 1998; Hussain *et al.*, 1999). Pan1 is a 1480 amino acid protein containing two N-terminal Eps15-homology (EH) domains, a central largely disordered region containing several predicted short coiled-coils, and a C-terminal actin regulatory domain.

Pan1 connects to the early coat through interactions found throughout the entire protein. Ede1 is one of the first proteins to arrive to an endocytic site and is thought to coordinate the initial steps of endocytosis; the C-terminus of Pan1 has been implicated in binding to the coiled-coil region of the early coat protein Ede1 (S. Barker and B. Wendland, unpublished observations). Next, adaptor proteins Yap1801/2 and Ent1/2 arrive to the patch; Pan1 binds these adaptors through the well-conserved Eps15-homology (EH) domains. Although Pan1 has two EH domains that are 72% similar, only the second domain can bind to EH ligands (Asn-Pro-Phe tripeptide motifs of the endocytic adaptor proteins Ent1/2 and Yap1801/2) (Salcini *et al.*, 1997; Paoluzi *et al.*, 1998; Wendland and Emr, 1998; Wendland *et al.*, 1999; Maldonado-Baez *et al.*, 2008). Finally, Pan1's central region can bind the adaptor/coat protein Sla2, which arrives to the patch slightly prior to Pan1 (Toshima *et al.*, 2005; 2007).

Once the early coat proteins are at the patch and the initial steps of endocytosis are complete, the late coat proteins, including Pan1, are recruited to the patch. Pan1 can form homo-dimers and homo-oligomers (Miliaras and Wendland, 2004; Pierce *et al.*, 2013). Additionally, Pan1 forms a trimeric complex with two late coat proteins, Sla1 and End3, which bind its N-terminus and central region, respectively (Tang *et al.*, 1997; 2000; Whitworth *et al.*, 2014). Throughout its N-terminus, Pan1 contains 19 Ark1/Prk1 consensus phosphorylation sites; it has been proposed that phosphorylation negatively

regulates Pan1's interactions with coat proteins and thus promotes dissociation of the coat from the vesicle (Zeng and Cai, 1999; Zeng *et al.*, 2001; Toshima *et al.*, 2005; 2007). However, recent evidence has shown that Pan1 and End3 remain tightly bound in the cytosol, thus phosphorylation may not affect this interaction (Boeke *et al.,* 2014). Before recycling back to the plasma membrane for another round of endocytosis, Pan1 must be dephosphorylated by Glc7 (Zeng *et al.*, 2007).

Lastly, Pan1 is involved with actin polymerization at the endocytic patch. Pan1 binds F-actin and the type I myosins Myo3/5 through motifs in its C-terminal region (Duncan *et al.*, 2001; Barker *et al.*, 2007). Pan1 has a weak actin nucleating promoting activity through binding of the Arp2/3 complex (Toshima *et al.*, 2005). However, Pan1 is not known to interact directly with the Wiskott-Aldrich syndrome protein (WASp) homolog, Las17, which is one of the strongest actin nucleating promoting factors at the patch (Li, 1997, Rodal et al 2003, Sun et al, 2006, Galletta et al, 2008). Pan1 and Las17 do share several common binding partners, including Arp2/3, Myo3/5, and Sla1 (Tang *et al,* 1997, 2000; Toshima *et al.,* 2005; Duncan *et al.,* 2001; Barker *et al*., 2007; Feliciano and Di Pietro, 2012). Due to Pan1's numerous interactions throughout the endocytic process, it is well suited to be key coordinator for maturation of the endocytic coat from early to late stages.

The timeline of recruitment of proteins to endocytic sites was determined by fluorescently tagging proteins and observing their localization and lifetimes at patches (Kaksonen *et al.*, 2003). To characterize the endocytic role of a protein, the gene is deleted and the differences in the behavior of other fluorescently tagged proteins at the endocytic patch are observed. Upon deletion of late coat proteins, other coat proteins

have increased lifetimes at the endocytic patch, indicating an arrest or a stall in the pathway (Kaksonen *et al.*, 2005). Additionally, actin polymerization is often aberrant, leading to increased or unregulated actin polymerization at the patch. Deletion of either Sla2 or the epsins Ent1 and Ent2 does not prevent the formation of endocytic patches, but it does halt the patch at the point of actin polymerization and causes an "uncoupling" between the forces resulting from actin polymerization and the subsequent changes of membrane shape (Kaksonen *et al.*, 2003; Skruzny *et al.*, 2012). In these mutants, short actin "comet tails" are anchored at the patch, but they are thought to be improperly attached to the membrane and thus the F-actin is unable to promote the invagination of the membrane to form a vesicle, ultimately inhibiting invagination of cargo. A distinct actin misregulation phenotype is seen with the double deletion of the Las17 inhibitors Bbc1 and Sla1 or Bbc1 and End3; Las17, the yeast homolog of WASP, is a strong Arp2/3 activator and thus without Las17 inhibition, there a significant increase of actin nucleation at endocytic sites (Naqvi *et al.*, 1998; Rodal *et al.*, 2003). These deletions do not prevent the formation of patches, but the lifetime of coat proteins is increased and is accompanied by highly exaggerated Arp2/3 actin polymerization. Large plumes of F-actin form at patches and extend far into the cell. In contrast to the Sla2/epsin deletes, the coat remains attached to both the membrane and actin, causing membrane to invaginate with the plumes, resulting in a successful endocytic event (Kaksonen *et al.*, 2005).

Due to Pan1's essential nature, deletion experiments to study the function of Pan1 have not been possible. Previous work with Pan1 mutant alleles has suggested roles for Pan1 in endocytosis, actin cytoskeletal organization, polarized cell growth, and mitochondrial sorting (Zoladek *et al.*, 1995; Tang and Cai, 1996; Wendland *et al.*, 1996;

Bidlingmaier *et al.*, 2001; Kamińska *et al.*, 2005). However, its exact mechanistic role(s) in these pathways is unknown, nor is it known which of these, if any, is Pan1's essential function(s). Pan1 binds early and late endocytic factors, thus we hypothesized that its deletion would cause misregulation of the recruitment and lifetimes of endocytic proteins at the patch. In addition, although Pan1 is itself not a strong regulator of actin polymerization, it does bind several proteins involved in actin regulation (both promotion and inhibition of actin polymerization) and therefore it is predicted that deletion could cause aberrant actin structures (Duncan *et al.*, 2001; Toshima *et al.*, 2005; Barker *et al.*, 2007). A Pan1 mutant allele, *pan1-20*, has a disrupted proline-rich domain (PRD); these cells had an increased number of invaginations, suggesting a misregulation of vesicle scission (Wendland *et al.*, 1996; Barker *et al.*, 2007). Pan1's PRD interacts with Myo3/5, which have an increased lifetime at the patch in the *pan1-20* strain (Barker *et al.*, 2007). This indicates that the Pan1 PRD is not critical for Myo3/5 recruitment to endocytic sites, but without proper binding to Pan1, their function at the endocytic site is abnormal or delayed. While this Pan1 mutant allele provided some information about the function of Pan1 at the endocytic patch, it does not completely eliminate the function of Pan1; thus, experiments using a Pan1-degron strain were imperative to uncover all of Pan1's roles at the patch.

In Pan1-AID, CME is quickly arrested and the progression between the early and late coat is delayed in the absence of the scaffolding function of Pan1. At the patches that did form, actin polymerization was aberrant, leading to the formation of dynamic actin flares containing coat components, but no invaginating membrane, suggesting a role for Pan1 in actin regulation and the coordination of coat interactions.

MATERIALS AND METHODS

Media and Growth Conditions

Yeast cells were grown in rich (yeast extract-peptone) or synthetic (yeast nitrogenous base with amino acids selection of plasmid maintenance) medium with 2% dextrose. For experiments using auxin in liquid cultures, 3-Indoleacetic acid (Sigma I2886) was added to synthetic media to a final concentration of 500μm. For experiments using auxin on solid media, 1-Naphthaleneacetic acid (Sigma N0640) was added to synthetic media to a final concentration of 1mM. Cycloheximide was added at a final concentration of 0.1M in indicated experiments.

Strains and Plasmids

The yeast strains and plasmids used in this study are listed in Tables 3.1 and 3.2, respectively. Strains were constructed using PCR-based genomic integration as described previously (Longtine *et al.,* 1998; Goldstein and McCusker, 1999; Nishimura *et al.,* 2009). DNA manipulations for plasmid construction were preformed using standard techniques, employing either T4 DNA polymerase-mediated ligations in *Escherichia coli* or homologous recombination with overlapping DNA fragments followed by plasmid rescue in *S. cerevisiae*. All restrictions enzymes were purchased from New England Biolabs (Ispwich, MA).

TABLE 3.1 Yeast strains used in this chapter

Strain	Genotype	Source
W303	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2*	Laboratory Strain
BY25598	*MATa ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ADH1-OsTIR1-9myc::URA3*	Yeast Genetic Resource

		Center
BWY1488	*MATa his3-Δ200 trp1-Δ901 leu2-3,112 ura3-52 lys2-801 suc2-Δ9 BAR1 sjl1::HIS3, sjl2::HIS3*	Laboratory Strain
BWY5393	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA*	This Study
BWY5394	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ADH1-OsTIR1-9myc::URA3 ade2::ADE2 HisΔ::URA Pan1-AID::G418*	This Study
BWY5890	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU*	This Study
BWY5891	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU*	This Study
BWY5892	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU*	This Study
BWY6051	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU Mup1-pHl::HPH*	This Study
BWY6052	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Mup1-pHl::HPH*	This Study
BWY6053	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEUMup1-pHl::HPH*	This Study
BWY6191	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU Ent2-GFP::G418*	This Study
BWY6192	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Ent2-GFP::G418*	This Study
BWY6193	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU Ent2-GFP::G418*	This Study
BWY6146	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU YAP1801-GFP::G418*	This Study
BWY6147	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU YAP1801-GFP::G418*	This Study
BWY6148	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU YAP1801-GFP::G418*	This Study
BWY6054	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEURVS167-GFP::HPH*	This Study
BWY6055	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU RVS167-GFP::HPH*	This Study

BWY6056	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU RVS167-GFP::HPH*	This Study
BWY6061	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418*	This Study
BWY6062	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418*	This Study
BWY6063	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418*	This Study
BWY6111	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU Sla2-GFP::HPH Sac6-RFP::G418*	This Study
BWY6112	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Sla2-GFP::HPH Sac6-RFP::G418*	This Study
BWY6113	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU Sla2-GFP::HPH Sac6-RFP::G418*	This Study
BWY6071	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU End3-GFP::HPH Sac6-RFP::G418*	This Study
BWY6072	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU End3-GFP::HPH Sac6-RFP::G418*	This Study
BWY6073	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU End3-GFP::HPH Sac6-RFP::G418*	This Study
BWY6190	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418, prk1::NAT*	This Study
BWY6206	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418, prk1::NAT*	This Study
BWY6207	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418, prk1::NAT*	This Study
BWY6286	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA*	This Study

	Pan1-AID::G418 Ade8Δ::LEU Sla2-GFP::HPH Sac6-RFP::G418, ede1::NAT	
BWY6349	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 ade2::ADE2 Ade8Δ::LEU Sla2-GFP::HPH Sac6-RFP::G418, ede1::NAT*	This Study
BWY6350	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Sla2-GFP::HPH Sac6-RFP::G418 ede1::NAT*	This Study
BWY6412	*MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 sla1::G418 bbc1::G418 Sac6-RFP::HIS*	This Study

TABLE 3.2 Plasmids used in this chapter

Plasmid	**Details**	**Description**	**Source**
pBW373	pRS426::GFP-PH of PLCδ	GFP-PH.426	Emr Lab

Fluorescence microscopy

GFP/RFP timelapse collections:

Log phase cells were incubated with/without 500μM IAA for 45min-1 hr at 30°C and then imaged using the Zeiss AxioObserver Yokogawa CSU-X1 spinning disk confocal. Movies were captured with 150ms-200ms exposure, 0.5-4 seconds/frame for 90-120 frames. Lifetimes of >50 individual patches for each strain were recorded and averaged together using ImageJ. Patch number per μm cell perimeter was counted for >30 cells/strain and averaged using ImageJ. Representative kymographs were generated with Zen software.

Image/Statistical Analysis

Statistical significance between populations was determined by one-way ANOVA followed by Tukey's Multiple Comparison post hoc analysis.

RESULTS

Endocytic patch initiation is independent of Pan1, while later steps require Pan1

To determine how CME is affected by the absence of Pan1, several known endocytic proteins were tagged with fluorescent markers and their patch dynamics were observed in the Pan1-AID strain after 45-60 min in the absence or presence of auxin. For all proteins tested, lifetimes and patch dynamics in control strains (WT and TIR1) in the presence and absence of auxin, as well as in Pan1-AID in the absence of auxin, were similar to previously published results (Tables 3.3 and 3.4, Figure 3.1, A and B) (Gagny *et al.*, 2000; Kaksonen *et al.*, 2003; 2005; Barker *et al.*, 2007; Stimpson *et al.*, 2009). This indicated that neither the addition of auxin, the AID tag, nor TIR1 affected endocytic dynamics when applied individually. Therefore, any patch number or lifetime discrepancies between Pan1-AID cells incubated in the presence or absence of auxin would suggest that Pan1 has a role in that protein's recruitment and regulation at the endocytic patch. If a protein localized to the patch and had a similar patch lifetime in Pan1-AID cells plus auxin, this would indicate that Pan1 was not required for that protein's recruitment and regulation at the patch.

Pan1 is not required to initiate early events in endocytosis

We first present our findings pertaining to the proteins that arrive prior to Pan1 at the endocytic patch. In Pan1-AID plus auxin cells, Ede1-GFP was observed in patches that were stable for at least 6 min, the length of the image acquisition period (Tables 3.3 and 3.4; Figure 3.1, C and D). In WT cells, Ede1-GFP binds and colocalizes with several endocytic proteins, including Pan1, and then dissociates from the endocytic patch prior to

Table 3.3 Endocytic patch lifetime(s) of fluorescently tagged proteins, Mean ± SD

	Wildtype		TIR1		Pan1-AID	
	- Auxin	**+ Auxin**	**- Auxin**	**+ Auxin**	**- Auxin**	**+ Auxin**
Ede1	77.5 ± 32.8	83.5 ± 34.2	82.4 ± 28.3	84.0 ± 30.5	82.4 ± 29.5	360 +
Yap1801	42.5 ± 10.9	44.2 ± 12.9	44.9 ± 13.6	43.3 ± 12.1	43.2 ± 11.6	67.9 ± 29.2
Ent2	54.7 ± 13.8	56.5 ± 16.2	54.1 ± 16.3	54.6 ± 12.8	53.2 ± 13.8	161.6 ± 66.2
Sla2	34.8 ± 9.0	40.1 ± 11.2	38.0 ± 10.7	40.7 ± 10.0	36.5 ± 9.9	98.6 ± 47.8
Sla1	27.8 ± 6.2	30.6 ± 7.0	27.5 ± 6.4	31.4 ± 5.6	25.1 ± 6.6	36.3 ± 17.7
End3	29.8 ± 6.8	32.8 ± 7.5	28.5 ± 6.3	29.9 ± 7.4	27.9 ± 6.0	42.5 ± 14.0
Las17	32.0 ± 8.1	34.3 ± 8.9	28.6 ± 6.7	32.1 ± 8.6	31.6 ± 9.4	60.7 ± 39.7
Myo5	10.3 ± 2.1	10.6 ± 2.2	10.4 ± 1.5	10.1 ± 1.9	9.5 ± 2.0	12.5 ± 6.7
Rvs167	11.2 ± 3.1	9.4 ± 2.3	9.9 ± 2.9	8.5 ± 2.4	10.0 ± 2.9	8.8 ± 3.9
Sac6	13.6 ± 3.8	13.4 ± 3.5	12.0 ± 2.7	14.5 ± 3.4	12.5 ± 3.9	23.3 ± 14.3 (Total)
						16. 8 ± 5.3 (Patches)
						42.8 ± 15.1 (Flares)

Table 3.4 Endocytic patch number per micron plasma membrane of fluorescently tagged proteins. Mean ± SD

	Wildtype		TIR1		Pan1-AID	
	- Auxin	**+ Auxin**	**- Auxin**	**+ Auxin**	**- Auxin**	**+ Auxin**
Ede1	0.87 ± 0.3	0.84 ± 0.1	0.85 ± 0.2	0.79 ± 0.1	0.80 ± 0.2	0.84 ± 0.2
Yap1801	0.35 ± 0.1	0.37 ± 0.1	0.33 ± 0.1	0.35 ± 0.1	0.36 ± 0.1	0.34 ± 0.1
Ent2	0.30 ± 0.1	0.29 ± 0.1	0.32 ± 0.1	0.33 ± 0.1	0.31 ± 0.1	0.31 ± 0.1
Sla2	0.57 ± 0.1	0.60 ± 0.1	0.54 ± 0.1	0.55 ± 0.1	0.51 ± 0.1	0.40 ± 0.1
Sla1	0.36 ± 0.1	0.36 ± 0.1	0.37 ± 0.1	0.34 ± 0.1	0.32± 0.1	0.10 ± 0.1
End3	0.60 ± 0.2	0.54 ± 0.1	0.53 ± 0.1	0.56 ± 0.1	0.50 ± 0.1	0.07 ± 0.1
Las17	0.41 ± 0.1	0.41 ± 0.1	0.44 ± 0.1	0.40 ± 0.1	0.36 ± 0.1	0.10 ± 0.1
Myo5	0.22 ± 0.1	0.21 ± 0.1	0.22 ± 0.1	0.21 ± 0.1	0.23 ± 0.1	0.08 ± 0.1
Rvs167	0.32 ± 0.1	0.34 ± 0.1	0.28 ± 0.1	0.24 ± 0.1	0.33 ± 0.1	0.07 ± 0.1
Sac6	0.28 ± 0.1	0.23 ± 0.1	0.29 ± 0.1	0.25 ± 0.1	0.27 ± 0.1	0.07 ± 0.1 (Total)
						0.08 ± 0.1 (Patches)
						0.01 ± 0.01 (Flares)

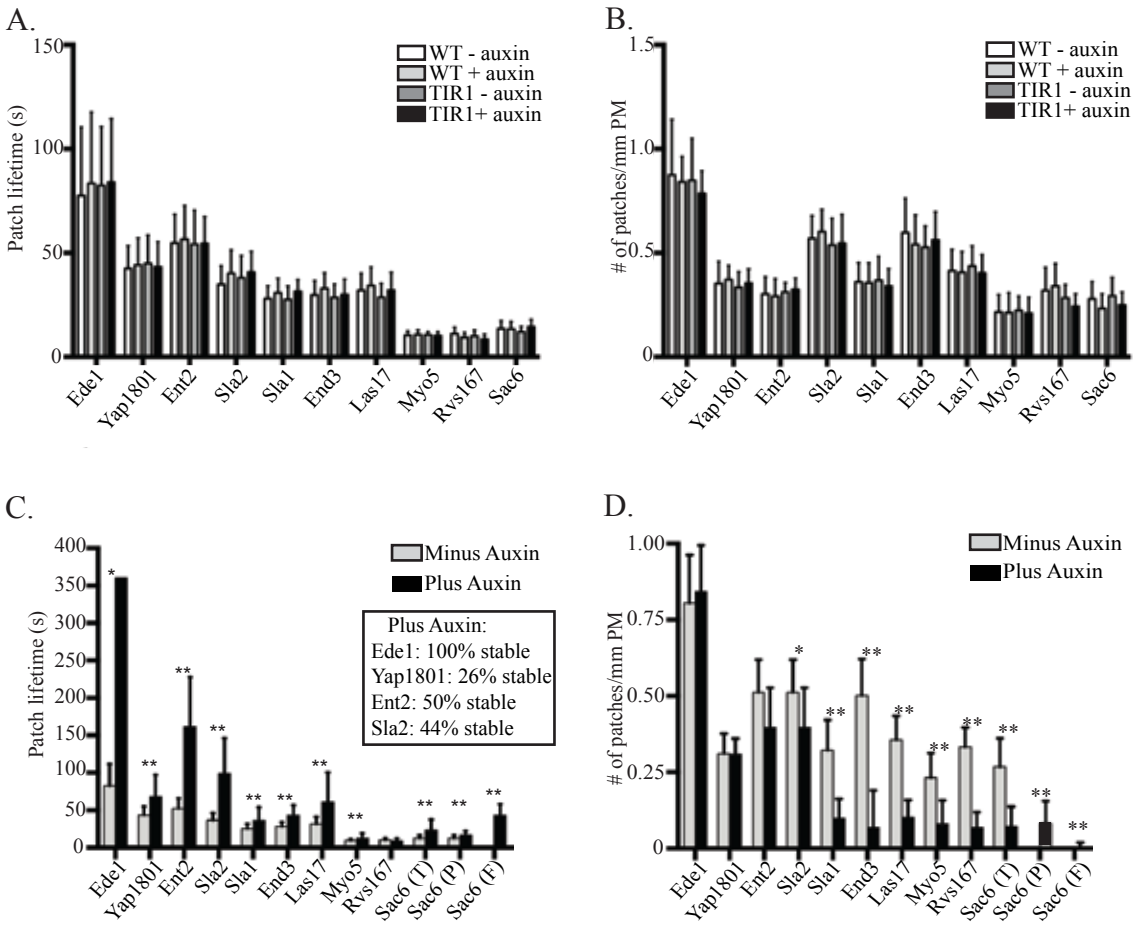


Figure 3.1 Endocytic proteins tagged with GFP have altered dynamics in Pan1-AID in the presence of auxin, but not in WT or TIR1 cells.

Patch lifetime (sec) (A and C) and number of patches per micron of plasma membrane (B and D) of endogenously GFP and RFP-tagged proteins in WT and TIR1 cells (A and B) and Pan1-AID cells (C and D) after 45-60 min ± auxin. Movies were taken with intervals of 0.5-4 s, 90-120 frames, with an exposure of 150 ms. Lifetimes: N > 50 patches/strain, percent stable is number of patches that remained present for entire duration of image acquisition. Patch #: N > 30 cells/strain. Sac6-RFP is split into patches (P), flares (F), and total (T) Sac6-RFP structures. *P ≤ 0.001, **P ≤ 0.0001, minus vs. plus auxin (Mean ± SD).

the recruitment of actin (Gagny *et al.*, 2000; Stimpson *et al.*, 2009). In Pan1-AID plus auxin, Sac6-RFP actin patches and flares did not overlap with Ede1-GFP patches even though the Ede1 patches were stable, suggesting that the early and late coat may have become unlinked (Figure 3.1 and 3.2). The adaptor proteins Yap1801 and Ent2 both arrive to the patch prior to Pan1 and bind to Pan1's second EH domain (Wendland and Emr, 1998; Wendland *et al.*, 1999). In Pan1-AID plus auxin, Yap1801-GFP and Ent2-GFP had increased lifetimes relative to controls, and 26% or 50% of patches, respectively, remained stable for the duration of image acquisition (Tables 3.3 and 3.4; Figure 3.1, C and D).

Sla2, a coat/adaptor protein, is thought to arrive slightly prior to Pan1 at the patch and binds several endocytic proteins including Pan1's central region (Toshima *et al.*, 2007). In Pan1-AID plus auxin, Sla2-GFP had an increased lifetime relative to controls, with 44% of patches remaining stable for the duration of image acquisition (Table 3.3 and 3.4, Figure 3.1 and 3.2). Although Sla2-GFP was recruited to patches, there were slightly fewer Sla2-GFP patches in the presence of auxin (Table 3.4; Figure 3.1D). Similar to WT cells, Sla2-GFP patches that were not stable moved in with the patch (Figure 3.2A).

Late arriving proteins are present at fewer patches and have extended lifetimes in the absence of Pan1

The remaining coat and actin proteins we observed all arrive at endocytic patches at the same time or after Pan1 in WT cells. In Pan1-AID plus auxin, Sla1, End3, Las17, Myo5, Rvs167, and Sac6 had decreased numbers of patches and all except Rvs167 had

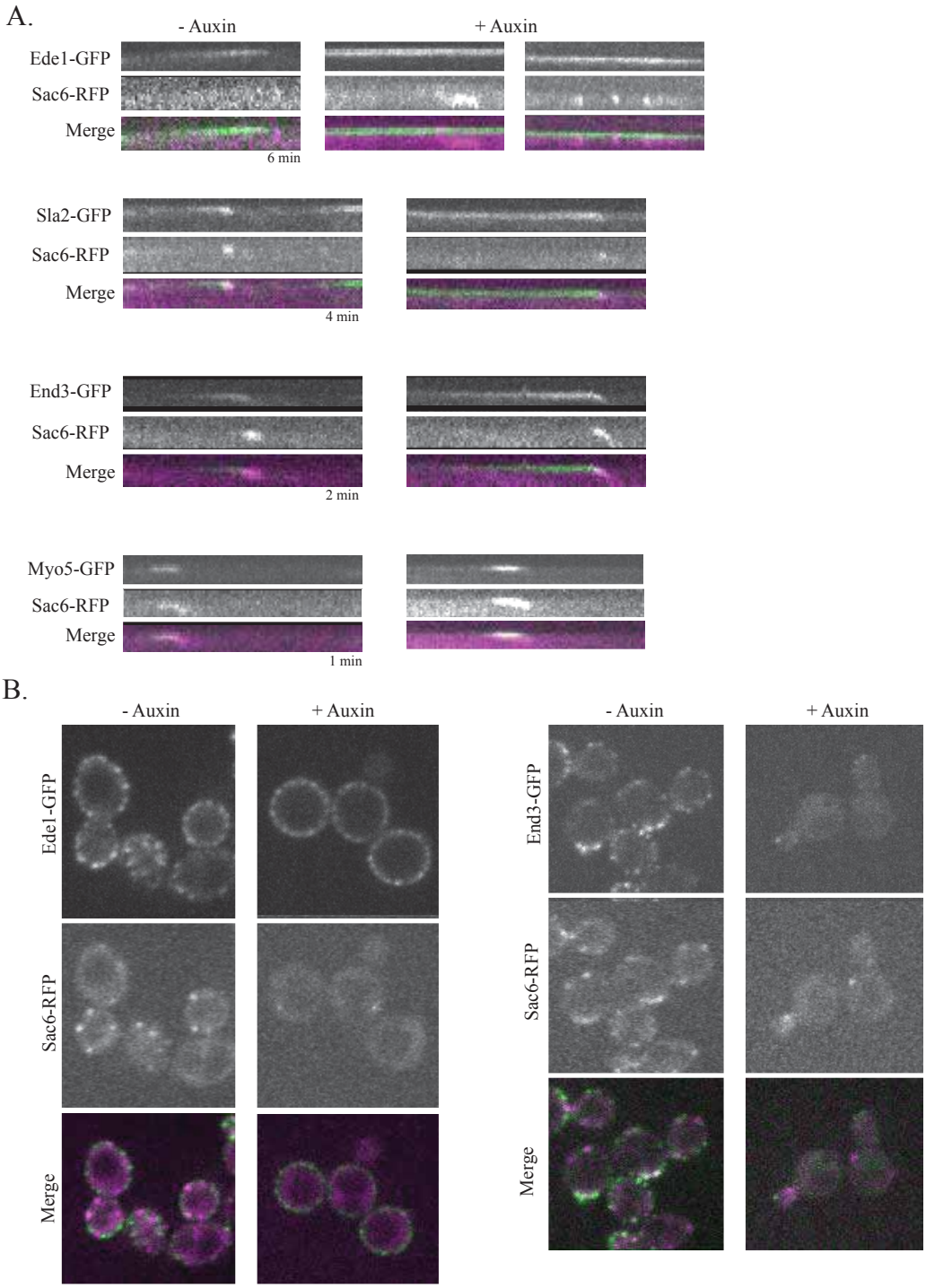


Figure 3.2 Endocytic proteins have longer lifetimes and late arriving proteins have fewer patches in Pan1-AID cells in the presence of auxin

(A) Representative kymographs of GFP-tagged proteins and Sac6-RFP in Pan1-AID cells grown in ± auxin for 45-60 min. All kymographs are oriented with cell interior at the bottom. Kymographs made from movies used for quantification in Figure 3.1. (B) Images of representative cells from Pan1-AID strains grown in ± auxin for 45-60 min. Scale bar, 2μm.

increased lifetimes at the patch relative to controls (Tables 3.3 and 3.4, Figure 3.1, C and D). Previous work has shown a decrease in patch number and an increase in Myo5 lifetime upon deletion of Pan1's PRD to which Myo5 binds (Barker *et al.*, 2007). Sla1, End3, and Myo5 bind Pan1, but they localized to endocytic patches in the absence of Pan1, so their localization to patches must depend on their interactions with other proteins and/or the membrane (Tang *et al.*, 1997; 2000; Barker *et al.*, 2007; Whitworth *et al.*, 2014). Las17 and Rvs167 are not known to bind Pan1 directly and they were also able to localize independently of Pan1. In patches that did not result in actin flares (see below), Sla2, Sla1, and End3 internalized with the patch while Myo5, Las17, and Rvs167 remained at the plasma membrane in a manner comparable to a wildtype endocytosis event.

Actin flares that contain late coat proteins, but not membrane, form in the absence of Pan1

Pan1's C-terminus is thought to contribute to actin polymerization at endocytic patches via several actin-regulatory domains and direct binding to F-actin (Duncan *et al.*, 2001; Toshima *et al.*, 2005). In Pan1-AID cells plus auxin we observed a significant decrease in the number of actin patches (Sac6-RFP was used to label branched F-actin); those that formed had an increased lifetime and some patches had the appearance of elongated structures of polymerized actin which were named "actin flares" (Tables 3.3 and 3.4; Figure 3.1, C and D, Figure 3.3, Movies 1-3). These actin flares originated from an endocytic patch and extended into the cytoplasm, sometimes flowing parallel to the plasma membrane. The coat components Sla2, Sla1, and End3 moved with the tip of the

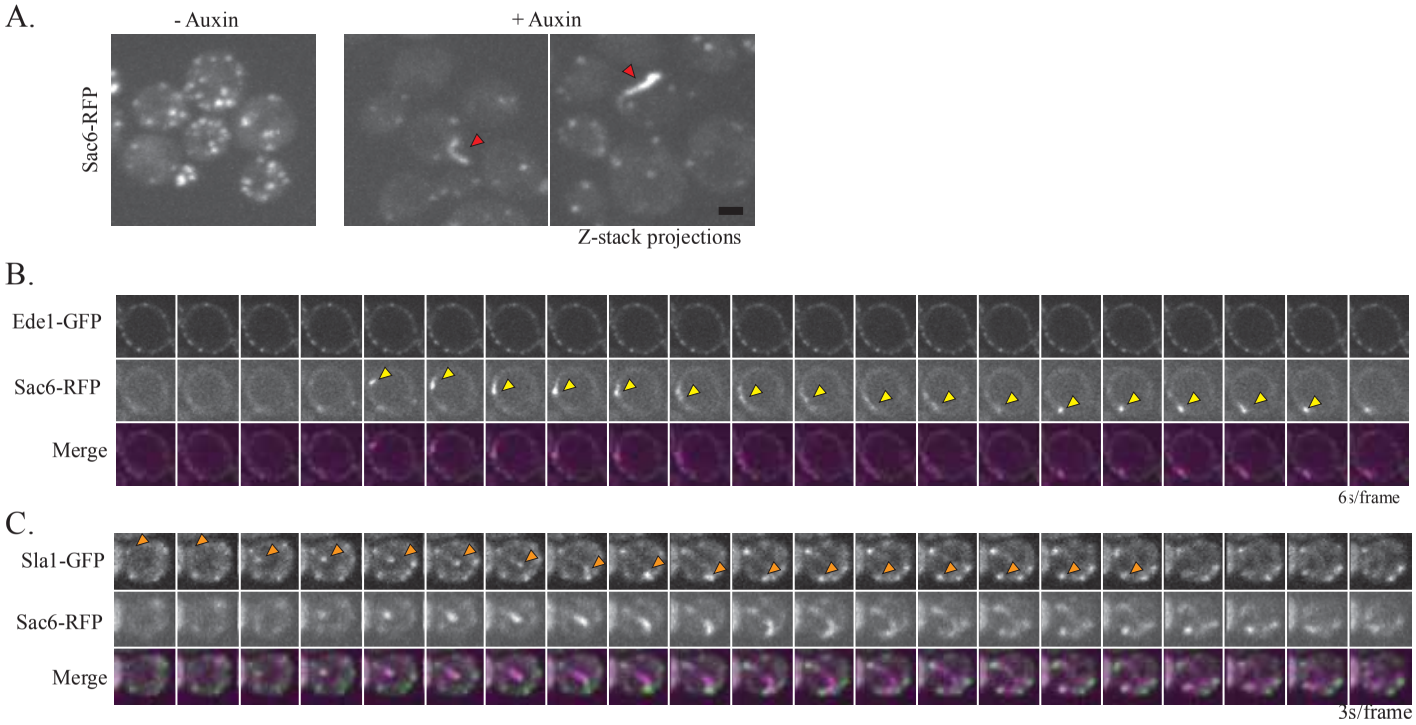


Figure 3.3 Dynamic actin flares appear in Pan1-AID cells in the presence of auxin. (A) Z-stack projections (maximum intensity) of Sac6-RFP Pan1-AID cells grown in ± auxin for45-60 min. Intervals of 0.27 μm. Red arrow: Sac6-RFP flare. (B and C) Time series of movies of Pan1-AID cells with indicated GFP/RFP proteins grown plus auxin for 45-60 min. Yellow arrow: Sac6-RFP flare moving along the PM without overlapping Ede1-GFP. Orange arrow: Sla1-GFP patch moving into the cell with a Sac6-RFP flare. Scale bar, 2μm.

actin flare. To visualize plasma membrane dynamics in relation to the actin flares, we used GFP-pleckstrin homology domain chimeras (GFP-PH), which bind the $PI(4,5)P_2$ lipids found in abundance on the plasma membrane. As a control, we confirmed that GFP-PH does label internalized membrane in synaptojanin mutants as seen in previous work (Figure 3.4A) (Stefan *et al.*, 2002). The Pan1-AID actin flares did not contain membrane (Figure 3.4, B and C), suggesting the coat/actin machinery has weakened connections to the membrane. The actin flares also did not contain internalized transmembrane cargo (Figure 3.4D). These flares differed from the aberrant actin phenotypes observed previously in other endocytic mutants. The actin-uncoupling phenotype observed in *sla2Δ* or *ent1/2Δ* cells is characterized by short actin comet tails anchored at the plasma membrane that do not invaginate the plasma membrane or coat components (Kaksonen *et al.*, 2003; Skruzny *et al.*, 2012). In contrast, the uncontrolled Arp2/3 actin plumes seen in *sla1Δ/bbc1Δ* or *end3Δ/bbc1Δ* cells are long and contain invaginated plasma membrane with coat components at the tip (Figure 3.4C) (Kaksonen *et al.*, 2005). In Pan1-AID plus auxin, the actin flares were not stable at patches, contained the coat components Sla2, Sla1, and End3, but did not contain membrane (Figure 3.3 and 3.4). The Pan1-AID actin flares were longer than the *sla2Δ* or *ent1/2Δ* actin comet tails, but not as elongated as the actin plumes seen in *sla1Δ/bbc1Δ* or *end3Δ/bbc1Δ*. It is known that Pan1 binds several actin regulators, such as the Las17 inhibitor Sla1 and the Arp2/3 activators Myo3 and Myo5 (Tang *et al.*, 2000; Duncan *et al.*, 2001; Toshima *et al.*, 2005; Barker *et al.*, 2007). Our data suggest that Pan1 may be involved in the coordination of actin regulatory elements at the patch and is important for

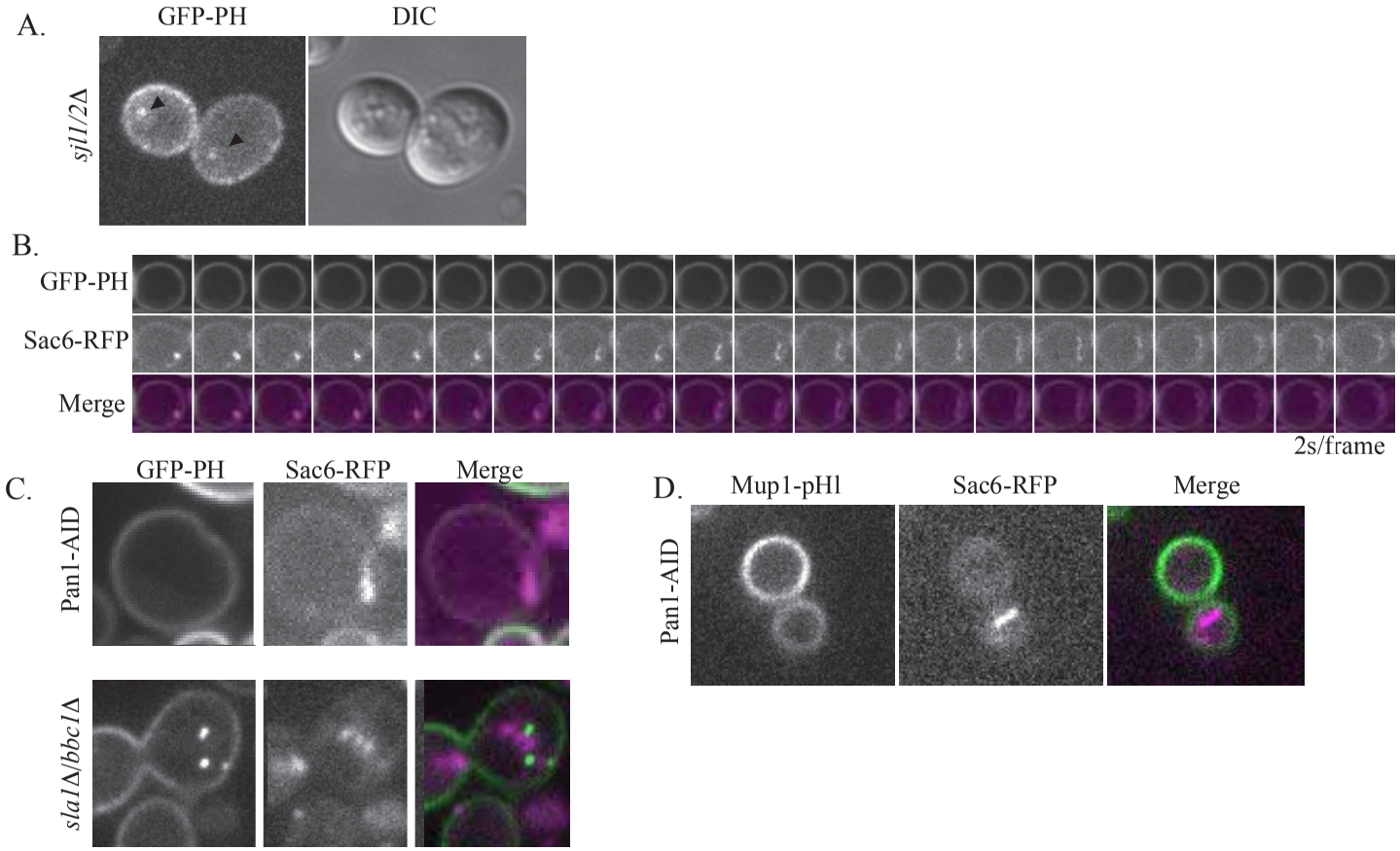


Figure 3.4 Actin flares do not contain invaginated membrane or transmembrane cargo
(A) Representative images of *sjl1/2*Δ cells with GFP-PH plasma membrane label. (B) Time series of movies of Sac6-RFP Pan1-AID cells with GFP-PH plasma membrane label; grown in plus auxin for 45-60 min. (C) Representative image of Pan1-AID and *sla1*Δ/*bbc1*Δ cells with Sac6-RFP and GFP-PH plasma membrane label; grown in plus auxin for 45-60 min. (D) Representative image of Mup1-pHl/Sac6-RFP Pan1-AID cells grown in auxin for 30 min and methionine for 1 hour.

proper linkage of the coat to the membrane in order transmit the force of actin polymerization needed to invaginate the membrane (Figure 3.5).

To briefly summarize our findings in Pan1-AID cells plus auxin, the endocytic factors that arrive prior to Pan1 (Ede1, Ent2, Yap1801, and Sla2) had longer lifetimes, with some of the patches remaining stable for the duration of the data collection period (Table 3.3 and Figure 3.1, C and D). The CME components that arrive at the same time or after Pan1 (Sla1, End3, Las17, Myo5, Rvs167, and Sac6) also had increased lifetimes whereas, unlike the earlier arriving proteins, they had decreased patch numbers (Tables 3.3 and 3.4; Figure 3.1, C and D). Overall, longer lifetimes and fewer late coat patches suggested inefficient progression of the endocytic patch to later stages and fewer productive endocytic events. The most dramatic phenotypic difference observed in the Pan1-AID strain in the presence of auxin (relative to the absence of auxin), was a significant decrease in the number of Sac6-RFP patches and the appearance of actin flares as labeled by Sac6-RFP (Figure 3.3). These flares contained late coat proteins, but not membrane or transmembrane cargo, and are thus thought not to be successful sites of endocytosis (Figure 3.3 and 3.4).

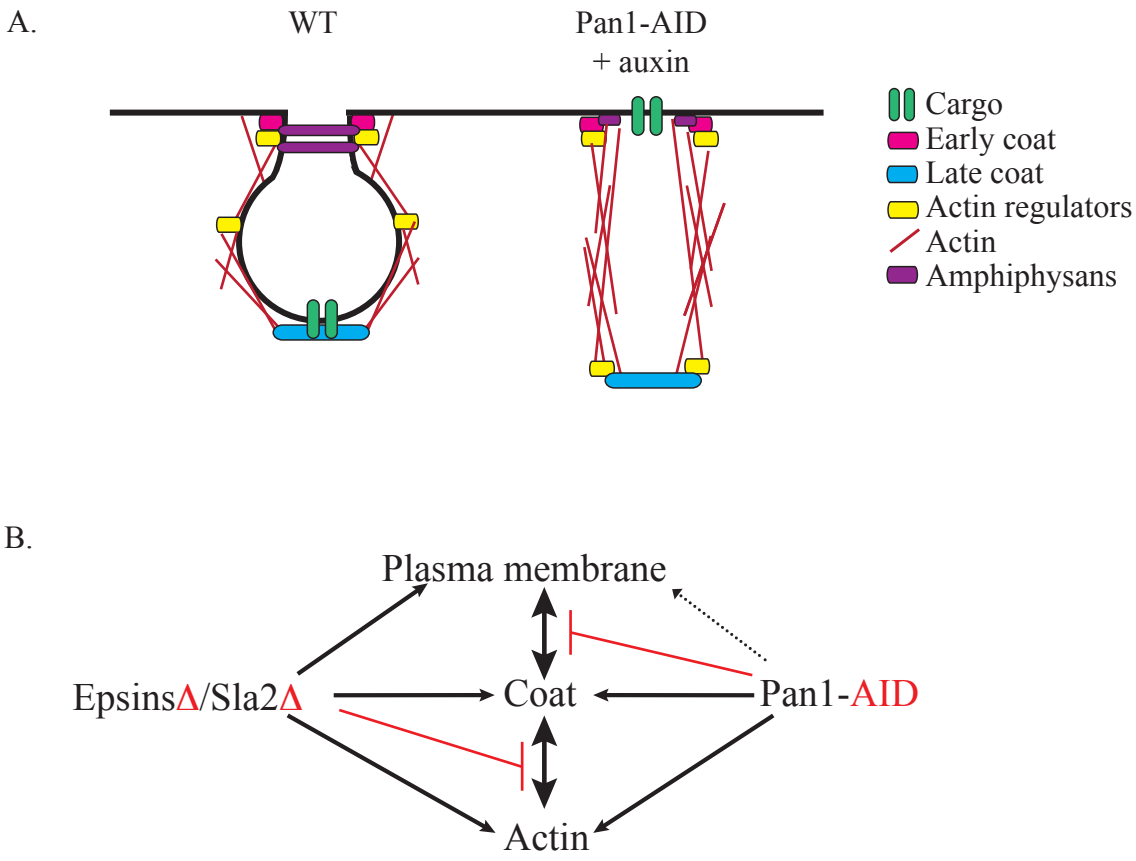


Figure 3.5 Sla2/Epsins and Pan1 strengthen critical interactions between membrane, coat, and actin in CME.
(A) Schematic of the final steps of CME in WT and Pan1-AID, + auxin cells.
(B) Model of Pan1 and Epsins/Sla2 interactions with the plasma membrane, endocytic coat, and actin machinery. EpsinΔ/Sla2Δ and Pan1-AID phenotypes shown in red. Dashed arrow represents binding between Pan1 and lipids (D. Pierce and B. Wendland, unpublished observations).

Deletion of Pan1 kinase, Prk1, in Pan1-AID cells does not restore growth or endocytic defects

Through phosphorylation and dephosphorylation of both proteins and lipids, the clathrin coat dissociates from the vesicle and proteins are recycled through the cytosol and back to the membrane to participate in further rounds of CME. Pan1 is heavily phosphorylated by the evolutionarily conserved protein kinases Ark1/Prk1, which is thought to negatively regulate Pan1's interactions with other coat proteins to promote dissociation of the endocytic protein coat (Zeng and Cai, 1999; Zeng *et al.*, 2001; Toshima *et al.*, 2005; 2007). To allow reuse at endocytic patches, Pan1 is dephosphorylated by Glc7 (Zeng *et al.*, 2007). Deletion of *PRK1* in a wild-type background results in the appearance of large cytosolic clumps of actin, but deletion in a *pan1-4* background rescues actin cytoskeletal defects (Cope *et al.* 1999; Zeng and Cai, 1999). However, *prk1Δ* does not restore the viability of a *pan1Δ* strain, suggesting that Prk1 does not play a role in Pan1's essential function (Zeng and Cai 1999). Therefore, we hypothesized that deletion of *PRK1* in the absence of Pan1 would not restore growth, but may restore some of the endocytic defects. Pan1-AID, *prk1Δ* cells were not able to grow in the presence of auxin (Figure 3.6A). Although the lifetimes of Sac6-RFP patches were slightly longer in *prk1Δ* cells, the number of patches did not change and actin clumps were observed in both conditions (Figure 3.6, B and C). These data indicate that Prk1 does not have a role in Pan1's essential condition, but may help Pan1 regulate the lifetimes of endocytic proteins.

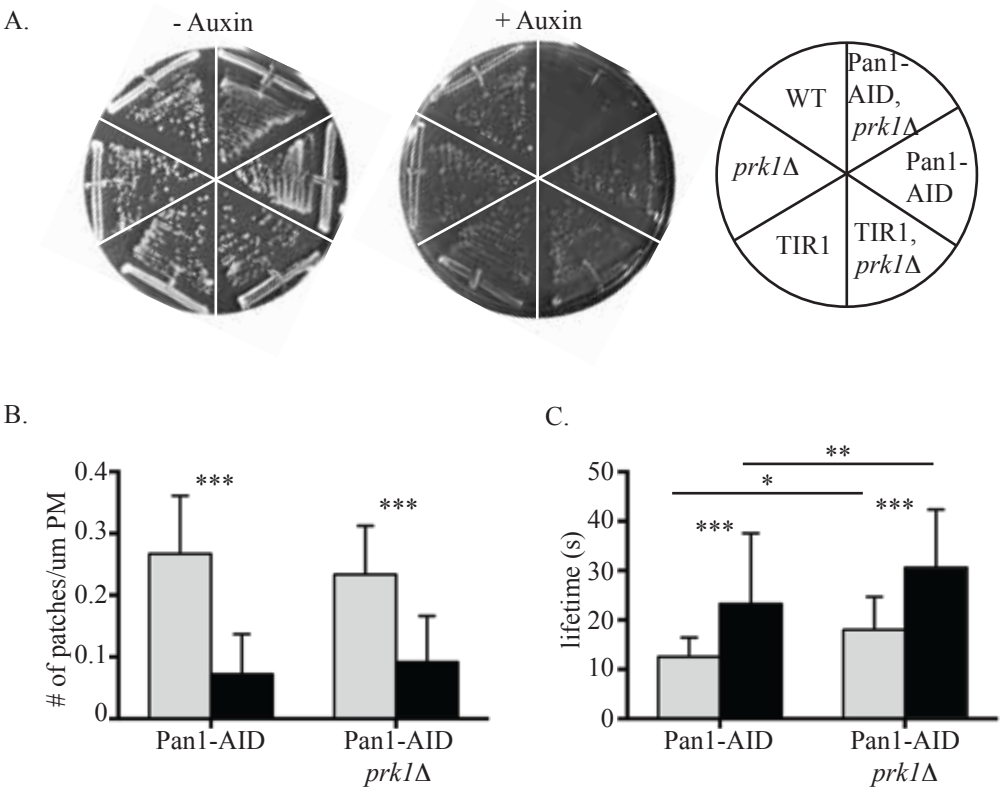


Figure 3.6 Deletion of Prk1 does not restore defects caused by loss of Pan1.
(A) WT and *prk1Δ* cells were streaked onto plates ± auxin and grown for 3 d at 30°C. (B) Number of Sac6-RFP patches per micron plasma membrane and (C) lifetime (sec) in Pan1-AID and Pan1-AID, *prk1Δ* cells incubated for 1 ± auxin. Movies were taken with intervals of 1000s, 120 frames, with an exposure of 150 ms. Patch #: N > 30 cells/condition. Lifetimes: N > 50 patches/condition. Mean ± SD.

Remaining endocytic patches do not likely contain remnant Pan1 protein

While the AID system does deplete targeted protein quickly, at the one-hour time point there may be some residual Pan1-AID protein remaining in the cell. This remaining pool of protein may localize and function at endocytic patches prior to being degraded, aiding the small percentage patches that mature and internalize normally. If this residual protein is eliminated, we hypothesized that the delayed maturation phenotypes we observed would be exacerbated in the complete absence of Pan1. If mature endocytic patches still form in the complete absence of Pan1, this indicates that endocytic patches can mature independently of Pan1, but at a severely reduced rate. To eliminate remaining Pan1-AID protein, endocytic patches were observed after 2 hours in the presence of auxin. At this time point, Pan1 protein is undetectable by western blot and about 10% of cells are dead (Figure 2.1D and 2.2B). After 2 hours in the presence of auxin, Sla1-GFP localizes to patches in similar numbers to 1 hour plus auxin (Figure 3.7A). However, the lifetime of Sla1-GFP increases even more than it did after 1 hour (Figure 3.7B). This suggests that after 1 hour, some Pan1 may be at the patches to help the coat mature, but once this pool of Pan1 is reduced even further, the lifetimes increase. Therefore, Sla1 does not depend on Pan1 to localize to patches, but it does require Pan1 to regulate its lifetime at the patch.

In AID experiments, Pan1-AID is continuously translated before being recognized by TIR1 and degraded. To eliminate this newly translated Pan1-AID protein pool, cycloheximide was added at the same time as auxin to shut-off all protein translation while degrading all previously synthesized Pan1-AID protein. Cycloheximide alone did have a slight, but not significant, impact on End3-GFP patch numbers and lifetimes; both

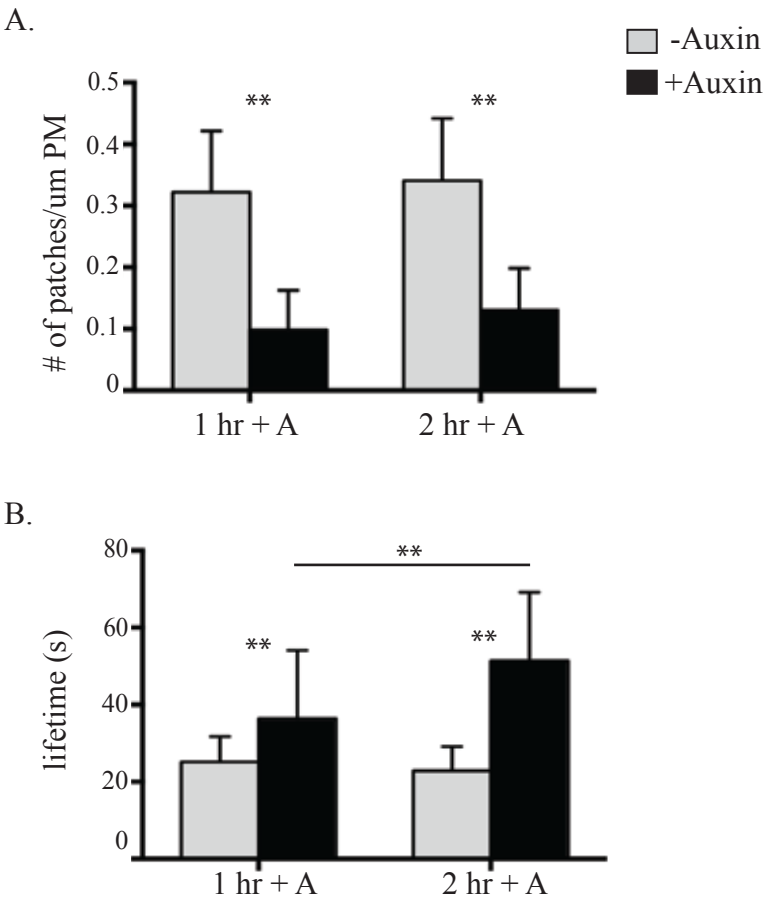


Figure 3.7 Incubating Pan1-AID cells in auxin for 2 hours does not exacerbate Sla1-GFP patch number or lifetime phenotype

(A) Number of Sla1-GFP patches per micron plasma membrane and (B) lifetime (sec) in Pan1-AID cells incubated for 1 or 2 hours ± auxin. Movies were taken with intervals of 1000s, 120 frames, with an exposure of 150 ms. Patch #: N > 30 cells/condition. Lifetimes: N > 50 patches/condition. Mean ± SD. ** = P < 0.0001

were reduced as compared to cells without auxin or cycloheximide (Figure 3.8, A and B). In the presence of auxin and cycloheximide, End3-GFP patch numbers were reduced similar to numbers observed in the absence of cycloheximide, and the lifetime increased. The lifetime of End3-GFP in the presence of auxin and cycloheximide was slightly reduced compared to auxin alone, but the trend was similar. In the presence of auxin and cycloheximide, mature endocytic patches containing late coat proteins were still observed, suggesting that these patches do not contain residual Pan1-AID protein (Figure 3.8). Therefore, these data, along with Figure 3.7, confirm that Pan1 is not necessary for late coat maturation, but it is critical for proper efficiency of this transition.

Endocytic patches are still able to form in the absence of two key endocytic regulators

Recent work implicated the early coat protein Ede1 as a critical coordinator for the early stages of endocytosis (Boeke *et al.,* 2014). It was suggested that the coiled-coil region of Ede1 needed to bind to an unknown late coat protein for proper maturation of the patch. Unpublished work from our lab found that the coiled-coil region of Ede1 binds to Pan1's N-terminus (S. Barker and B. Wendland). In the absence of Ede1, early arriving proteins have reduced or altered patch numbers, while later arriving proteins, including Sla2, Sla1, Las17, Ent1/2, have decreased lifetimes and decreased patch numbers (Kaksonen *et al.,* 2005; Reider *et al.,* 2009; Stimpson *et al.,* 2009; Carroll *et al.,* 2012). It was hypothesized that removal of both Ede1 and Pan1 would further exacerbate the endocytic defects seen in the individual mutants and perhaps inhibit all endocytic patches from forming. Deletion of *EDE1* does not restore growth of Pan1-AID cells in the

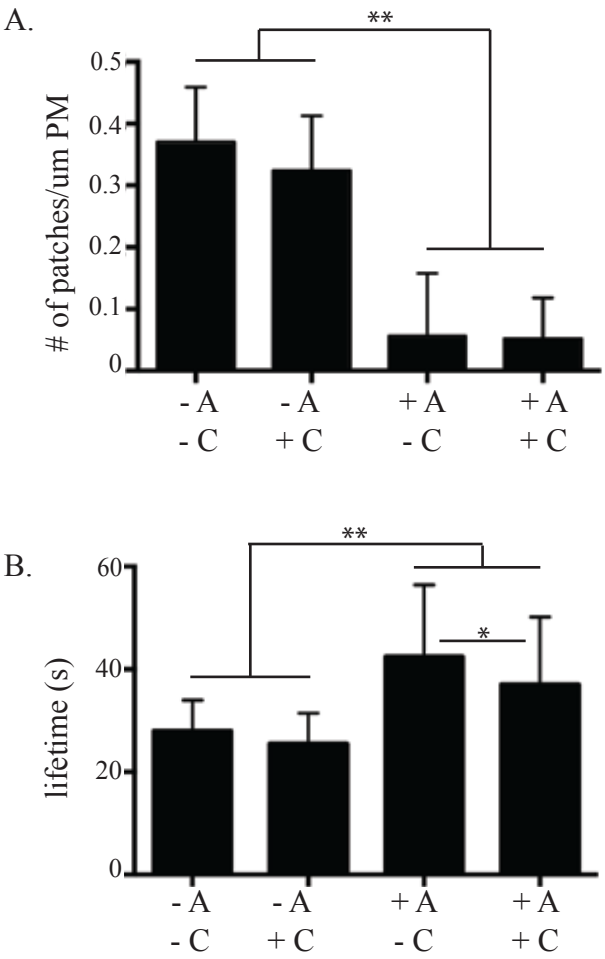


Figure 3.8 Cycloheximide does not exacerbate the phenotype of the absence of Pan1.

(A) Number of End3-GFP patches per micron plasma membrane and (B) lifetime (sec) in Pan1-AID cells incubated for 60 min ± auxin and ± 0.1M cycloheximide. Movies were taken with intervals of 1000s, 120 frames, with an exposure of 150 ms. Patch #: N > 30 cells/condition. Lifetimes: N > 50 patches/condition. Mean ± SD. * = $P < 0.05$, ** = $P < 0.0001$

presence of auxin (Figure 3.9A). In Pan1-AID *ede1Δ* cells, Sla2-GFP was able to localize to patches, but with altered dynamics as compared to Pan1-AID and *ede1Δ* alone (Figure 3.9, B and C). Both in the presence and absence of auxin, Pan1-AID *ede1Δ* cells had fewer Sla2-GFP patches than Pan1-AID cells in the absence of auxin, but there was no difference in the lifetime. This decrease in Sla2 patch number in the absence of Ede1 has been previously reported in the literature (Stimpson *et al.*, 2009). However, in Pan1-AID *ede1Δ* cells, Sla2-GFP patches did not further decrease in patch number or increase in lifetime with the addition of auxin, as seen in Pan1-AID cells. Thus, deletion of *EDE1* restored the Sla2 localization and lifetime defects due to absence of Pan1. This suggests that Ede1 and Pan1 may function together to coordinate the transition between early and late endocytic stages.

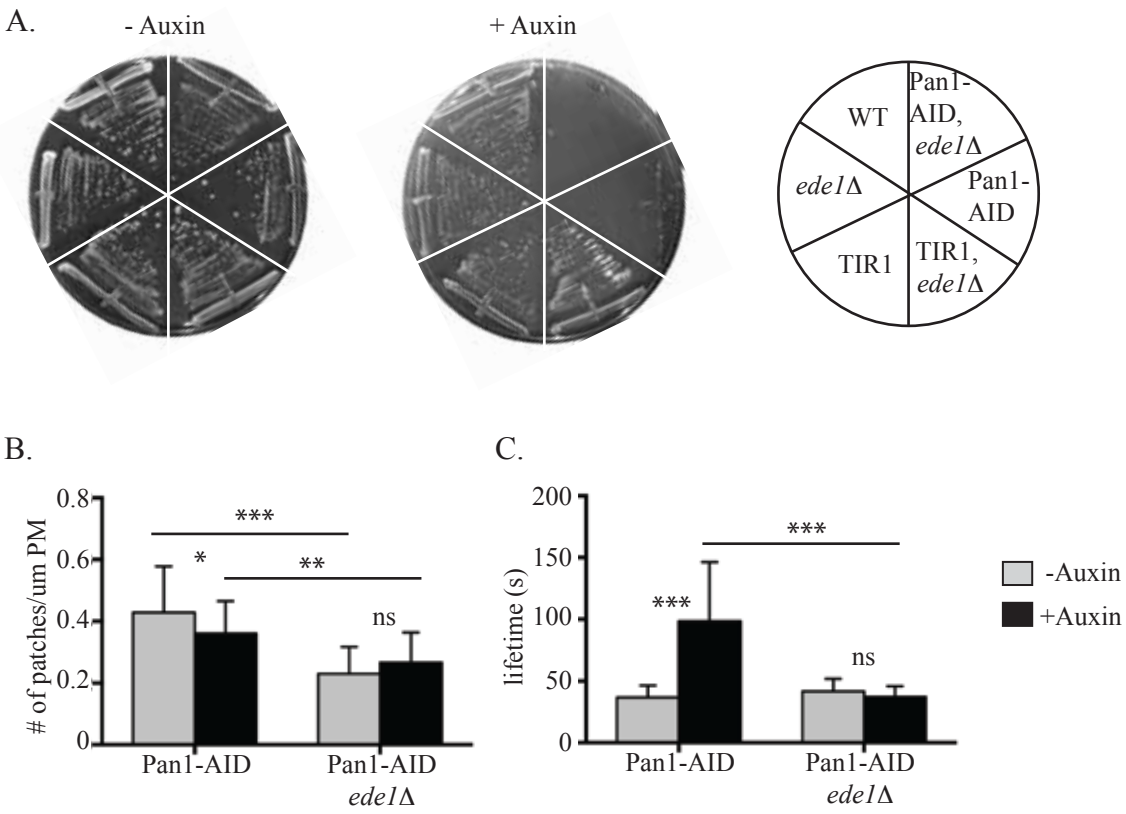


Figure 3.9 Deletion of *EDE1* partially restores defects due to absence of Pan1.
(A) WT and *ede1Δ* cells were streaked onto plates ± auxin and grown for 3 d at 30°C. (B) Number of Sla2-GFP patches per micron plasma membrane and (C) lifetime (sec) in Pan1-AID and Pan1-AID *ede1Δ* cells incubated for 1 ± auxin. Movies were taken with intervals of 1000s, 120 frames, with an exposure of 150 ms. Patch #: N > 30 cells/condition. Lifetimes: N > 50 patches/condition. Mean ± SD. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.0001, ns = no significance.

DISCUSSION

An individual CME event requires the temporal and spatial coordination of hundreds of interactions between many different proteins. Each event can be temporally separated into stages: early, late, actin polymerization, and scission. Endocytic proteins are classified according to the stage of endocytosis they arrive to the patch. Pan1 stands out among endocytic proteins because it interacts with numerous proteins throughout all stages of endocytosis, making Pan1 an ideal candidate as a scaffold to hold the coat together as it transitions between stages (Sachs and Deardorff, 1992; Wendland *et al.*, 1996). However, the exact mechanism of Pan1's function in endocytosis has never been fully realized due to experimental challenges. Pan-AID provided a unique opportunity to deduce Pan1's mechanisms of endocytic regulation and to characterize endocytosis in the absence of Pan1.

In Pan1-AID cells in the presence of auxin, both bulk and receptor-mediated endocytosis were arrested (Figure 2.3). The phenotypes of the dynamics of endocytic proteins in the absence of Pan1 can be clearly separated into the early and late stages of endocytosis. The early coat proteins localize with WT efficiency to patches, but their lifetimes are significantly extended and many patches are stable (Tables 3.3 and 3.4, Figure 3.1). Therefore, Pan1 is not required to initiate endocytic sites but is important for coat maturation. The few late coat patches that did form had extended lifetimes. The decrease in the number of late coat protein patches could either be the result of the individual proteins having a reduced binding efficiency and/or an overall decrease in the number of endocytic patches that mature to later stages. If the latter were true, this would implicate Pan1 as a critical regulator of the transition from early to late coat, where Pan1

coordinates and strengthens the interactions that are crucial for the early coat proteins to link with late coat proteins (i.e., Ede1-Pan1, Syp1-Las17, Sla2-late coat proteins and actin).

To rule out the possibility of un-degraded Pan1-AID protein functioning at these remaining mature patches, two experiments were performed to eliminate residual Pan1 protein: extension of time in auxin to degrade more Pan1-AID protein and addition of cycloheximide to inhibit protein translation. Late coat patches were found in both conditions in comparable numbers to the one hour in auxin condition, confirming that these late coat patches could form in the absence of Pan1 (Figures 3.7 and 3.8). Therefore, while Pan1 is not absolutely required for maturation to late coat, it is a very critical component for efficient endocytosis.

Abnormal actin polymerization phenotypes are commonly seen in endocytosis mutants (Kaksonen *et al.*, 2003; 2005; Skruzny *et al.*, 2012). Although Pan1's direct role in actin regulation may be small, its indirect role in the coordination of other actin inhibitors and regulators is important (Duncan *et al.*, 2001; Toshima *et al.*, 2005; Barker *et al.*, 2007). In the absence of Pan1 we observed significantly fewer actin patches, some of which developed dynamic actin flares that contain late coat proteins, but do not invaginate membrane or transmembrane cargo (Figures 3.1- 3.4). These flares represent a distinct "in-between" phenotype relative to the previously described actin comet tails and actin plumes of other endocytic mutants (Kaksonen *et al.*, 2003; 2005; Skruzny *et al.*, 2012). We hypothesize that in the absence of Pan1, early acting inhibitors of Arp2/3/actin polymerization, such as Syp1 and Sla1, cannot properly engage Las17. This leads to increased Arp2/3 actin polymerization that resembles *sla1Δ/bbc1Δ* actin plumes

(Kaksonen *et al.*, 2005). And, similar to Sla2 we propose that Pan1 is critical for coupling the membrane to the coat/actin machinery in order to transmit the forces of actin polymerization to invaginate the membrane (Figure 3.4) (Kaksonen *et al.*, 2003; Skruzny *et al.*, 2012).

Past results have shown that deletion of the Pan1 kinase Prk1 can restore actin cytoskeletal defects in a Pan1 mutant background, but cannot restore the growth defects of a *pan1Δ* strain (Zeng and Cai 1999). Deletion of *PRK1* did not rescue growth or endocytic defects caused by the absence of Pan1 (Figure 3.6). Thus, phosphorylation of Pan1 may be important for Pan1's actin regulatory role, but not its general endocytic or essential functions.

Recent work identified Ede1 as an organizer of the initiation of endocytosis and suggested that Ede1's coiled-coil region binds unknown late coat protein(s) (Boeke *et al.*, 2014). Ede1's homolog, Eps15, binds Intersectin through its coiled-coil region, similar to our unpublished work that found that Pan1 binds to Ede1's coiled-coil region (Sengar *et al.*, 1999; Wong *et al.*, 2012, S. Barker and B. Wendland, unpublished observations). Together, this strongly suggests that Pan1 and Ede1 are likely to work together to mediate endocytic progression from early to late stages. In the absence of Ede1, Sla2 patches have shorter lifetimes and fewer patches; in the absence of Pan1, Sla2 patches have increased lifetimes and fewer patches; and in absence of both, Sla2 has fewer patches with wild-type lifetime (Figure 3.9B) (Stimpson *et al.,* 2009). Thus, *ede1Δ* partially restores the defects due to the absence of Pan1. In reference to the lifetime, Ede1 may be important for the arrival of Sla2 to the patch, thus in the absence of Ede1, Sla2 arrives later, resulting in a shorter lifetime. Pan1 is important for the mid-to-late stages of

Sla2's lifetime, thus in the absence of Pan1, Sla2 patches have a longer lifetime. In the absence of both Ede1 and Pan1, Sla2 may both arrive later and leave later, resulting in a lifetime that appears normal, but is actually shifted later in the timeline of CME. Therefore, this confirms that proper coat maturation depends on both Ede1 and Pan1 for coordination of the early, middle, and late stages of CME.

Our study of Pan1 confirmed that Pan1 is an essential endocytic scaffold that regulates the interactions important for transitions between endocytic stages: the early coat to the late coat, actin regulatory proteins to actin machinery, and the membrane to the coat/actin machinery (Figure 3.5). Further studies are required to determine which regions of Pan1 are important for its endocytic role (discussed in Chapter 4) and what other proteins interact with Pan1 at each transition point.

CHAPTER 4: DEFINING THE REGIONS OF PAN1 CRITICAL FOR ITS VARIOUS FUNCTIONS

Some of the work presented in this chapter is published at Molecular Biology of the Cell (Bradford *et al.,* 2015).

ABSTRACT

Pan1 is a large protein that has several known conserved binding domains, including two Eps15-homology domains (EH), coiled-coiled regions, an F-actin binding domain, and a proline-rich domain. The central region of Pan1 has remained elusive due to the experimental difficulties of working with an unstructured amino acid sequence. All previous work defining Pan1's regions has been completed using Pan1 mutant alleles that depend on temperature sensitivity; a variable that can alter endocytic phenotypes. Additionally, the work has all been done in reference to Pan1's role in CME, leaving little to no evidence of which regions are important for Pan1's essential role. Thus, a complete, unbiased, study of Pan1 regional functionality for CME and viability is needed to determine which interactions and motifs are important for either function and whether or not these functions could be separated.

By expressing various regions of Pan1 on single-copy plasmids in Pan1-AID, it was determined which regions of Pan1 are crucial for its various functions. We identified a region of Pan1 spanning the second EH domain and central region that was critical for viability and endocytosis. Additionally, we defined regions of Pan1 that supported viability and not endocytosis, suggesting Pan1 may have an additional role(s) independent of endocytosis.

INTRODUCTION

Pan1 and its mammalian homolog, Intersectin, are both large proteins that contain several binding sites enabling them to interact with numerous proteins throughout the endocytic process, thus giving rise to their characterization as scaffolding proteins (Sachs and Deardorff, 1992; Wendland *et al.*, 1996, Yamabhai *et al.*, 1998; Hussain *et al.*, 1999). Much has been done to define the various regions, motifs, and binding sites within the proteins; however, due to their essential nature, a more careful definition of their functions was technically difficult, if not impossible, in the past. The development of the Pan1-AID strain allows, for the first-time, the ability to observe the contribution of each region and motif of Pan1 in live cells.

Pan1 is a 1480 amino acid protein containing two N-terminal Eps15-homology (EH) domains, a central largely disordered region containing several coiled-coils, and a C-terminal actin regulatory domain. The N-terminus is made up of two long repeat regions (LR1 and LR2) that each contain an EH domain. The two EH domains are 72% similar, yet only the second domain can bind to EH ligands (Asn-Pro-Phe (NPF) tripeptide motifs of the endocytic adaptor proteins Ent1/2 and Yap1801/2) (Salcini *et al.*, 1997; Paoluzi *et al.*, 1998; Wendland and Emr, 1998; Wendland *et al.*, 1999; Maldonado-Baez *et al.*, 2008). Pan1 forms a complex with Sla1 and End3, which bind its N-terminus and central region, respectively (Tang *et al.*, 1997; 2000; Whitworth *et al.*, 2014). Through its central region, Pan1 can form homodimers and homo-oligomers and binds the coat protein Sla2 (Miliaras and Wendland, 2004; Toshima *et al.*, 2005; 2007; Pierce *et al.*, 2013). In addition, it has been proposed that the central region contains three nuclear localization signals (NLS) and a lipid-binding region (Kamińska *et al.*, 2007, D.

Pierce and B. Wendland, unpublished results). Pan1 regulates actin polymerization through motifs in its C-terminal region, which bind F-actin and the type I myosins Myo3/5 (Duncan *et al.*, 2001; Barker *et al.*, 2007). Through binding the Arp2/3 complex, the C-terminus has a weak actin nucleating promoting activity (Toshima *et al.*, 2005). The C-terminus has also been implicated in binding to the coiled-coil region of the early coat protein Ede1 (S. Barker and B. Wendland, unpublished observations). Additionally, Pan1 is phosphorylated by the evolutionarily conserved protein kinases Ark1/Prk1 through 19 consensus phosphorylation sites; it has been proposed that this phosphorylation negatively regulates Pan1's interactions with other coat proteins and thus promotes dissociation of the coat from the vesicle (Zeng and Cai, 1999; Zeng *et al.*, 2001; Toshima *et al.*, 2005; 2007). Before recycling back to the plasma membrane for another round of endocytosis, Pan1 must be dephosphorylated by Glc7 (Zeng *et al.*, 2007). Due to Pan1's numerous interactions throughout the endocytic process, it is well suited to be a key coordinator for maturation of the endocytic coat from early to late stages.

Recent work with Pan1 containing mutated or deleted EH domains found that Pan1's EH domains are not critical for patch formation or efficient endocytosis, but may be important for the patch lifetime regulation of some coat proteins (Suzuki *et al.*, 2011). The mild phenotype of these mutant alleles is most likely due to the redundancy of EH domains in End3 and Ede1, which are also found at endocytic patches. In fact, when all EH domains in these three proteins are mutated, endocytic efficiency is significantly decreased. In these EH mutants, early coat proteins, such as Syp1, had a similar number of patches but with an increased lifetime, while late coat proteins, such as Abp1, had a decreased number of patches and a similar lifetime to WT. These data suggest that while

patches can form in the absence of functional EH domains, there is a decreased success rate of endocytic internalization.

The Pan1-AID strain is an ideal system to test the functional important of the various Pan1 regions and domains. Previous attempts to define regions depended on plasmid shuffling experiments in a *pan1Δ* background strain; thus these experiments were limited to study of portions of Pan1 that could support viability (Sachs and Deardorf 1992, Miliaras *et al.,* 2004, Duncan *et al.,* 2001, Suzuki *et al*., 2012). In Pan1-AID, full-length Pan1-AID protein was acutely depleted within one hour by the addition of auxin, allowing characterization of the functionality of the plasmid-expressed Pan1 fragment. Using this method, it was determined that the C-terminal actin-regulatory region of Pan1 is not critical for patch localization, CME, or viability. Portions of N-terminus and central region were necessary for localization, CME, and viability. Additionally, two fragments were found that restore viability, but not CME, suggesting they may function in Pan1's essential function, which can be separated from its endocytic role.

MATERIALS AND METHODS

Media and Growth Conditions

Yeast cells were grown in rich (yeast extract-peptone) or synthetic (yeast nitrogenous base with amino acids selection of plasmid maintenance) medium with 2% dextrose. For experiments using auxin in liquid cultures, 3-Indoleacetic acid (Sigma I2886) was added to synthetic media to a final concentration of 500μm. For experiments using auxin on solid media, 1-Naphthaleneacetic acid (Sigma N0640) was added to synthetic media to a final concentration of 1mM.

Strains and Plasmids

The yeast strains and plasmids used in this study are listed in Tables 4.1 & 4.2, respectively. Strains were constructed using PCR-based genomic integration as described previously (Longtine *et al.,* 1998; Goldstein and McCusker, 1999; Nishimura *et al.,* 2009). DNA manipulations for plasmid construction were preformed using standard techniques, employing either T4 DNA polymerase-mediated ligations in *Escherichia coli* or homologous recombination with overlapping DNA fragments followed by plasmid rescue in *S. cerevisiae*. All restrictions enzymes were purchased from New England Biolabs (Ispwich, MA).

TABLE 4.1 Yeast strains used in this chapter

Strain	Genotype	Source
BWY5892	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU*	This Study
BWY6232	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU Sac6-RFP::G418*	This Study
BWY6422	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-*	This Study

	100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418 end3::NAT	
BWY6423	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU Sla1-GFP::HPH Sac6-RFP::G418 end3::NAT*	This Study
BWY6502	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU Mup1-pHl::HPH end3::NAT*	This Study
BWY6564	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA Pan1-AID::G418 Ade8Δ::LEU, end3::NAT*	This Study

TABLE 4.2 Plasmids used in this chapter

Plasmid	Details	Description	Source
pRS414	CEN, TRP1		Laboratory plasmid
pBW626	pRS414::PAN1 (CEN, TRP1)	pPan1.414	Sachs et al., 1992
pBW628	pRS414::pan1(AA1-97, 390-1480) (CEN, TRP1)	pPan1 AA: 1-97, 390-1480.414	Sachs et al., 1992
pBW630	pRS414::pan1(AA1-855) (CEN, TRP1)	pPan1 AA: 1-855.414	Sachs et al., 1992
pBW1438	pRS414::pan1 W312A W642A (CEN, TRP1)	pPan1-EH1/2.414	Laboratory plasmid
pBW1443	pRS414::PAN1-GFP (CEN, TRP1)	pPan1-GFP.414	Laboratory plasmid
pBW2308	pRS415::pan1 K/R→E (CEN, LEU2)	pPan1-K/R→E.415	Laboratory plasmid
pBW2309	pRS415::pan1 K/R→A (CEN, LEU2)	pPan1-K/R→A.415	Laboratory plasmid
pBW2508	pRS414::pan1(AA250-855) (CEN, TRP1)	pPan1 AA: 250-855.414	This study
pBW2535	pRS414::pan1 (AA250-855 W312A) (CEN, TRP1)	pPan1 AA: 250-855 EH1.414	This study
pBW2537	pRS414::pan1 (AA250-855 W312A W642A) (CEN, TRP1)	pPan1 AA: 250-855 EH1/2.414	This study
pBW2550	pRS415::pan1 (AA402-1050 K/R→E) (CEN, LEU2)	pPan1 AA: 402-1050 K/R→E.415	This study
pBW2551	pRS415::pan1 (AA402-1050 K/R→E) (CEN, LEU2)	pPan1 AA: 402-1050 K/R→A.415	This study
pBW2581	pRS414::pan1(AA1-702)-GFP (CEN, TRP1)	pPan1 AA: 1-702-GFP.414	This study

pBW2582	pRS414::pan1(AA1-1050)-GFP (CEN, TRP1)	pPan1 AA: 1-1050-GFP.414	This study
pBW2583	pRS414::pan1(AA702-1480)-GFP (CEN, TRP1)	pPan1 AA: 702-1480-GFP.414	This study
pBW2584	pRS414::pan1(AA1050-1480)-GFP (CEN, TRP1)	pPan1 AA:1050-1480-GFP.414	This study
pBW2594	pRS414::pan1(AA702-1050)-GFP (CEN, TRP1)	pPan1 AA: 702-1050-GFP.414	This study
pBW2597	pRS414::pan1(AA1-97,402-855) (CEN, TRP1)	pPan1 AA: 1-97, 402-855.414	This study
pBW2618	pRS414::pan1 (AA250-855 W642A) (CEN, TRP1)	pPan1 AA250-855 EH2.414	This study
pBW2623	pRS414::pan1(AA402-1050)-GFP (CEN, TRP1)	pPan1 AA: 402-1050-GFP.414	This study
pBW2624	pRS414::pan1(AA1-855)-GFP (CEN, TRP1)	pPan1 AA: 1-855-GFP.414	This study
pBW2626	pRS414::pan1(AA402-855)-GFP (CEN, TRP1)	pPan1 AA: 402-855-GFP.414	This study
pBW2627	pRS414::pan1(AA1-97, 402-855)-GFP (CEN, TRP1)	pPan1 AA: 1-97, 402-855-GFP.414	This study
pBW2629	pRS414::pan1(AA1-97, 402-855 W642A) (CEN, TRP1)	pPan1 AA: 1-97, 402-855 EH2.414	This study
pBW2642	pRS414::pan1(AA1-390, 680-1480) (CEN, TRP1)	pPan1 AA: 1-390, 680-1480.414	This study
pBW2645	pRS414::pan1(AA402-855) (CEN, TRP1)	pPan1 AA: 402-855.414	This study
pBW2667	pRS414::pan1-15TA (CEN, TRP1)	pPan1-15TA.414	This study
pBW2670	pRS414::pan1(AA402-1050 K/R→E) (CEN, TRP1)	pPan1 AA:402-1050 K/R→E.414	This study
pBW2671	pRS414::pan1-K/R→A (CEN, TRP1)	pPan1 K/R→A	This study
pBW2673	pRS414::pan1(AA402-1050 K/R→A) (CEN, TRP1)	pPan1 AA:402-1050 K/R→A.414	This study
pBW2675	pRS414::pan1 (250-855 15TA) (CEN, TRP1)	pPan1 AA:250-855 15TA.414	This study
pBW2679	pRS414::pan1 (402-1050 15TA) (CEN, TRP1)	pPan1 AA:402-1050 15TA.414	This study
pBW2696	pRS414::pan1(AA1-401, 856-1480 (CEN, TRP1)	pPan1 AA: 1-401, 856-1480.414	This study
pBW2723	pRS414::pan1(AA402-1050) (CEN, TRP1)	pPan1 AA: 402-1050.414	This study
pBW2779	pRS414::pan1 (AA402-1050 W642A) (CEN, TRP1)	pPan1 AA: 402-1050 EH2.414	This study
pBW2780	pRS414::pan1(AA402-1480) (CEN, TRP1)	pPan1 AA: 402-1480.414	This study

pBW2789	pRS414::pan1(AA1-97, 680-1480) (CEN, TRP1)	pPan1 AA: 1-97, 680-1480.414	This study
pBW2795	pRS414::pan1(AA1-702) (CEN, TRP1)	pPan1 AA: 1-702.414	This study
pBW2797	pRS414::pan1(AA250-855)-GFP (CEN, TRP1)	pPan1 AA: 250-855-GFP.414	This study
pBW2814	pRS414::pan1 W312A W642A-GFP (CEN, TRP1)	pPan1-EH1/2-GFP.414	This study
pBW2815	pRS414::pan1 (1-97, 402-855 W642A)-GFP (CEN, TRP1)	pPan1 AA: 1-97, 402-855 EH2-GFP.414	This study
pBW2817	pRS414::pan1 (250-855 W312A)-GFP (CEN, TRP1)	pPan1 AA: 250-855 EH1-GFP.414	This study
pBW2818	pRS414::pan1 (250-855 W642A)-GFP (CEN, TRP1)	pPan1 AA: 250-855 EH2-GFP.414	This study
pBW2819	pRS414::pan1 (250-855 W312A W642A)-GFP (CEN, TRP1)	pPan1 AA: 250-855 EH1/2-GFP.414	This study
pBW2841	pRS414::pan1(AA1-401, 856-1480)-GFP (CEN, TRP1)	pPan1 AA:1-401, 856-1480-GFP.414	This study
pBW2842	pRS414::pan1 W312A-GFP (CEN, TRP1)	pPan1-EH1-GFP.414	This study
pBW2844	pRS414::pan1(AA1-401, 856-1480 W312A)-GFP (CEN, TRP1)	pPan1 AA:1-401, 856-1480 EH1-GFP.414	This study
pBW2911	pRS414::pan1 (402-1050 W642A)-GFP (CEN, TRP1)	pPan1 AA: 402-1050 EH2-GFP.414	This study

Protein Expression

TCA Precipitation**:**

Mid to late-log phase cells -/+ Auxin at indicated time points were harvested and resuspended in 1ml of 10% trichloroacetic acid (TCA) with 1mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, a protease inhibitor), incubated on ice for 20 min, and precipitates were centrifuged at 14,000 rpm for 10 min at 4°C. Precipitates were washed twice with cold acetone, resuspended by sonication, and air-dried. Protein samples were processed for SDS-PAGE separation by adding 2x protein sample buffer with 1mM AEBSF and solubilized by bead disruption.

SDS-PAGE and Immunoblotting:

Proteins were separated on polyacrylamide mini gels (7.5%) at 27mA in SDS running buffer (3mM SDS, 25mM Tris base, 192mm glycine) and then transferred onto nitrocellulose membranes at 80V for 90 min in cold transfer buffer (20% methanol, 0.0375% SDS, 48mM Tris base, 30mM glycine). The membranes were blocked in 5% milk in TBST (10mM Tris, pH 7.5, 0.25M NaCl, 0.025% Tween-20). Blots were incubated in the specified primary antibody overnight at the following concentrations: Rabbit-anti-Pan1 was a gift from D.G. Drubin and was used at 1:10,000; Rabbit-anti-GFP is from Molecular Probes and was used at 1:5,000. The blots were washed 3 times in TBST, incubated with secondary antibodies conjugated to HRP (Pierce, Rockford, IL) diluted 1:2,000 in milk solution for 45 min. Blots were washed again 3 times in TBST, and then developed with chemiluminescent substrate (Solution 1:2.5mM Luminol, 400uM paracoumaric acid, 100mM Tris-HCl, pH 8.5. Solution 2: 5.4mM H_2O_2, 100mM Tris-HCl, pH 8.5) for 2 min at room temperature. Chemiluminescence images were acquired using a FluorChem M FM0455 imager.

Growth Assays

Serial Dilutions: Log phase cells were diluted to OD600 of 0.25, from which four 1:5 serial dilutions were plated on YNB-tryptophan minus or plus 1mm NAA and grown for three days at 30°C.

Propidium Iodide Staining

Aliquots of cells from each culture were taken at the indicated times after incubation in minus or plus 500μm IAA at 30°C and labeled with 1μg/ml propidium iodide. 5,000 cells were immediately analyzed on a BD FACS-Calibur (Becton Dickinson & Co., Mountain View, CA) flow cytometry system to quantify the population of cells with fluorescent propidium iodide staining.

Fluorescence microscopy

Mup1-pHl:

Mup1-pHlourin images were collected using a Zeiss Axiovert 135TV inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a Sensicam QE CCD camera (Cooke, Romulus, MI), Zeiss 100x 1.4 NA Plan-Apochromat objective, motorized filter wheels, fluorescein isothiocynate (FITC) and Texas Red filter sets (Semrock, Rochester, NY), and Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO). Images were captured with 500ms exposure, identical binning, intensification and illumination intensity. For quantification of fluorescence intensity, 16-bit image files were analyzed in Image J v1.41n. Background subtraction was performed before measurement of integrated density, and values were corrected for cell size.

To perform kinetic analysis of endocytosis, cells expressing Mup1-pHlourin were grown overnight in synthetic YNB media lacking methionine (YNB-met). Cells were then diluted to a density of 0.35 OD/ml in YNB-met, and were grown to a density of 0.6 OD/ml. Cells were then seeded onto concanavalin A-coated 8-well glass-bottomed chamber slides (LabTek, Scotts Valley, CA) containing YNB-Met and were allowed to

settle before imaging. Cells were/were not treated with 500μM IAA for 30 min, then methionine was added at a concentration of 20μg/ml and images were captured after 30 min or one hr. During image acquisition, cells were maintained at a constant temperature of 30°C. Experiments with Pan1 plasmids were performed in YNB-trp-met media.

GFP/RFP timelapse collections:

Log phase cells were incubated with/without 500μM IAA for 45min-1 hr at 30°C and then imaged using the Zeiss AxioObserver Yokogawa CSU-X1 spinning disk confocal. Movies were captured with 150ms-200ms exposure, 0.5-4 seconds/frame for 90-120 frames. Lifetimes of >50 individual patches for each strain were recorded and averaged together using ImageJ. Patch number per μm cell perimeter was counted for >30 cells/strain and averaged using ImageJ. Representative kymographs were generated with Zen software.

Image/Statistical Analysis

Statistical significance between populations was determined by one-way ANOVA followed by Tukey's Multiple Comparison post hoc analysis.

RESULTS

Regions of Pan1 important for localization to the endocytic patch

Pan1's central region is critical for localization and stabilization at endocytic patches

We predicted that Pan1 must localize to the patch in order to perform its endocytic function. We identified the regions of Pan1 that are critical for proper Pan1 localization to the endocytic patch by C-terminally tagging Pan1 fragments with GFP and observing their localization. Pan1 binds several other endocytic proteins, some of which arrive to the patch before Pan1 and may recruit Pan1. Pan1 also binds itself, so by expressing the fragments in the Pan1-AID strain in the absence and presence of auxin, we were able to determine if the presence of full-length Pan1 was necessary for proper localization of the fragments (Miliaras and Wendland, 2004; Toshima *et al.*, 2005; Pierce *et al.*, 2013). All Pan1 fragments used expressed protein of expected size in both the absence and presence of auxin (Figure 4.1).

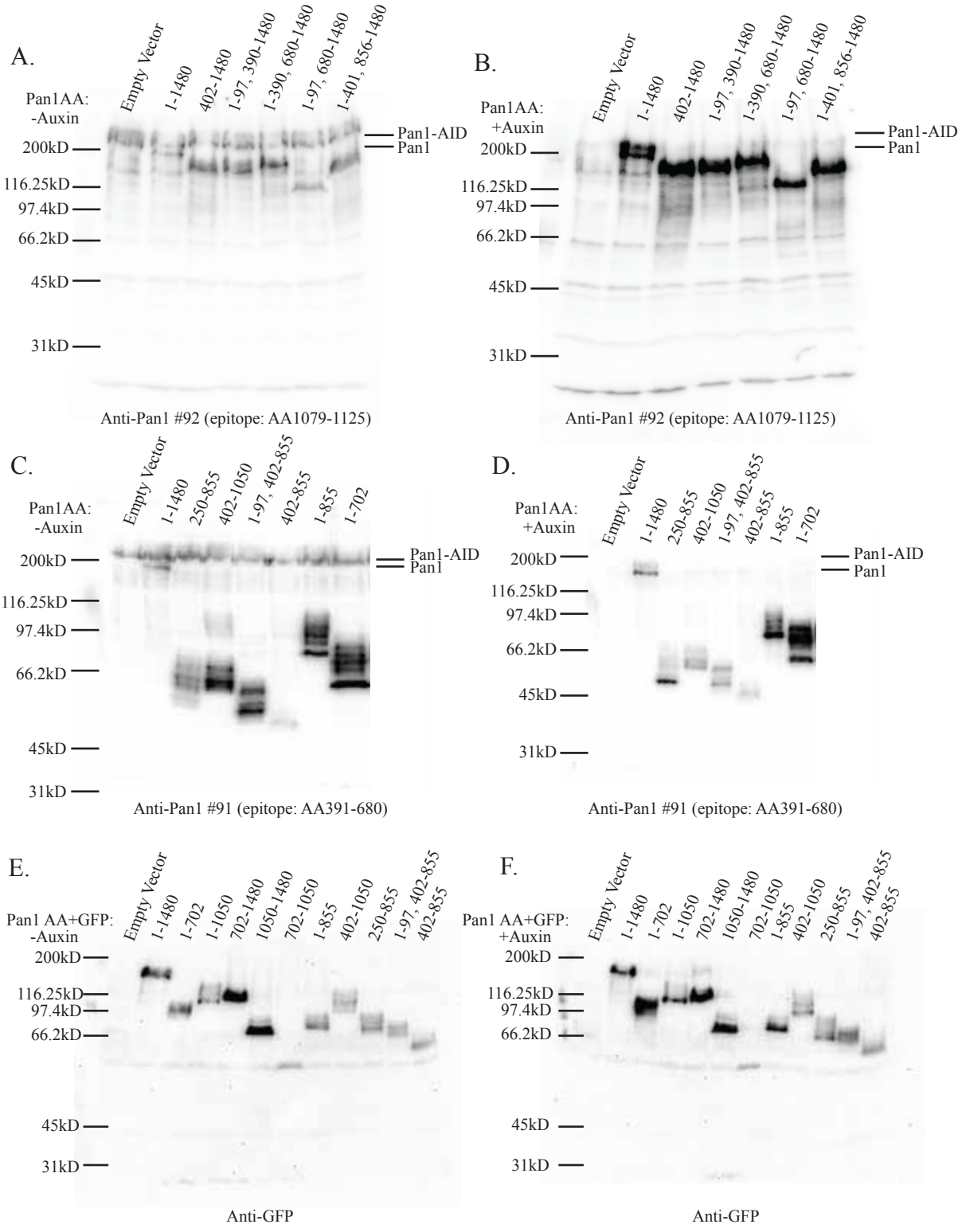


Figure 4.1 All plasmids used in this study express protein of expected mass. (A-F) Immunoblots detecting Pan1 in cell lysates of Pan1-AID cells containing indicated plasmids grown at 30°C ± auxin for 2 h.

All Pan1-GFP fragments tested were able to localize to endocytic patches in the absence and presence of auxin except for Pan1AA402-855-GFP and Pan1AA1050-1480-GFP (Figure 4.2). This confirmed that the C-terminal actin regulatory region of Pan1 was not necessary for localization, and also could not localize to patches without the *in cis* support of the central region. The N-terminal (Pan1AA1-702-GFP) and central region (Pan1AA702-1050-GFP) were individually sufficient to localize to the patches. Both the N-terminal and central regions of Pan1 have endocytic binding partners that arrive before or at the same time as Pan1 to patches, suggesting these interactions may be important for Pan1's recruitment to patches.

In the absence of auxin, Pan1-GFP fragments had a decreased patch number and lifetime compared to full-length Pan1. Upon removal of full-length Pan1 by the addition of auxin, all Pan1-GFP fragments (except Pan1AA702-1050-GFP) trended toward more patches; this increase was statistically significant for Pan1AA1-855-GFP, AA250-855-GFP, and AA402-1050-GFP (Figure 4.2). This suggests that these fragments were competing with full-length Pan1 for binding at the patch. The lifetimes of all fragments also increased in the presence of auxin; this may be due to either decreased competition with full-length Pan1 and/or an overall increase in endocytic patch lifetime observed in Figure 3.1C.

Although a majority of Pan1 fragments were able to localize to endocytic patches, some had significantly decreased lifetimes at the patch compared to full-length Pan1 (Figure 4.2B). There are several interpretations for the shortened lifetimes, one of which is that these fragments are missing key binding elements that strengthen Pan1's interactions at the patch. Similar to Pan1 localization, Pan1's central region was critical

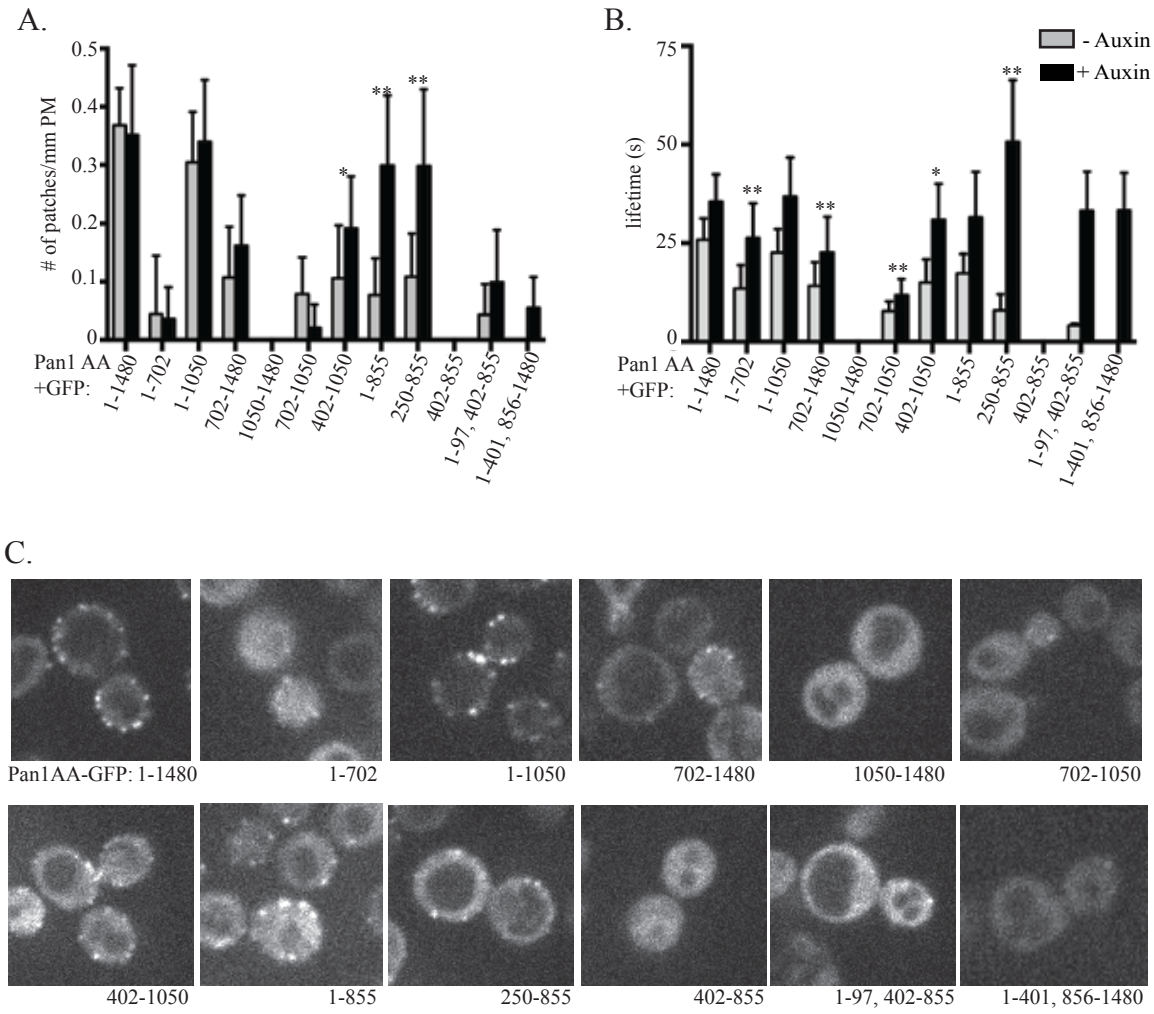


Figure 4.2 Pan1's central region is critical for localization to endocytic patch. (A) Number of Pan1-GFP patches per micron plasma membrane in Pan1-AID strain ± auxin for 1 h. No patches were observed for Pan1 AA1050-1480-GFP and Pan1AA402-855-GFP. N > 30 cells/strain. (B) Lifetime (s) of GFP-tagged proteins at endocytic patch in Pan1-AID cells ± auxin for 1 h. (C) Representative images of Pan1-AID cells plus indicated Pan1-GFP fragments grown in the presence of auxin for 1 h. *P ≤ 0.01, **P ≤ 0.0001 minus vs. plus auxin (Mean ± SD).

for Pan1 fragment stabilization/lifetime at the patch, as highlighted by the dramatic difference in lifetime and patch number between Pan1AA1-702 and Pan1AA1-855/1050. Although Pan1AA1-702-GFP and Pan1AA1-855/1050-GFP can both localize to patches, AA1-702-GFP had a significantly shorter lifetime, suggesting that Pan1AA703-855/1050 contains a patch stabilizing property. However, the central region alone, Pan1AA702-1050-GFP, also had a short lifetime, indicating that this region required another Pan1 region for normal temporal behavior. Additional N- or C-terminal sequences appended to the Pan1AA702-1050-GFP fragments increased its lifetime, but only the addition of the N-terminus increased the lifetime to WT levels. Therefore, the N-terminal and central regions together restore normal lifetime dynamics of Pan1 at endocytic patches.

In conclusion, the central region (Pan1AA702-1050) alone was sufficient for Pan1 localization to the endocytic patch, but the N-terminus was needed to ensure Pan1 had normal lifetime dynamics.

Regions of Pan1 important for endocytosis

Pan1's EH domains and central region are necessary for its endocytic function

In the absence of full-length Pan1, endocytosis was arrested (Figure 2.3). We identified the regions of Pan1 necessary for its endocytic role using the Mup1-pHl assay with Pan1 fragments in the Pan1-AID strain. After pretreatment with auxin for 30 min, methionine was added to induce internalization of Mup1-pHl and cells were observed one hour later. If a Pan1 fragment contained all regions necessary for endocytosis, Mup1-pHl would be efficiently internalized and the cells would be dim, similar to cells with full-length Pan1. However, if a Pan1 fragment could not support endocytosis, Mup1-pHl would remain at the plasma membrane and the cells would be bright, similar to Pan1-AID cells with EV. In the case where an intermediate brightness value of the population was obtained, there are two potential explanations. First, there may be a mixture of cells with Mup1-pHl at the plasma membrane, in endosomes or MVBs, or dim in the vacuole, part of which may be due to heterogeneous expression of plasmids from cell to cell. Alternatively, this could be due to all the cells having an overall intermediate brightness. When quantified for percentage of Mup1-pHl internalized, these samples would have values between those seen for EV and for full-length Pan1. An intermediate phenotype is indicative of slow or inefficient endocytosis, suggesting the fragment only partially restored endocytosis.

Similar to results for Pan1 localization, efficient Mup1-pHl internalization required Pan1's central region supported by portions of the N-terminus; the C-terminal actin region was not required (Figure 4.3). It was previously thought that Pan1's first EH domain was less important for Pan1's endocytic role as it does not bind adaptor proteins

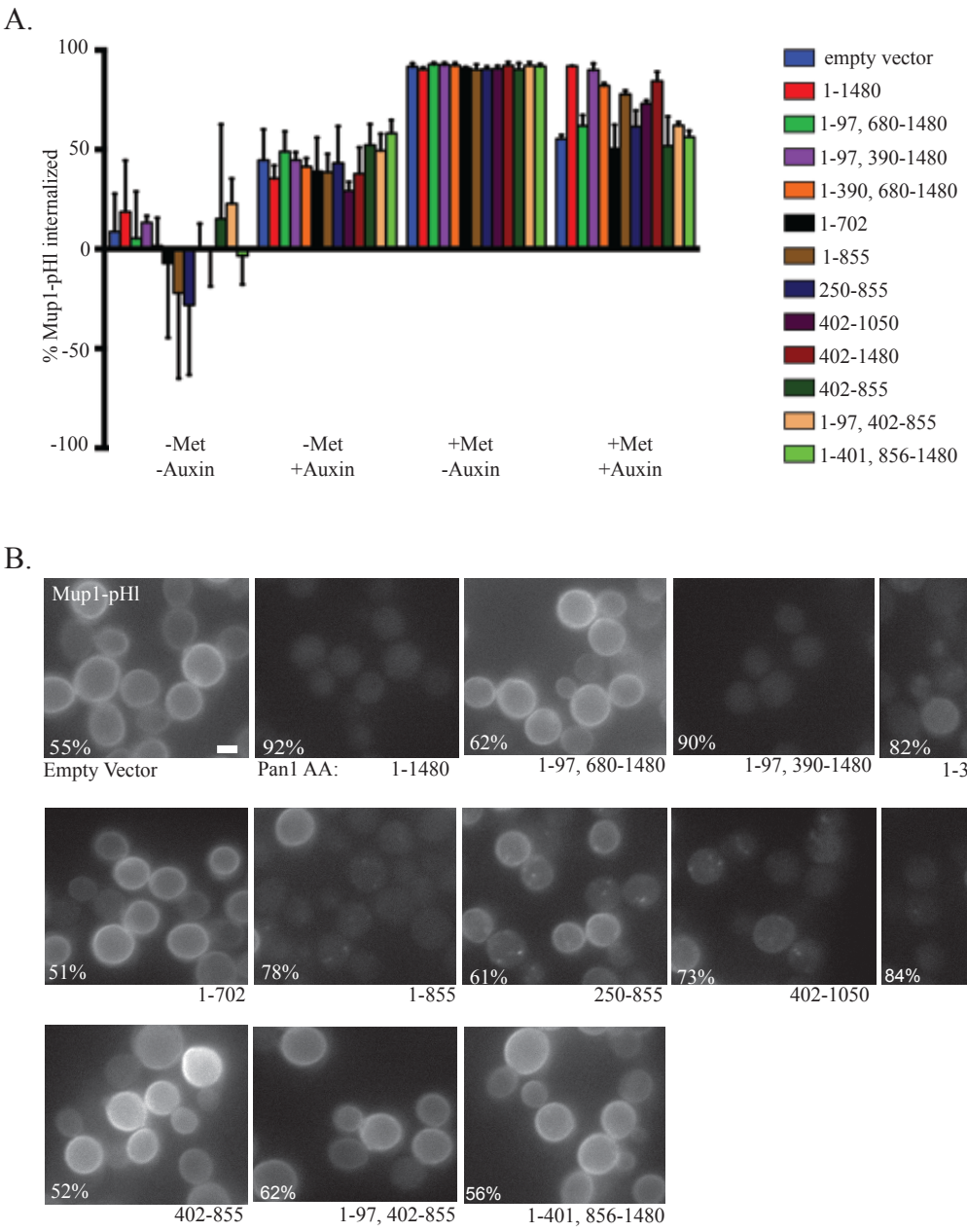


Figure 4.3 Pan1's EH domains and central region are important for its endocytic function.

(A) Quantification of the amount of Mup1-pHl fluorescence internalized after 30 min ± auxin pre-treatment and 1 h ± methionine. Percent Mup1-pHl internalized was calculated by measuring the amount of fluorescence at the end of the assay compared to the amount at the beginning. Graph represents average of three replicate experiments. Mean ± SD. (B) Representative images of Pan1-AID strains plus indicated plasmids after 30 min plus auxin and 1 h plus methionine. Percentage is average Mup1-pHl internalized calculated in (A). Scale bar, 2μm.

and was found to be dispensable for viability (Sachs and Deardorff, 1992; Wendland and Emr, 1998; Wendland *et al.*, 1999). Surprisingly, we found that the first EH domain could in fact support endocytosis in the absence of EH2, as Pan1AA1-390, 680-1490 internalized most Mup1-pHl (Figure 4.3). However, when both EH domains were deleted as in Pan1AA1-97, 680-1480, Mup1-pHl was not internalized. Although the N-terminus of Pan1 (Pan1AA1-702) could localize to endocytic patches, this region was unable to promote internalization of Mup1-pHl. Extending the fragment to include some of the central region (Pan1AA1-855) restored the endocytic function, further confirming Pan1's central region as critical for Pan1's localization and function at the endocytic patch. Additionally, we defined a region of Pan1, AA402-855, that was necessary, but not sufficient, for endocytosis, as Pan1AA1-401, 856-1480 and AA402-855 did not internalize Mup1-pHl. This region spans the EH2 domain and some of the central region. Interestingly, adding to this AA402-855 fragment either the rest of the central region (Pan1AA402-1050) or the N-terminus (Pan1AA1-855) restored the endocytic function. Pan1AA250-855, which includes both EH domains, only barely restored endocytosis (as seen by the punctae present in Figure 5E), while the far N-terminal portion of LR1 (Pan1AA1-97, 402-855) did not promote internalization of Mup1-pHl at all. These data suggest that Pan1AA402-855 is critical for CME, but it requires LR1 (including EH1) or the rest of the central region for full endocytic function.

In summary, Pan1AA402-855, which contains EH2 and a portion of the central region, was critical for endocytic function and patch formation and requires either LR1 or the central region for full restoration of endocytic defects.

Regions of Pan1 that restore viability

Pan1 contains three overlapping minimal essential fragments that localize to patches, but only one restores endocytosis

Pan1 is an essential protein whose primary role is in CME, which is not thought to be an essential process; therefore, this raises the questions of what is Pan1's essential role and can it be separated from its role in endocytosis. To explore these questions, the essential regions of Pan1 were defined by expressing fragments of Pan1 on plasmids in Pan1-AID cells and observing which fragments restored growth and viability in the presence of auxin. Pan1 AA402-855 is necessary, but not sufficient for growth or viability; this "minimal" essential region requires the addition of one of three regions of Pan1: AA250-401, AA856-1050, or AA1-97 to rescue growth and viability (Figure 4.4). The minimal region contains the second EH domain as well as a portion of the central region that is important for Pan1-Pan1 binding and Pan1-End3 binding. Pan1's C-terminal actin-regulatory region is not essential for viability, confirming data from the literature (Duncan *et al.*, 2001).

In addition, we found that Pan1's first EH domain could support growth in the absence of the second EH domain (and vice versa), contradicting previous results (Figure 4.4) (Sachs and Deardorff, 1992). This disparity was resolved by our finding that the original plasmid encoding Pan1AA: 1-390, 680-1480 does not express protein. Adaptor proteins have only been shown to bind the second EH domain; however, our data suggest that either adaptor proteins can interact with the first EH domain, or these interactions with Pan1 are not essential for growth or endocytosis.

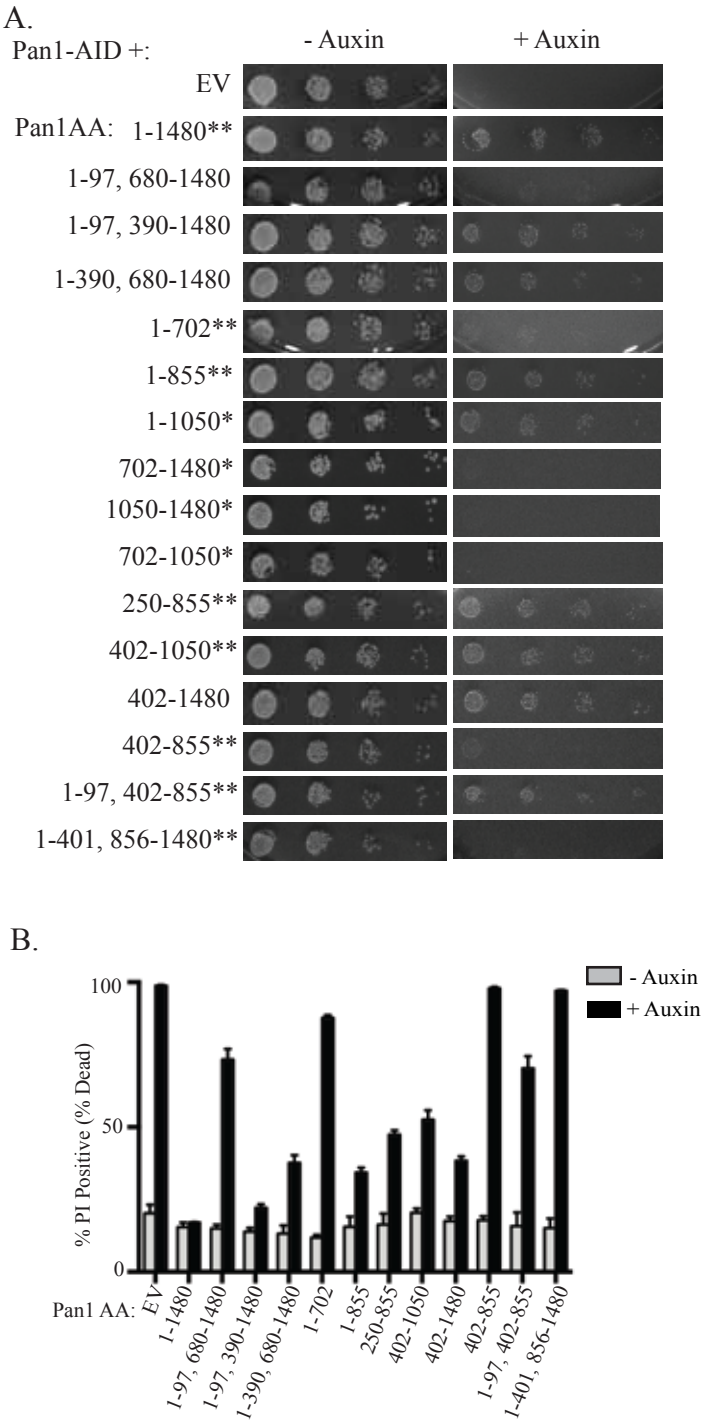
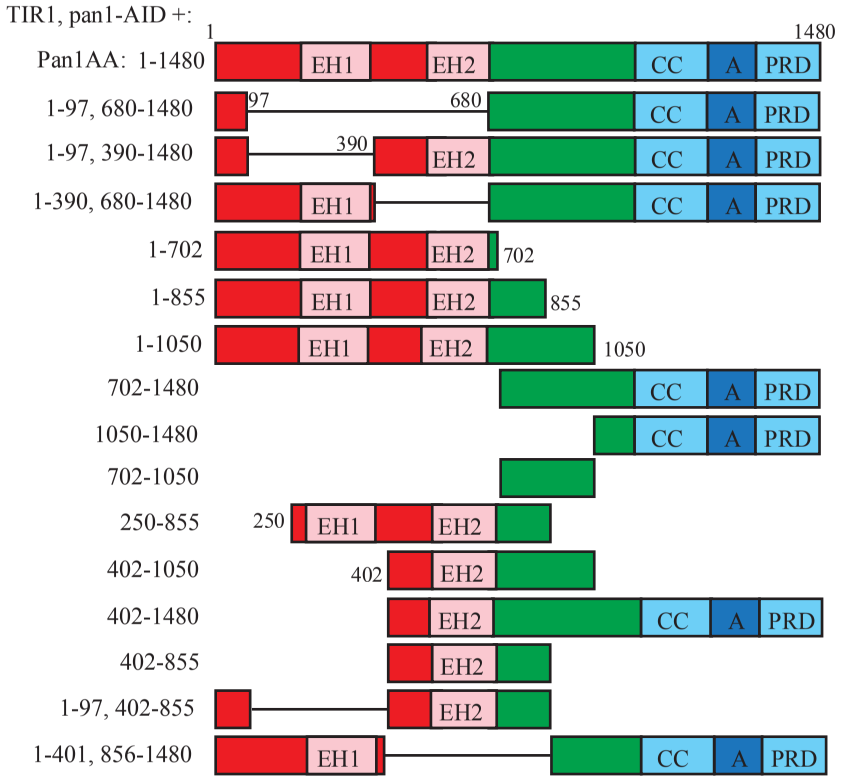


Figure 4.4 Pan1 contains three overlapping minimal essential regions.
(A) Serial dilutions of Pan1 fragments grown ± auxin for 3 d at 30°C. * = Pan1 fragment is C-terminally GFP tagged, ** = both GFP and untagged versions of Pan1 fragment are used in experiments, both grow similarly in ± auxin. (B) Percentage of Pan1-AID cells with indicated Pan1 plasmids that were positively stained with PI after grown for 24 h ± auxin at 30°C was quantified by flow cytometry. Graph represents the average of three replicate experiments. Mean ± SE.

All Pan1 fragments tested that did not support growth also did not restore viability of the Pan1-AID strain in the presence of auxin (Figure 4.4B). The essential regions of Pan1 partially restored viability, but the increased death suggests that the cells are in a compromised state, perhaps due to partial loss of Pan1's non-essential function.

In summary, Pan1's minimal common region, AA402-855, is critical for its localization and stability at the endocytic patch and necessary for Pan1's endocytic and essential roles (Figure 4.5). This common minimal region requires additional Pan1 regions to stably localize to endocytic patches (Pan1AA1-97, AA250-401, or AA856-1050), restore endocytosis (Pan1AA1-401 or AA856-1050), or support cell viability (Pan1AA1-97, AA250-401, or AA856-1050). The exact mechanism of action that each of these three additional regions contributes to the common minimal region is currently under investigation. Of the Pan1 fragments tested, all fragments that did not support growth also did not restore endocytosis or viability. Pan1AA1-855 and Pan1AA402-1050 are the minimal regions necessary for endocytosis, but the mechanism of how either the addition of the LR1 and LR2 or the remaining central region function to restore endocytosis is unknown. Potential explanations are that the Sla1 binding site in LR1 and the lipid-binding activity in the central region could be critical for Pan1's endocytic functions (Tang *et al.*, 2000, D. Pierce and B. Wendland, unpublished results). Two fragments, Pan1AA1-97, 402-855 and Pan1AA250-855 supported growth, but were not able to fully restore endocytosis of Mup1-pHl, suggesting that either: 1) these fragments support a basal level of bulk endocytosis, but not endocytosis of the specific cargo or, 2) these fragments have an additional, essential, role in the cell.



	Localizes to endocytic patch, +A	Lifetime at endocytic patch (-A/+A)	Growth in TIR1, Pan1-AID, + A	Endocytosis in TIR1, Pan1-AID, +A
Pan1AA: 1-1480	+	25.8s/35.6s	+	+
1-97, 680-1480	NA	NA	-	-
1-97, 390-1480	NA	NA	+	+
1-390, 680-1480	NA	NA	+	+
1-702	+	13.4s (N=19)/26.3s (N=16)	-	-
1-855	+	17.4s/31.6s	+	+
1-1050	+	22.5s/36.8s	+	N/A
702-1480	+	14.1s/22.6s	-	N/A
1050-1480	-	0s	-	N/A
702-1050	+	7.7s/11.9s	-	N/A
250-855	+	7.9s/50.7s	+	+/--
402-1050	+	15.0s (N=25)/31.0s	+	+/-
402-1480	NA	NA	+	+
402-855	-	0s	-	-
1-97, 402-855	+	4.0s (N=7)/33.2s	+	-
1-401, 856-1480	+	0s/33.3s(N=25)	-	-

Figure 4.5 Summery of functions of Pan1 pieces

Schematic of Pan1 fragments. C-terminally GFP tagged proteins localization to endocytic patch: + = localizes to patch, - = no patches present; lifetime(s) of GFP-tagged proteins at endocytic patch ± auxin for 1 h, N> 50 patches for all except those indicated, movies were taken with 1 s frames for 120 frames, 150ms exposure; rescue of growth of Pan1-AID by Pan1 protein fragments in the presence of auxin: + = rescue, - = no rescue; rescue of Mup1-pHl endocytosis in Pan1-AID by Pan1 protein fragments in the presence of auxin: + = rescue, - = no rescue.

Regions of Pan1 important for actin regulation

The contribution of Pan1 fragments to endocytic patch formation was observed with Sac6-RFP in Pan1-AID cells expressing various Pan1 fragments (both GFP-tagged and untagged) in the presence of auxin. All Pan1-GFP fragments arrived to the patch prior to Sac6-RFP, similarly to full-length Pan1. As summarized previously for Pan1-AID cells, in the presence of auxin there was a decrease in Sac6-RFP patch numbers and Sac6-RFP actin flares were observed (Figure 3.1 and 3.3). In contrast, no actin flares were seen in the presence of Pan1-GFP or untagged Pan1 fragments, suggesting that these fragments all contain some portion of Pan1 that is able to directly or indirectly regulate actin polymerization (Figure 4.6). However, Pan1AA1-97, 390-1480; AA1-390, 680-1480; AA1-855; AA1-1050; and AA402-1480 were able to rescue the decreased Sac6-RFP patch number phenotype. All of these fragments contain a portion of the N-terminus and central region, again confirming their importance for Pan1 function. Additionally, these data also indicated that the C-terminal actin regulatory region of Pan1 is neither necessary nor sufficient for Sac6-RFP dynamics at the endocytic patch. This implies that Pan1's role in actin regulation may be more indirect through its interactions with other actin-regulatory proteins.

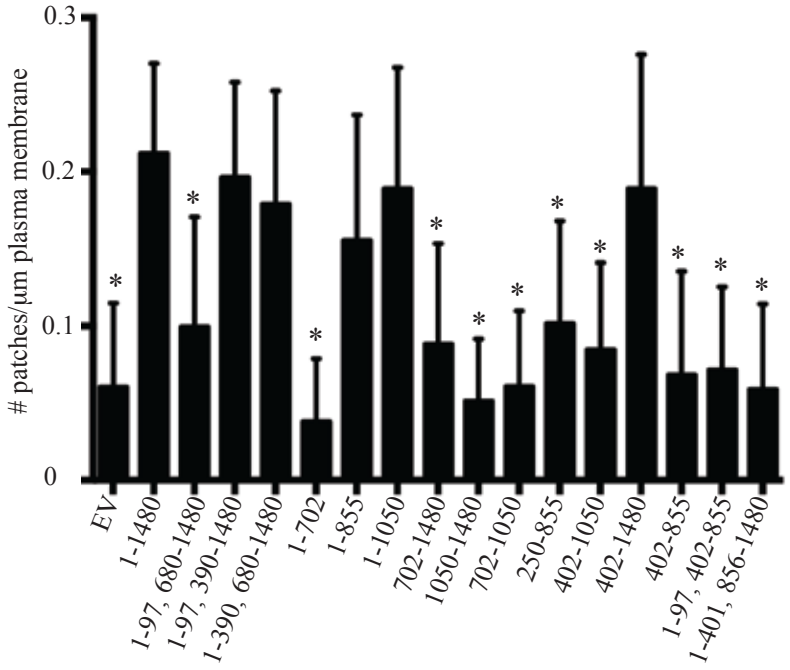


Figure 4.6: Pan1's N-terminus and central region are necessary for actin patch formation.

(A) Number of Sac6-RFP patches per micron plasma membrane in Pan1-AID cells plus indicated plasmid. Cells were incubated in auxin for 45-60min before imaging. N > 30 cells/strain. *P ≤ 0.0001 vs. full-length Pan1 (1-1480). Mean ± SD.

At least one Pan1 EH domain is necessary to support growth, localization, and endocytosis in minimal Pan1 fragments

Pan1 contains two EH domains within the two N-terminal LR regions; these EH domains contain well-conserved binding pockets for NPF motif containing proteins (Paoluzi *et al.,* 1998). Recent work indicated that Pan1's EH domains are not necessary for endocytosis due to the redundancy of EH domains present in Ede1 and End3 (Boeke *et al.,* 2014). We confirmed these results by mutating the tryptophan in both Pan1 EH domains in full-length Pan1 and found that this construct was able to restore growth and endocytosis to Pan1-AID cells in the presence of auxin (Figure 4.7, A and B). However, due to the evidence in my previous results that indicates at least one EH domain is important for endocytosis, I tested the necessity of each EH domain in the context of the minimal essential fragments. Pan1 AA1-97, 402-855 and 402-1050 each contain only the second EH domain; when the NPF binding pocket is mutated, these regions can no longer support growth, but this mutation did not affect the ability of AA402-1050 to restore endocytosis. AA402-1050 contains most of the central region, which is also critical for endocytosis, so perhaps the presence of the central region helps support endocytosis. Pan1 AA250-855 contains both EH domains. Interestingly, mutation of each individual EH domain only slightly decreases restoration of growth and endocytosis in Pan1-AID, while mutation of both EH domain renders the fragment useless for both growth and endocytosis. This again confirms that at least one EH domain and some of the central region is necessary for Pan1 to support endocytosis.

Presumably, localization to the endocytic patch is required for Pan1 to support endocytosis. To test the necessity of the EH domains for localization to the patch, Pan1

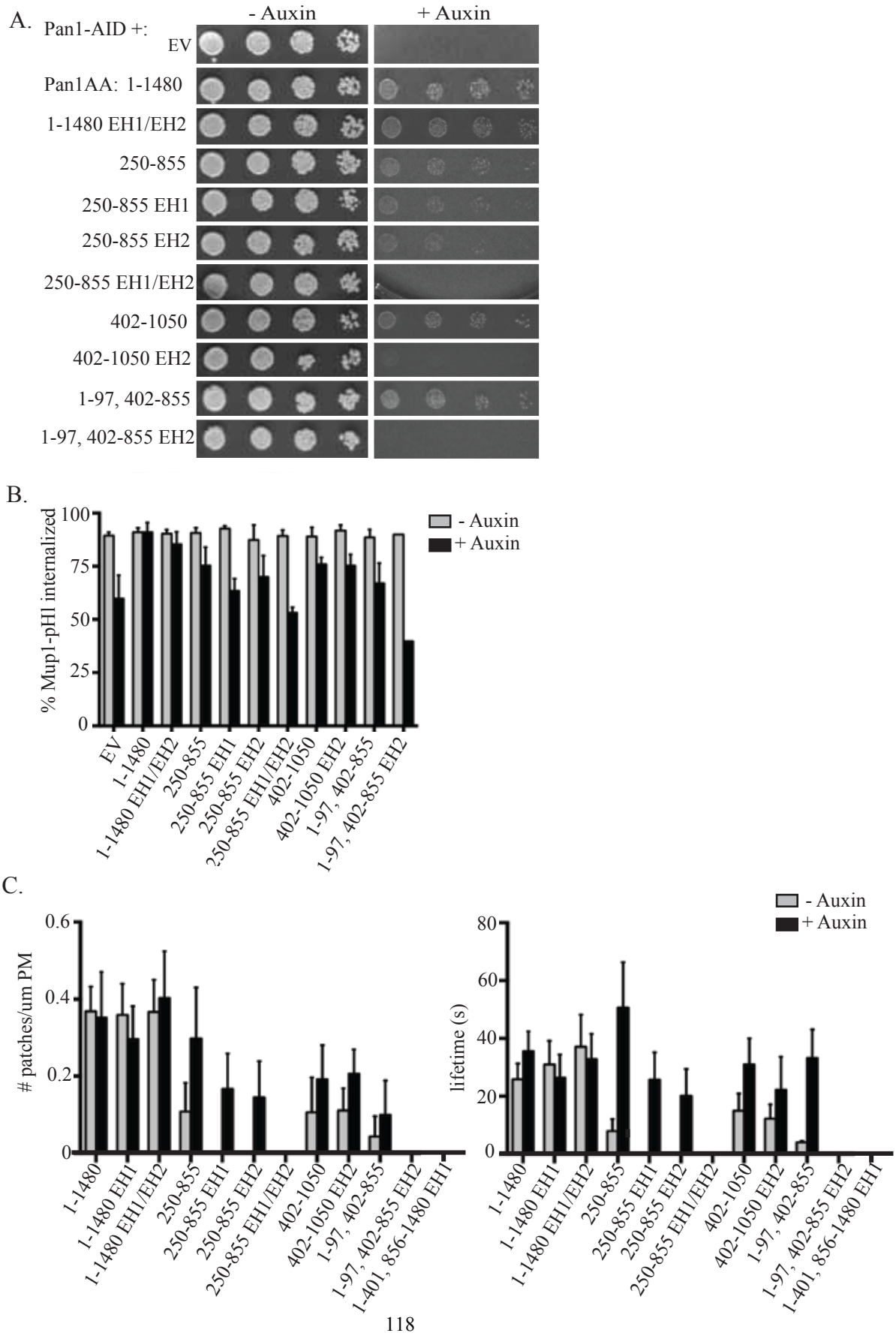

A.
- Auxin
+ Auxin
Pan1-AID +:
EV
Pan1AA: 1-1480
1-1480 EH1/EH2
250-855
250-855 EH1
250-855 EH2
250-855 EH1/EH2
402-1050
402-1050 EH2
1-97, 402-855
1-97, 402-855 EH2
B.
% Mup1-pHl internalized
100
75
50
25
0
- Auxin
+ Auxin
EV
1-1480
1-1480 EH1/EH2
250-855
250-855 EH1
250-855 EH2
250-855 EH1/EH2
402-1050
402-1050 EH2
1-97, 402-855
1-97, 402-855 EH2
C.
patches/um PM
0.6
0.4
0.2
0
lifetime (s)
80
60
40
20
0
- Auxin
+ Auxin
1-1480
1-1480 EH1
1-1480 EH1/EH2
250-855
250-855 EH1
250-855 EH2
250-855 EH1/EH2
402-1050
402-1050 EH2
1-97, 402-855
1-97, 402-855 EH2
1-401, 856-1480 EH1

Figure 4.7 At least one Pan1 EH domain is necessary for growth, CME, and localization in minimal Pan1 fragments

(A) Serial dilutions of Pan1 fragments expressed in Pan1-AID cells grown ± auxin for 3 d at 30°C. (B) Quantification of the amount of Mup1-pHl fluorescence internalized after 30 min ± auxin pre-treatment and 1 h ± methionine. Percent Mup1-pHl internalized was calculated by measuring the amount of fluorescence at the end of the assay compared to the amount at the beginning. Mean ± SD. (C) Number of Pan1-GFP patches per micron plasma membrane (left) and lifetime in seconds (right) in Pan1-AID strain ± auxin for 1 h. Absence of bar indicates no patches formed. N > 30 cells/strain for patch number, N> 20 patches/strain for lifetime.

EH1 = W312A mutation, EH2 = W642A mutation, EH1/EH2 = W312A and W642A mutations.

fragments containing mutations in the NPF-motif binding pocket of EH1, EH2, or both EH domains tagged with GFP were observed in Pan1-AID cells in the absence and presence of auxin. In full-length Pan1, mutation of either the first or both EH domains did not affect localization of Pan1 (Figure 4.7C). The NPF-motif binding pocket is also not necessary for localization and lifetime in Pan1AA402-1050. However, at least one, wild type, EH domain is necessary for localization and lifetime in the context of the other two minimal fragments. Mutation of either EH1 or EH2 in AA250-855 decreased the patch number and lifetime, and mutation of both EH domains completely disrupted localization. In AA1-97, 402-855, mutation of the only EH domain present, EH2, disrupted localization.

These results suggest that the NPF-motif binding pocket does somewhat contribute to Pan1 patch localization and endocytosis in the context of the minimal fragments, but it is redundant with other regions that are present in full-length and Pan1AA402-1050. One possible region that could be critical for localization is the lipid-binding region. Interestingly, the Pan1AA402-1050 W642A mutant fragment can restore CME and localize to patches, but does not restore viability; once again confirming that Pan1's endocytic and essential roles can be separated.

Lipid binding region of Pan1 is critical for growth and endocytosis in minimal essential fragments

Previous work in our laboratory found a lipid-binding region in the central portion of Pan1 (D. Pierce and B. Wendland, unpublished observations). This region spanning AA1020-1040 is a basic patch that contains ten positively charged lysines and arginines and resembles the PI4,5P_2 consensus sequence (Yu *et al.,* 1992). Mutating seven of these lysines and arginines to glutamic acid (negative) or alanine (neutral) is predicted to maintain secondary structure while abolishing lipid binding (D. Pierce and B. Wendland, unpublished observations). Mutation to negatively charged amino acids was predicted to be a more severe mutation as this would repel the region from lipids, while the neutral mutation simply disrupts binding. In the context of full-length Pan1, either mutation did not interfere with growth or endocytosis. However, in the minimal fragment of AA402-1050 these mutations led to loss of restoration of both growth and endocytosis (Figure 4.8). Thus, the lipid-binding region is necessary for growth and endocytosis in the minimal Pan1 fragment. This fragment may be important for localization or lifetime of Pan1 at the endocytic patch. The region may also be important for Pan1's essential function(s).

It is important to note that this lipid-binding region overlaps with a predicted NLS region (Kaminska *et al.,* 2007). When four of the ten lysines were mutated to asparagine (neutral, uncharged), a reporter protein was unable to localize to the nucleus; however, this work was never confirmed in vivo in the context of full-length Pan1. Two of these lysines are mutated in our binding mutants; thus, our lipid-binding mutants may also be

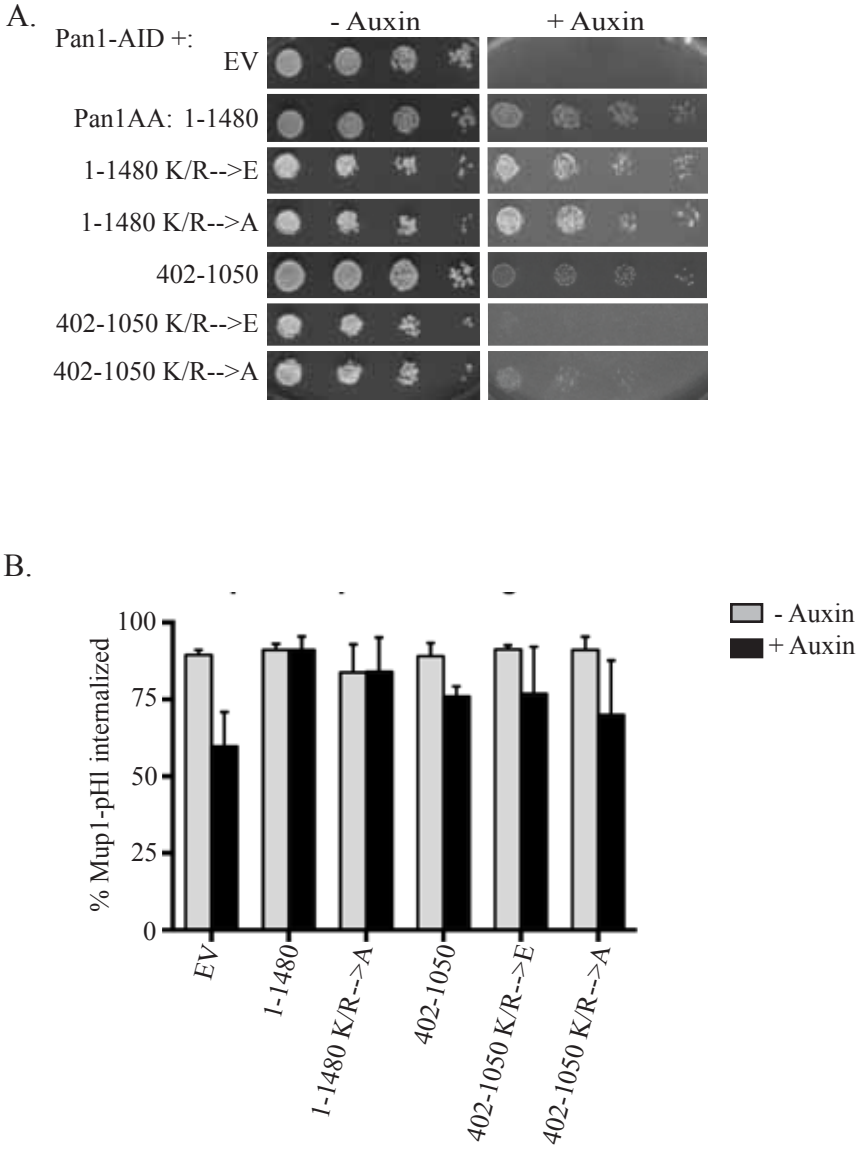


Figure 4.8 Pan1 lipid-binding domain is essential for growth and endocytosis in minimal Pan1 fragment

(A) Serial dilutions of Pan1 fragments expressed in Pan1-AID cells grown ± auxin for 3 d at 30°C. K/R→E = 7 lysines/arginines mutated to glutamic acid, K/R→A = 7 lysines/arginines mutated to alanine. (B) Quantification of the amount of Mup1-pHl fluorescence internalized after 30 min ± auxin pre-treatment and 1 h ± methionine. Percent Mup1-pHl internalized was calculated by measuring the amount of fluorescence at the end of the assay compared to the amount at the beginning. Mean ± SD.

disrupting the putative NLS region. Further experiments are needed to refine these regions and their functions.

Pan1 phosphorylation sites are not necessary for growth or endocytosis

Cycles of phosphorylation and dephosphorylation are critical for recycling endocytic proteins between internalization events. Pan1 contains 19 Ark1/Prk1 phosphorylation consensus sites throughout its N-terminus. Mutation of 15 of these sites from threonine to alanine mimics the phenotype seen in *ark1Δ/prk1Δ* cells, which are characterized by the presence of large clumps of actin in the cytosol and a slight defect in cell growth (Cope *et al.,* 1999; Toshima *et al*., 2005). It was proposed that phosphorylation of Pan1 negatively regulates its interactions with other coat proteins and F-actin, as well as inhibiting its activation of Arp2/3 actin polymerization (Zeng *et al.,* 2001, Toshima *et al.,* 2005). However, it was not know if these phosphorylation sites are critical for CME and/or viability.

Using plasmids that encode fragments of Pan1 on plasmids that have 15 of the Ark1/Prk1 phosphorylation sites mutated in Pan1-AID cells, it was confirmed that these sites are not necessary for restoration of growth in full-length Pan1, nor are they necessary for endocytosis (Figure 4.9). These sites do not become essential for growth or endocytosis in the context of the minimal fragments. Thus, it appears that Ark1/Prk1 phosphorylation of these 15 sites is not important for Pan1's endocytic or essential function; whether or not the other 4 sites or any unknown phosphorylation sites contribute to function needs to be investigated.

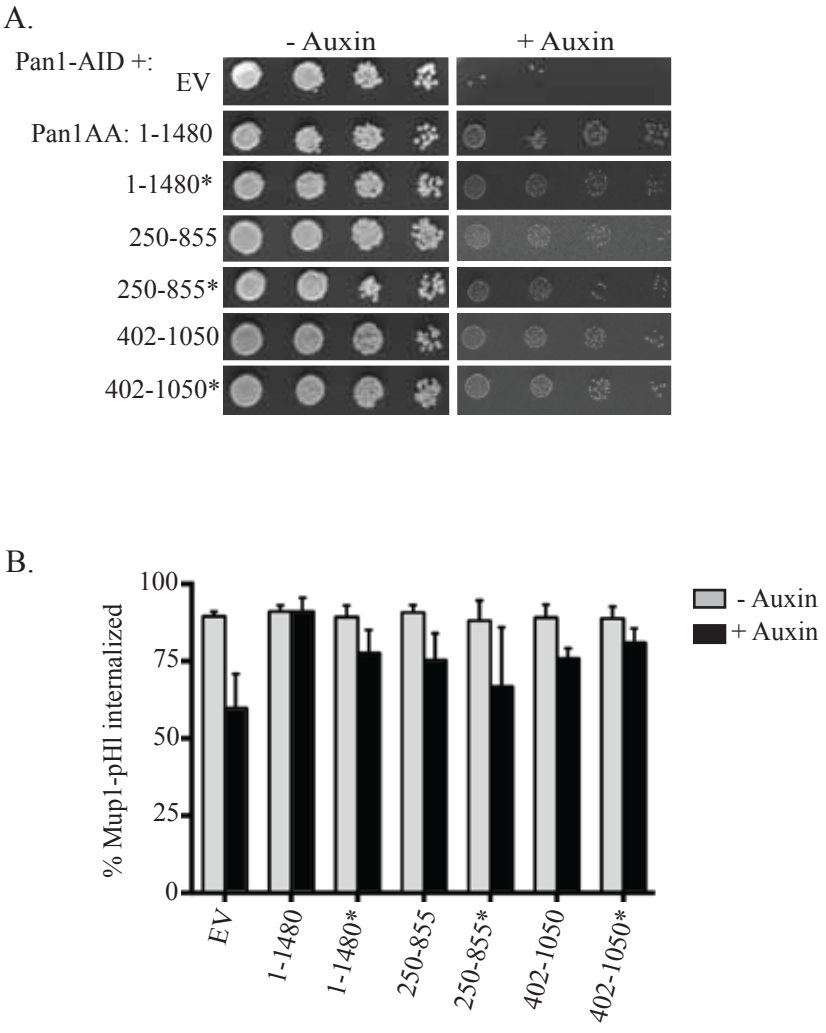


Figure 4.9 Ark1/Prk1 phosphorylation of Pan1 is not necessary for growth or endocytosis

(A) Serial dilutions of Pan1 fragments expressed in Pan1-AID cells grown ± auxin for 3 d at 30°C. * = 15TA mutation. (B) Quantification of the amount of Mup1-pHl fluorescence internalized after 30 min ± auxin pre-treatment and 1 h ± methionine. Percent Mup1-pHl internalized was calculated by measuring the amount of fluorescence at the end of the assay compared to the amount at the beginning. Mean ± SD.

End3 interactions with Pan1

As discussed in previous results, the first few hundred amino acids of the central region of Pan1 is necessary for all tested functions. This region contains a binding site for another late coat protein, End3, as well as regions implicated in Pan1 homo-dimers and homo-oligomers (Miliaras *et al*., 2004; Pierce *et al*., 2013; Whitworth *et al.,* 2014). The Pan1-End3 interaction is stable both at the patch and in the cytosol and is no longer thought to be regulated by phosphorylation as previously suggested (Zeng *et al.,* 2001; Toshima *et al.,* 2007; Boeke *et al.,* 2014). This suggests that the Pan1-End3 interaction is important, but is the central region that we found critical due to the End3 interaction or the Pan1 interaction (or both?). Do the minimal Pan1 fragments depend on the presence of End3 to localize and function at the endocytic patch or restore viability? To test this, *END3* was deleted in the Pan1-AID strain background and Pan1 fragments were tested for their ability to function in the absence of End3. Deletion of *END3* alone results in slow growth and a severe CME defect (Whitworth *et al.*, 2014). In the Pan1-AID *end3Δ* strain in the presence of auxin, only full-length Pan1 was able to restore growth, indicating that the minimal Pan1 fragments rely on the presence of End3 to function (Figure 4.10A). In Mup1-pHl experiments, Pan1-AID *end3Δ* plus EV plasmid has severely reduced CME due to the loss of end3 and this CME defect is exacerbated by the loss of Pan1 (Figure 4.10B). When a Pan1 plasmid is added in the absence of auxin, the two copies of Pan1 protein are able to slightly increase CME efficiency in Pan1-AID *end3Δ* cell, but this increase is lost upon loss of Pan1-AID protein in the presence of auxin.

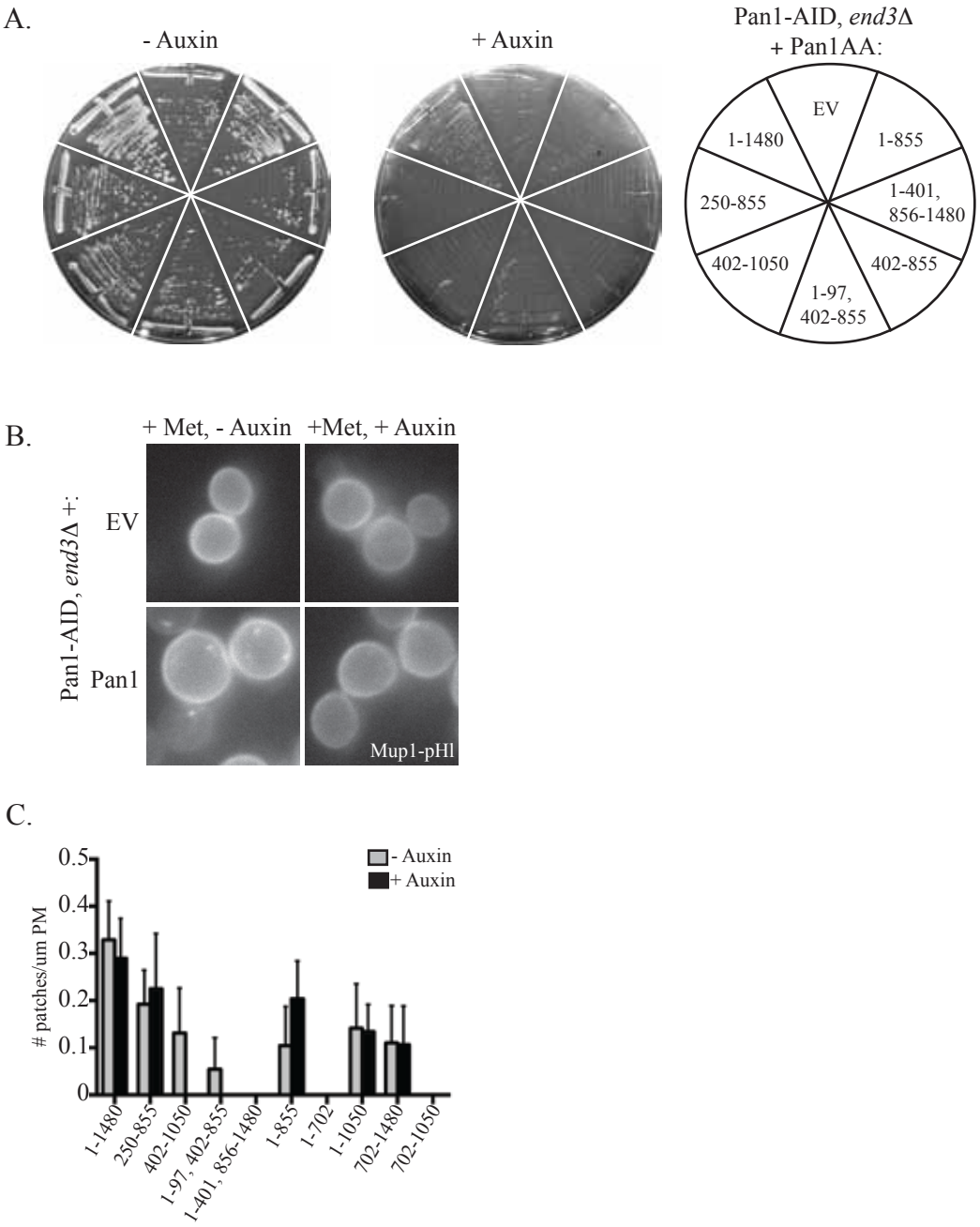


Figure 4.10 Pan1 essential fragments rely on End3 for viabilty and localization. (A) Pan1-AID *end3Δ* cells containing indicated plasmids were streaked onto plates ± auxin and grown for 4 d at 30°C. (B) Representative images of Pan1-AID,*end3Δ* strains plus indicated plasmids after 30 min ± auxin and 1 h plus methionine. (C) Number of Pan1-GFP patches per micron plasma membrane in Pan1-AID *end3Δ* cells ± auxin for 1 hr. Absence of bar indicates no patches formed.

Pan1 and End3 arrive and leave the endocytic patches in similar time frames. Loss of *END3* results in significantly increased Pan1 lifetimes (Whitworth *et al.*, 2014). In the absence of Pan1, there were fewer End3 patches and those patches had longer lifetimes, suggesting that End3 may depend on Pan1 for patch localization and lifetime regulation (Figure 3.1D). Pan1-GFP fragments were observed in Pan1-AID *end3Δ* cells in the absence and presence of full-length Pan1 (Figure 4.10C). The absence of End3 did not affect the localization of full-length Pan1. However, while the three essential regions could localize to the patch in the absence of End3, only Pan1AA205-855 could localize in the absence of both End3 and Pan1. Further characterization of Pan1 localization with other Pan1 fragments suggest that in the absence of End3 and Pan1, Pan1 needs at least 2 EH domains and the LR2 in order to localize to the patch properly. Therefore, although End3 is not absolutely necessary for full-length Pan1 localization, it's importance does increase in the context of smaller Pan1 fragments.

DISCUSSION

The Pan1-AID strain allowed for a more rigorous study of the necessity and sufficiency of all the various Pan1 domains and regions for Pan1 function. We found that the N-terminus of Pan1 only partially contributes to endocytosis and viability. For the functions we tested, the central domain is necessary and the C-terminus is neither necessary nor sufficient (summarized in Figure 4.5). Pan1's EH domains and lipid binding domain have varying levels of importance based on the function tested and presence of other regions. End3, Pan1's most stable binding partner, is necessary for viability in the absence of full-length Pan1.

We found that the most functionally important region of Pan1, AA702-1050, is the least-studied, leaving many questions open concerning the mechanisms for Pan1 function. The known activities and interesting features of this region include: homo-dimerization, End3 binding, Sla2 binding, a Prk1 consensus phosphorylation site, a lipid-binding region, and a putative NLS domain, many of which show conservation among fungal species (Tang *et al.*, 2000; Zeng *et al.*, 2001; Miliaras and Wendland, 2004; Toshima *et al.*, 2005; Kamińska *et al.*, 2007; Toshima *et al.*, 2007; Pierce *et al.*, 2013). Additionally, there appears to be two more areas (AA855-892 and AA918-975) that are well conserved among fungal species, but have no assigned function. More careful study of Pan1's central region is clearly warranted.

Although Pan1 has two well conserved EH domains, it was previously thought that Pan1's EH1 could not compensate for EH2 loss (Wendland and Emr, 1998; Wendland *et al.*, 1999; Maldonado-Baez *et al.*, 2008; Suzuki *et al.*, 2011). However, our work clearly shows that EH1 can function to support endocytosis and viability in the

absence of EH2 function (Figure 4.3, 4.4, and 4.7). Suzuki et al. showed that specific deletion of both Pan1 EH1 and EH2 does not abolish endocytosis, but our data using a fragment missing EH1, EH2, and the region between (LR2), does abolish the endocytic and essential function of Pan1, while just mutating these EH domains alone does not; this implicates a role for the LR2 region. Currently, the only known domains in LR2 are 10 Prk1/Ark1 phosphorylation sites (Tang *et al.*, 2000; Zeng *et al.*, 2001; Toshima *et al.*, 2005; 2007). Are these sites critical for Pan1 function or are there unknown binding partners in the LR2?

The proper localization of proteins to patches is critical for their endocytic function, but the domains that are critical for this localization were unknown for Pan1. We found that the N-terminal and central regions of Pan1 independently localized to the patch; each had significantly decreased lifetimes in comparison to full-length Pan1, but had normal lifetime dynamics when combined (Figure 4.2). The C-terminus of Pan1 was neither necessary nor sufficient for localization. Both the N-terminal and central regions of Pan1 interact with proteins that arrive at the patch before or at the same time as Pan1, suggesting that these interactions contribute to Pan1 recruitment (Tang *et al.*, 1997; Wendland and Emr, 1998; Wendland *et al.*, 1999; Tang *et al.*, 2000; Toshima *et al.*, 2007). Interestingly, some fragments, such as Pan1AA1-702 and 1-97, 402-855, could localize to the patch without supporting endocytosis. This demonstrated that regions important for endocytic localization and function could be separated. The exact mechanisms and critical domains of Pan1 localization are being further studied, but initial results indicate that two of the minimal fragments need at least one functioning EH domain for localization (Figure 4.7C).

Our work found two minimal regions of Pan1 that support viability and endocytosis: Pan1AA1-855 and AA402-1050 (Figure 4.3 and 4.4). They share the "common essential region" (AA402-855), which we showed is necessary for all Pan1 functions, while the non-overlapping portions are very different. Do these two regions support endocytosis in similar, or in different ways? Pan1AA855-1050 interacts with Sla2 and the lipid membrane, both of which could help Pan1 properly localize to endocytic sites to perform its function (Toshima *et al.*, 2007). The lipid binding site and the EH domain are not necessary for CME in Pan1 AA402-1050, suggesting that this fragment most likely can localize to the patch independently of these sites (Figure 4.7B and 4.8B). However, both sites in this minimal fragment are necessary for viability and are therefore necessary for Pan1's essential function (Figure 4.7A and 4.8A). Does this essential function occur at the plasma membrane or a different membrane compartment in the cell? In contrast, Pan1AA1-402, which contains LR1, EH1, and part of LR2, could help localize Pan1 to the patch through Sla1 interactions or phosphoregulation. However, data in Figure 4.9 suggests that the phosphorylation motifs are not critical for growth or CME. That Pan1AA250-855 and Pan1AA1-97, 402-855 do not support endocytosis suggests an endocytic role for LR1, which binds Sla1 (Figure 4.3) (Tang *et al.*, 2000; Zeng *et al.*, 2001; Toshima *et al.*, 2005; 2007). Thus, the contribution of Sla1 to the minimal Pan1 fragments needs to be tested. The exact binding domain of Sla1 also needs to be defined to determine if Pan1-Sla1 interaction is critical for endocytosis and/or viability.

Interestingly, we found two minimal regions of Pan1 that support viability and localize to patches, but do not fully restore endocytosis: Pan1AA250-855 and Pan1AA1-

97, 402-855 (Figure 4.2-4.4). Each contains the common essential region, but the two non-overlapping regions contain different portions of the N-terminus. Each fragment needs one functioning NPF-binding motif in the EH domain to restore growth and localize to patches (Figure 4.7, A and C). Pan1AA250-402 contains EH1 and LR2, while the other has a glutamine rich N-terminus with an NPY motif. How are these regions contributing to Pan1's essential function? Are there non-endocytic proteins that interact with Pan1 in these regions? Is Pan1's essential function carried out at the endocytic patch?

We will continue to define the regions important for Pan1's essential and endocytic functions.

CHAPTER 5: CONCLUSIONS

In this dissertation, I have described the construction and use of a novel Pan1-degron strain to uncover the mechanisms of Pan1's function at the endocytic patch. Pan1-AID was also used to further annotate the regions and domains of Pan1 that are critical for endocytic patch localization, endocytosis, and viability. The preceding chapters include data and in-depth discussion of these results. To conclude, I will discuss my results in the context of the endocytic field and hypothesize how our expanding knowledge of Pan1 can be applied to Intersectin and human disease.

Pan1 is a large, flexible scaffold protein that has a well-characterized role in CME and may have another, unknown, essential function in the cell. With Pan1-AID, Pan1 protein was specifically and acutely depleted in live cells and we observed the resulting phenotypes. In the absence of Pan1, CME is arrested and cells begin to die. We currently do not know how or why the cells die in the absence of Pan1 and whether or not this is due to the CME arrest. CME is arrested because of a delay in maturation of the endocytic vesicle coat, an over activation of actin polymerization, and a disconnect between the actin machinery, coat proteins, and plasma membrane. Thus, we can conclude that Pan1 is critical for strengthening these interactions during the vulnerable transition states of CME. The second EH domain and a portion of the central region of Pan1 are necessary, but not sufficient for all known Pan1 functions.

The Pan1-AID strain as a tool for studying Pan1 function

Novel scientific discoveries depend on constant updates to existing techniques and new technologies. Big discoveries in the endocytosis field followed soon after revolutionary techniques were invented—electron microscopy, molecular genetics,

fluorescent microscopy, and live cell imaging all played huge roles in elucidating the mysteries of the endocytic pathway. For the research of particular proteins within the process, the field relied heavily on gene deletion and mutation experiments in yeast. Much was learned from *pan1* mutant alleles, but the *PAN1* gene is essential and cannot be deleted. To expand the knowledge of Pan1's function, it was necessary to use a novel method to specifically degrade Pan1 protein in live cells using conditions that did not cause any off-target effects on the cells. In this dissertation, the Auxin-Inducible Degron was used to acutely deplete Pan1 protein from cells, observe how endocytosis dynamics changed and which regions of Pan1 were important for these dynamics.

The degron allele method was originally developed over a decade ago, but recent upgrades to this technique have allowed for cleaner, more direct assays. The AID system depends on the interaction of plant proteins and hormones which should not, in theory, have any endogenous targets in yeast. While it appears that TIR1, the AID tag, and auxin are innocuous to yeast cells, it is critical that bioinformatics techniques are used in the future to evaluate if the presence of these plant proteins changes the expression levels or alters signaling pathway dynamics in yeast cells. The AID tag used in Chapter 2 is small enough that it should not affect Pan1 binding dynamics; however upgraded, smaller, AID tags have been developed that may be necessary to use for future Pan1 studies. One particular advantage of these smaller AID tags is they allow for an additional GFP tag to be added so the localization of the remaining Pan1 protein that has not yet been degraded can be observed. This Pan1-GFP-AID strain would confirm whether or not the few endocytic patches that do mature to the later stages contain a trace amount of Pan1

protein. The strain can continue to be used in the future to more clearly define each of Pan1's roles and for a more careful characterization of Pan1's protein architecture.

Regulating transitions between stages of CME

One of the remaining open questions in the endocytic field is how each stage is coordinated in relation to the other stages. What proteins are important for the transitions, how are they recruited to the patch, and what signals are required for each stage? It was hypothesized that scaffolding proteins function to strengthen interactions between separate stages. However, studies of these scaffold proteins are inherently difficult due to their importance for growth and viability. Recent advances in methods have allowed for more precise observation of these scaffolding proteins. Boeke *et al*, 2014, identified the early coat protein Ede1 as the key coordinator for the initiation and early steps of endocytosis, but Ede1 was not critical for maintenance of the late coat. Sla2 and Ent1/2 are critical for coupling the endocytic coat and actin machinery (Kaksonen *et al.,* 2005; Skruzny *et al*., 2012). What coordinates the interactions between these two steps? Work in this dissertation provides evidence that Pan1 can coordinate the three transitions in CME: early coat to late coat, actin regulators to actin machinery, and coat/actin machinery to the plasma membrane (Chapter 3). As Pan1 interacts with Ede1, Ent1/2, and Sla2, it may function with these proteins to regulate a majority of the endocytic process (Figure 5.1) (Aguilar *et al.,* 2003; Toshima *et al.,* 2005, 2007; S. Barker and B. Wendland, unpublished observations). Another theory suggests that the type and amount of cargo present at the patch may provide a signal for maturation of the coat, but whether or not this is valid has yet to be proven. Regulation of the final stage of endocytosis,

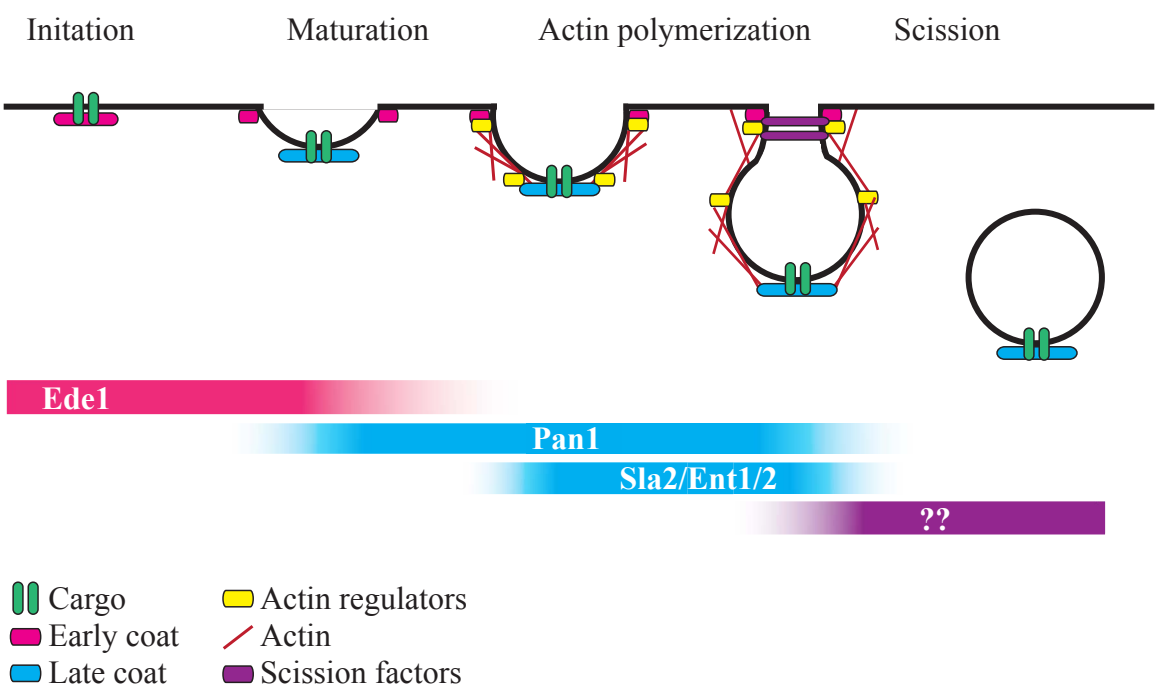


Figure 5.1: Key coordinators of CME transitions.
Top: Schematic of CME timeline. Bottom: Time periods during which Ede1, Pan1, and Sla2/Ent1/2 function to coordinate the transitions between CME stages. ?? = unknown regulator of transition.

scission and uncoating, is not as clear; it is hypothesized that amphiphysin proteins are recruited to the vesicle neck by a combination of the membrane curvature and dephosphorylation of the membrane (Liu *et al.,* 2006; 2009). Future work should focus on defining if this final stage is regulated by another scaffolding protein and/or the physiological properties of actin polymerization and membrane deformation.

Functional characterization of Pan1 regions and domains

For the first time, the contribution of individual regions and domains of Pan1 to Pan1's endocytic and essential functions could be determined using Pan1-AID. Previously, only fragments of Pan1 that supported viability could be studied. The work in Chapter 4 shows that there are three overlapping essential regions of Pan1; only one of these regions can fully support CME, suggesting that Pan1's essential and endocytic functions can be separated (Figure 5.2).

This work supports previous research that found that Pan1's C-terminal actin regulatory region is dispensable for endocytosis and viability (Duncan *et al*., 2001). Without the C-terminus, Pan1 resembles the short isoform of ITSN (Hussain *et al.,* 1999). If this actin regulatory region is not necessary for endocytosis or viability, why was it conserved? Is this region critical for another function of Pan1 through a yet undefined domain? Or is it there simply to ensure proper actin polymerization during CME?

We found that Pan1 AA402-855 is necessary, but not sufficient, for all Pan1 functions. This region spans the second EH domain and a portion of the central region. Not much is known about the central region of Pan1 because it is highly unstructured,

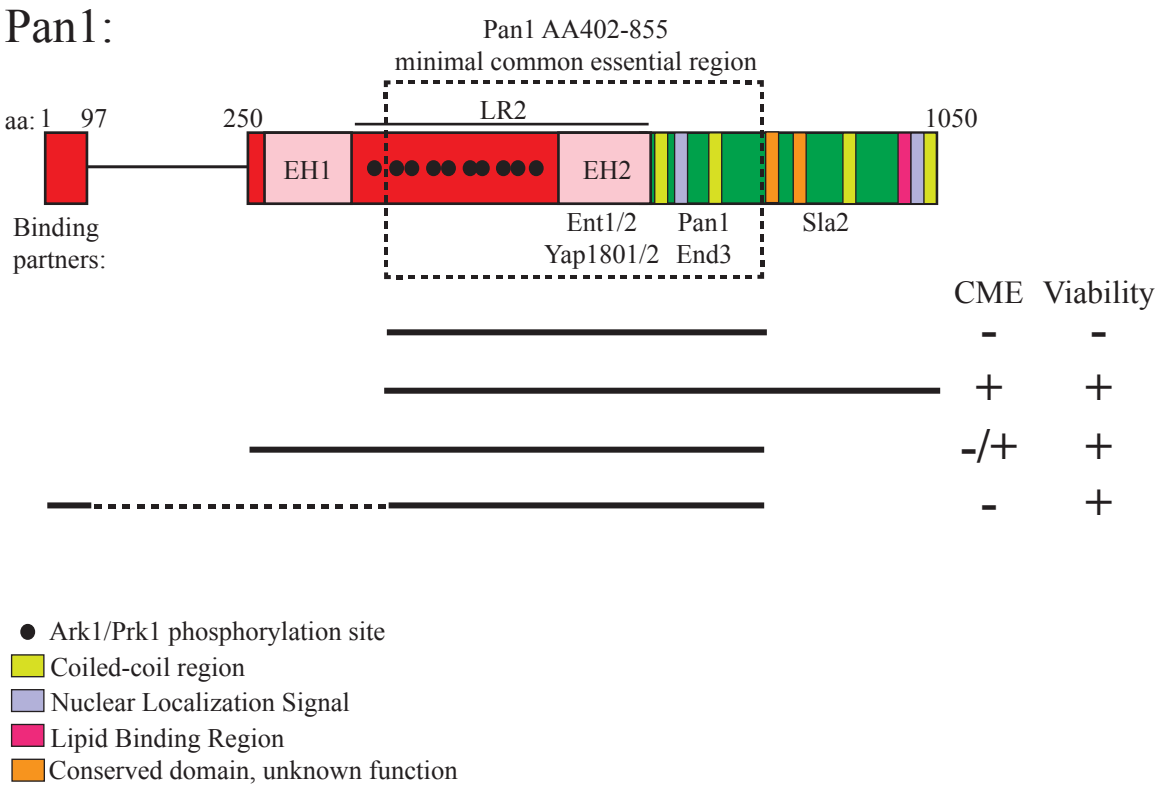


Figure 5.2: Three overlapping minimal essential regions of Pan1.
Schematic of the three regions of Pan1 that are critical for CME and/or viability.
+ indicates this Pan1 fragment can restore CME or viability in Pan1-AID plus auxin;
- indicates this Pan1 fragment cannot restore CME or viability in Pan1-AID plus auxin.

making biochemical experiments technically difficult. Currently, only three proteins are known to bind in this region—End3, Sla2, and Pan1 itself. Are these protein interactions critical for Pan1's endocytic and essential roles? This region also contains some putative NLS and phosphorylation domains whose contributions are yet to be studied. There are also two well-conserved regions that may contribute to Pan1's unknown essential function.

To learn more about the elusive central region and essential functions of Pan1, two screens should be done to identify the involved pathways and what protein interactions are important. A screen for novel binding partners of Pan1 AA402-855 may provide evidence of previously unknown endocytic proteins and/or essential pathway partners. A high-copy suppressor screen for proteins that can support viability in the presence of Pan1's minimal essential region may also show Pan1's essential function.

What is the essential function of Pan1?

Pan1 has historically been characterized as a CME scaffold protein, but CME is not an essential process (Chu *et al.,* 1996; Madania *et al.,* 1999; Kaksonen *et al.,* 2005; Newpher and Lemmon, 2006; Prosser *et al.,* 2011). Intersectin, Pan1's mammalian homologue, has signaling roles independent of its CME role; does Pan1 also have signaling roles and could this be its essential function? Or is Pan1 only involved in endocytosis? One way to deduce how a protein keeps cells alive is to observe how cells die in the absence of the protein. Chapter 2 shows that in the absence of Pan1, cells die quickly and conventional death inhibitor drugs cannot inhibit the death pathway(s). More

work into how and why these cells are dying is necessary to determine why Pan1 is essential.

Yeast have only two defined endocytic pathways: CME and Rho1-dependent clathrin-independent endocytosis (CIE). The presence of a CIE pathway in yeast is a relatively new discovery and not much is known about the proteins involved (Prosser *et al.,* 2011). So far, Pan1 has only been shown to function in CME, but its role in CIE has not yet been tested. Intersectin has been show to act in at least one CIE pathway (caveolin-dependent endocytosis); thus, it can by hypothesized that Pan1 may be involved in CIE as well (Predescu *et al.,* 2003; Klein *et al.,* 2009). If Pan1 were necessary for both pathways, then endocytosis would be completely abolished in the absence of Pan1 if these are the only two endocytic routes. A complete block of endocytic routes would be extremely detrimental to cells, as they could not uptake necessary nutrients, respond to stress signals, or regulate membrane composition. Does death due to a full block of endocytosis resemble death due to the absence of Pan1? Future work will be done to see if Pan1 functions in the yeast CIE pathway and if the block in endocytosis is what leads to cell death.

Pan1 could be acting similarly to Intersectin and act as a both a scaffold for signaling and endocytosis pathways. Pan1 has historically been considered only a CME protein, so very little research has been done to see if Pan1 has other roles in the cell. Two studies found putative domains of Pan1 that do not seem to be related to CME: a nuclear localization signal (NLS) and a stress-induced phosphorylation site (Kaminska *et al.,* 2007; Reiter *et al.,* 2012). Only one other endocytic protein in yeast, Sla1, has been shown to localize to the nucleus, but the function of this localization is not known

(Gardiner *et al.,* 2007). Could Pan1 and Sla1, which form a protein complex, be traveling to and functioning in the nucleus together? Using GFP localization, future work will need to confirm if, when, and how these proteins localize to the nucleus. Recent work found a novel Hog1-dependent phosphorylation site of Pan1 that is highly phosphorylated in response to osmotic stress (Reiter *et al*., 2012). Is this phosphorylation site important for Pan1's essential function? A cell's stress response system is intimately linked to endocytosis; cells will often uptake stress receptors and change the membrane composition via endocytosis when a harmful environmental factor sensed. Using Pan1-AID, the contribution of this Hog1 phosphorylation site to localization, endocytosis, and viability can be tested. The phosphorylation site is not within Pan1's essential region, so while Pan1 may have a role in this stress particular response, it is probably not Pan1's essential function. However, this does not rule out the possibility that Pan1 could be involved in other stress responses that could be essential.

The future of endocytosis studies

This dissertation focuses on Pan1's scaffolding role in endocytosis and which regions of Pan1 contribute to CME and viability. These experiments can be done with relative efficiency in yeast compared to studies of Intersectin in mammalian systems. Thus our novel findings of Pan1 can be used to design more specific experiments of ITSN function. The AID system can be used in mammalian systems and thus experiments with AID tagged ITSN1/2 in mammalian cells can confirm that Pan1 and ITSN have similar scaffolding roles. We hypothesize that without ITSN, CME would have a similar phenotype as in yeast cells without Pan1—delayed maturation of the endocytic coat, the

appearance of actin flares, and a disconnect between the coat, actin, and membrane. The ITSN-AID cell lines could also be used to see if there are similarities between which fragments of ITSN and Pan1 are critical for various functions. There is some knowledge about which regions of ITSN are important for its alternative signaling roles; if one of these regions turns out to be essential, then this knowledge can be applied to future Pan1 research on the mechanisms of its potential signaling roles.

The more that is known about the similarities and differences between ITSN and Pan1, the better we can use Pan1 and yeast as a model system for basic cellular functions in wild type and disease states. The most recent yeast model of Alzheimer's Disease shows deficiencies in CME (Treusch *et al.,* 2012). More work needs to be done to characterize how and why CME is misregulated and which proteins are involved. By combining Pan1-AID and the yeast AD model, the role of Pan1 in the AD can be assessed and genetic and drug screens can be performed to discover novel therapeutic targets. The development of more yeast models of human disease will complement work done in mammalian systems and lead to a better understanding of diseases and the discovery of therapeutic targets. Additionally, further characterization of endocytic trafficking is essential to develop therapeutics that can be targeted to specific intracellular organelles.

APPENDIX 1: PAN1-DHFR DEGRON ALLELE

The Pan1-DHFR allele was created by Karen Whitworth; all results presented in this chapter represent original work.

ABSTRACT

Although much research has been done to characterize the various binding regions and functions of Pan1 important in endocytosis, the exact mechanisms of Pan1 function have yet to be discovered due to Pan1's essential nature. Temperature-sensitive (*ts*) alleles and conditional expression of Pan1 were unsuccessful at completely removing all of Pan1 function; therefore it was critical to create a degron allele of Pan1 in which Pan1 protein could be conditionally and specifically depleted. Prior to creation of the Pan1-AID degron strain, another Pan1-degron allele was used to specifically degrade Pan1 protein in nonpermissive conditions. This strain, *pan1-td,* used the temperature-sensitive DHFR-degron tag combined with galactose-induced overexpression of the E3 ubiquitin ligase Ubr1 to degrade Pan1 in the non-permissive conditions: 37ºC and galactose media (Sanchez-Diaz *et al.*, 2004). Using this strain, it was confirmed that Pan1 is required for cell growth and endocytosis. A candidate high-copy suppressor screen using *pan1-td* found that Cell Wall Integrity pathway proteins could restore growth to *pan1-td* cells; but this was specific to the *pan1-td* strain and most-likely a false positive as these proteins did not rescue growth of Pan1-AID. Therefore, due to the slow degradation of Pan1 and the off-target effects of the non-permissive conditions in *pan1-td* cells, this strain will not be used for future studies of Pan1.

INTRODUCTION

In order to elucidate a protein's function, a common method in genetics is to simply delete the encoding gene from the organism's genome and observe the resulting phenotypes. However, essential genes cannot be deleted and instead must be mutated or conditionally inactivated. Although several temperature sensitive alleles of *PAN1* exist, these most likely do not eliminate all of Pan1's functions. Typically, to induce conditional deletion, a gene can be put under the control of the regulatable galactose promoter. However, Pan1 contains many methionines and internal start sites that allow truncated versions of Pan1 lacking any 5' UTR sequences to be expressed constitutively (Tang and Cai, 1996, K. Whitworth and B. Wendland, unpublished observations). There is also a phenotypic lag in galactose regulation experiments (and inactivation of *ts* mutants at nonpermissive temperatures) because it could take several hours for the existing protein to be degraded in the cell once the galactose promoter has been repressed. A more efficient and specific way to conditionally knock-out a gene in yeast is to promote protein degradation through the genomic fusion of the gene with a degron-fusion allele.

Our lab has used the methods of Sanchez-Diaz *et al.* (2004) to produce a degron-fusion allele of *PAN1* (*pan1-td*). In *pan1-td*, *PAN1* is fused with a mouse mutant allele of dihydrofolate reductase (DHFR) in which there is a P66L mutation and an N-terminal ubiquitin peptide. The ubiquitin is cleaved co-translationally from the N-terminus of DHFR exposing an internal residue that has been mutated from valine to arginine. These mutations cause a conformational change at the nonpermissive temperature (37°C) that exposes the N-terminal arginine on the surface. Due to the N-end rule, which relates the

stability of a protein to its N-terminal residue, the protein with the unstable N-terminal arginine is targeted for ubiquitination by the E3 ubiquitin ligase, Ubr1, resulting in the rapid degradation of DHFR and the protein to which it is fused. These conditions also provide internal controls for the strain because high-temperature or galactose induction alone are not responsible for the phenotypes observed. Using *pan1-td,* we found that cells are inviable and endocytosis is arrested under the nonpermissive conditions due to the loss of Pan1 protein.

One of the advantages of the *pan1-td* system is the ability to perform genetic screens to discover proteins that can bypass the loss of Pan1 to restore growth in the nonpermissive conditions. A candidate screen found that several components of the Cell Wall Integrity (CWI) pathway were able to restore growth of *pan1-td* cells in non-permissive conditions when overexpressed. In cells, the CWI pathway is used to sense extracellular environments and stressors; specific stressors, such as temperature and pH, can activate the pathway and signal for the cell to upregulate expression of proteins that protect the cell from harmful environments. It was originally thought that Pan1 might be involved in the stress signaling; however, high-copy expression of the CWI components was not able to restore growth in another Pan1 degron system, Pan1-AID. It was concluded that the CWI pathway components were restoring growth by over activating the CWI pathway, which protected the cells in the harmful non-permissive conditions of *pan1-td*. Therefore, all future genetic screens will be conducted in strains, such as Pan1-AID, that have less stressful non-permissive conditions.

MATERIALS AND METHODS

Media and Growth Conditions

Permissive conditions: yeast cells were grown in rich (yeast extract-peptone) or synthetic (yeast nitrogenous base with amino acids selection of plasmid maintenance) medium with 2% dextrose or raffinose at 30°C.

Pan1-td Non-permissive conditions: yeast cells were grown in rich or synthetic medium with 2% galactose at 37°C. For pACT-GEV experiments, B-estradiol was added at a final concentration of 1μM.

Pan1-AID non-permissive conditions: 1mM NAA was added to synthetic media plates.

Strains and Plasmids

The yeast strains and plasmids used in this study are listed in Tables AI.1 & AI.2, respectively. Strains were constructed using PCR-based genomic integration as described previously (Longtine *et al.,* 1998; Goldstein and McCusker, 1999; Nishimura *et al*., 2009). DNA manipulations for plasmid construction were preformed using standard techniques, employing either T4 DNA polymerase-mediated ligations in *Escherichia coli* or homologous recombination with overlapping DNA fragments followed by plasmid rescue in *S. cerevisiae*. All restrictions enzymes were purchased from New England Biolabs (Ispwich, MA).

TABLE AI.I Yeast strains used in this chapter

Strain	Genotype	Source
W303	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100*	Laboratory Strain
BWY3781	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100 HIS::pGAL-UBR1*	Laboratory Strain
BWY4150	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100 HIS::pGAL-UBR1 URA::ub-DHFRts-HA-Pan1*	Laboratory Strain
BWY5270	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100 LEU2::pACT-GEV*	This Study
BWY5271	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100 HIS::pGAL-UBR1 LEU2::pACT-GEV*	This Study
BWY5273	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100 HIS::pGAL-UBR1 URA::ub-DHFRts-HA-Pan1 LEU2::pACT-GEV*	This Study
BWY5274	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100 HIS::pGAL-UBR1 URA::ub-Leu-DHFRts-HA-Pan1*	This Study
BWY5275	*MATa ura3-1 his3-11 leu2,3112 trp1-1 can1-100 HIS::pGAL-UBR1 URA::ub-Ile-DHFRts-HA-Pan1*	This Study
BWY5394	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ADH1-OsTIR1-9myc::URA3 ade2::ADE2 HisΔ::URA Pan1-AID::G418*	This Study

TABLE AI.2 Plasmids used in this chapter

Plasmid	Details	Description	Source
pRS426	2μ, URA3	Empty Vector (EV)	Laboratory plasmid
pBW1473	pRS426::MID2 (2μ, URA3)	pMid2.426	(Prosser et al., 2011)
pBW1623	pRS426::ROM2 (2μ, URA3)	pRom2.426	Laboratory plasmid
pBW1821	pSH24, pSEY18: PCK1 (2μ, URA3)	pPkc1.426	(Helliwell et al., 1998)
pBW1953	pRS426::RHO2 (2μ, URA3)	pRho2.426	Laboratory plasmid
pBW2053	Yep24::ROM1 (2μ, URA3)	pRom1.426	(Prosser et al., 2011)
pBW2054	Yep24::RHO1 (2μ, URA3)	pRHO1.426	(Prosser et al., 2011)
pBW2240	pACT1-GEV (leu2)	pACT-GEV	Goschling Lab
pBW2299	pCup-Ub-DHFRts-HA, H2L (ura3)	DHFR-Leu	This study
pBW2300	pCup-UB-DHFRts-HA, H2I (ura3)	DHFR-Ile	This study

Protein Expression

TCA Precipitation**:**

Mid to late-log phase cells in permissive or non-permissive conditions at indicated time points were harvested and resuspended in 1ml of 10% trichloroacetic acid (TCA) with 1mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, a protease inhibitor), incubated on ice for 20 min, and precipitates were centrifuged at 14,000 rpm for 10 min at 4°C. Precipitates were washed twice with cold acetone, resuspended by sonication, and air-dried. Protein samples were processed for SDS-PAGE separation by adding 2x protein sample buffer with 1mM AEBSF and solubilized by bead disruption.

SDS-PAGE and Immunoblotting:

Proteins were separated on polyacrylamide mini gels (7.5%) at 27mA in SDS running buffer (3mM SDS, 25mM Tris base, 192mm glycine) and then transferred onto nitrocellulose membranes at 80V for 90 min in cold transfer buffer (20% methanol, 0.0375% SDS, 48mM Tris base, 30mM glycine). The membranes were blocked in 5% milk in TBST (10mM Tris, pH 7.5, 0.25M NaCl, 0.025% Tween-20). Blots were incubated in the specified primary antibody overnight at the following concentrations: Rabbit-anti-Pan1 was a gift from D.G. Drubin and was used at 1:10,000; Rabbit-anti-Act1 was a gift from D. Pruyne and was used at 1:5,000. The blots were washed 3 times in TBST, incubated with secondary antibodies conjugated to HRP (Pierce, Rockford, IL) diluted 1:2,000 in milk solution for 45 min. Blots were washed again 3 times in TBST, and then developed with chemiluminescent substrate (Solution 1:2.5mM Luminol, 400uM paracoumaric acid, 100mM Tris-HCl, pH 8.5. Solution 2: 5.4mM H_2O_2, 100mM

Tris-HCl, pH 8.5) for 2 min at room temperature. Chemiluminescence images were acquired using a FluorChem M FM0455 imager.

Growth Assays

Serial Dilutions: Log phase cells were grown in permissive conditions and diluted to OD600 of 0.25, from which four 1:5 serial dilutions were plated in permissive and non-permissive conditions and grown for three days at 30°C.

FM4-64 staining

FM4-64 images were collected using a Zeiss Axiovert 135TV inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a Sensicam QE CCD camera (Cooke, Romulus, MI), Zeiss 100x 1.4 NA Plan-Apochromat objective, motorized filter wheels, fluorescein isothiocynate (FITC) and Texas Red filter sets (Semrock, Rochester, NY), and Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO). Images were captured with 500ms exposure, identical binning, intensification and illumination intensity. Log phase cells were grown in permissive conditions and were switched to permissive or non-permissive conditions for 60 m. For FM4-64 labeling, cell aliquots were transferred to an eppendorf tube with 10ug/ml FM4-64 (Molecular Probes, Eugene, OR) and incubated for 15 min at 30°C or 37°C. The cells were washed twice with fresh media to remove excess FM4-64, resuspended in 100ul fresh media, and then put on ice until visualization at the microscope. Cells were spotted onto uncoated glass slides and covered with a coverslip.

RESULTS

In *pan1-td* cells, Pan1 protein is depleted and cell growth is inhibited upon the switch to nonpermissive conditions

In the *pan1-td* strain, *PAN1* is N-terminally tagged with the temperature sensitive DHFR mutant and the expression of the E3 ubiquitin ligase, Ubr1, is placed under galactose regulation (Sanchez-Diaz *et al.,* 2004). In the non-permissive conditions, 37°C and galactose as the carbon source, Ubr1 is overexpressed and recognizes the N-terminal arginine of the DHFR tag (Figure AI.1A). Neither over activation of Ubr1, nor temperature shifts alone affect the levels of Pan1 or cell growth (Figure AI.1, B and C). However, when both nonpermissive conditions are combined in *pan1-td* cells, Pan1 protein is depleted within 4 hours and the cells cannot grow. Therefore, *pan1-td* can be used to assess the acute phenotypes of the loss of Pan1 on cellular processes, which will uncover the function of Pan1.

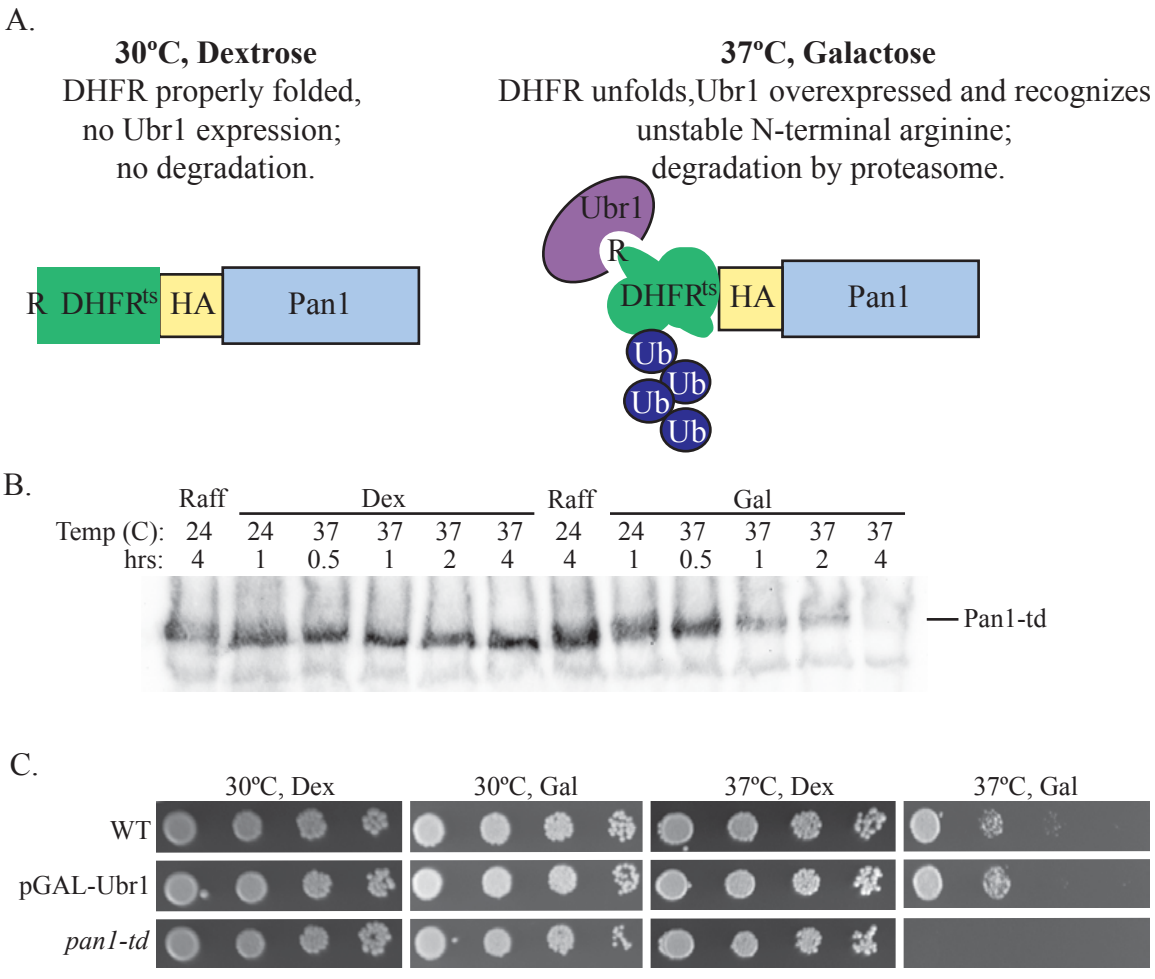


Figure AI.1. *Pan1-td* protein is depleted and cells do not grow in nonpermissive conditions.
(A) Schematic of the DHFR degron tag attached to Pan1 in the permissive and non-permissive conditions. (B) Immunoblot detecting Pan1 of *pan1-td* cell lysates grown for the indicated times in the permissive or non-permissive conditions. (C) Serial dilutions of indicated strains grown 3 d in labeled conditions. EV = empty vector.

Endocytosis is arrested in the absence of Pan1

Pan1 is presumed to play a critical role as an endocytic scaffolding protein; thus, we hypothesized that upon removal of Pan1, endocytosis would be negatively affected. The lipophilic dye FM4-64 binds to the plasma membrane and becomes fluorescent and marks membranes as they are internalized through endocytosis and targeted to the vacuole. In *pan1-td* cells in the permissive conditions, FM4-64 is internalized normally, but upon the switch to nonpermissive conditions, FM4-64 is trapped at the plasma membrane (Figure AI.2). This endocytic arrest confirms that Pan1 is essential for endocytosis and supports results using Pan1-AID (Chapter 2).

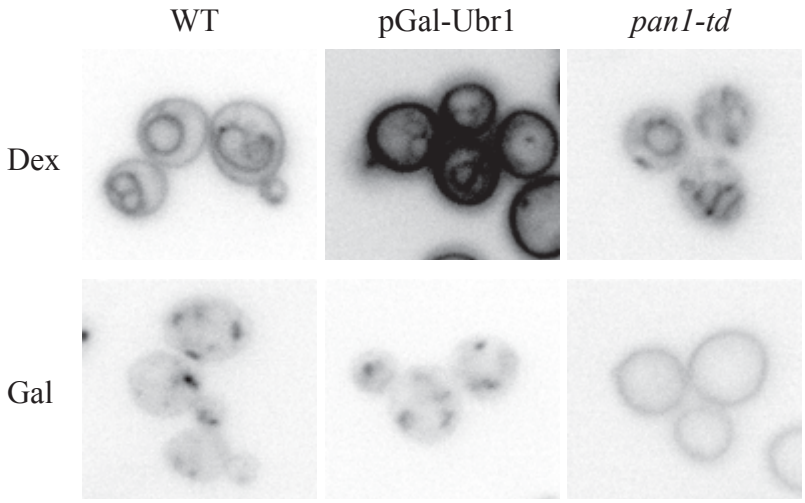
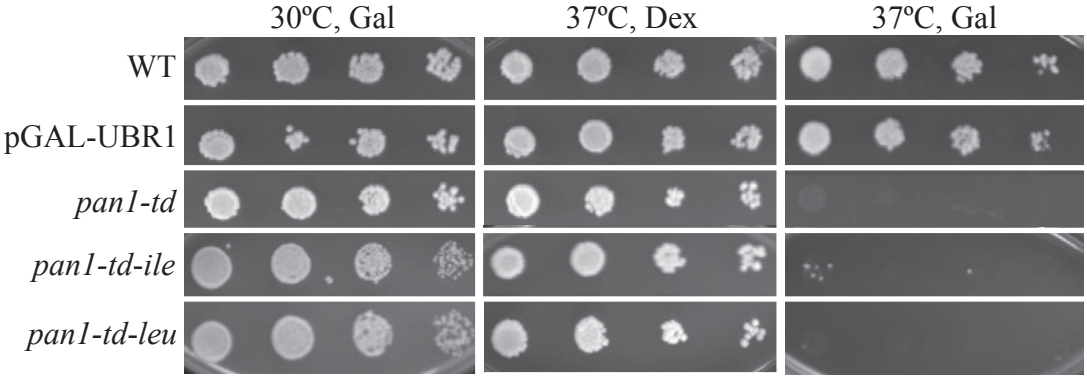


Figure AI.2. Endocytosis is arrested in *pan1-td* cells in the nonpermissive conditions.
Representative images of log-phase cultures grown at 37ºC in permissive or non-permissive conditions for 60 min before labeling with FM4-64 for 15 min.

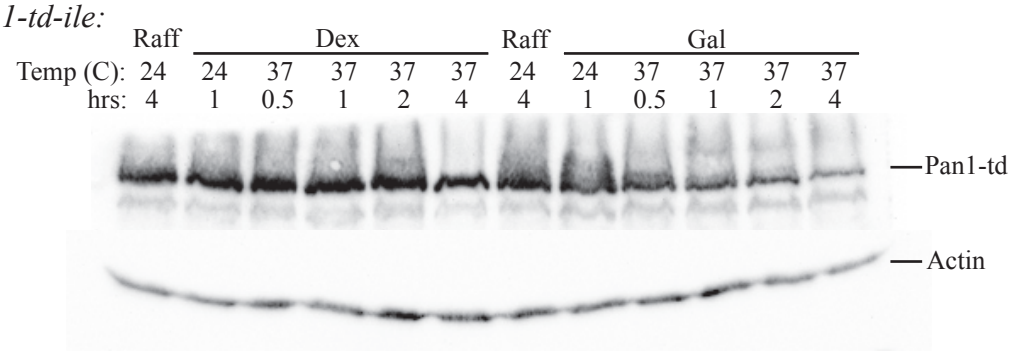
Optimization of Ubr1 recognition of DHFR did not increase Pan1 degradation in *pan1-td*

Degradation of Pan1 in *pan1-td* is dependent upon the temperature sensitive conformational change of DHFR, which exposes an N-terminal arginine that is recognized by Ubr1 for degradation. In *pan1-td*, the recognition and subsequent degradation of Pan1 takes about 3-4 hours, which is significantly slower than the published DHFR model, which degrades the target protein in less than 30 mins (Sanchez-Diaz *et al.,* 2004). Recent work studied the importance of the second amino acid after the first arginine for N-end rule degradation and found that hydrophobic residues are ideal at the second position for efficient degradation (Choi *et al*. 2010). Additionally, they found that proline and histidine were the least efficient; DHFR's second amino acid is histidine. To increase efficiency of *pan1-td* degradation, the second amino acid of DHFR was mutated from histidine to either leucine (*pan1-td-leu*) or isoleucine (*pan1-td-ile*). Both *pan1-td-leu* and *pan1-td-ile* displayed similar growth phenotypes to *pan1-td* in the permissive and nonpermissive conditions (Figure AI.3A). However, Pan1 was degraded in a similar time frame to *pan1-td* in *pan1-td-leu* and *pan1-td-ile* strains (Figure AI.3, B and C). Thus, optimization of the second amino acid did not increase N-end rule degradation efficiency.

A.



B. *pan1-td-ile:*



C. *pan1-td-leu:*

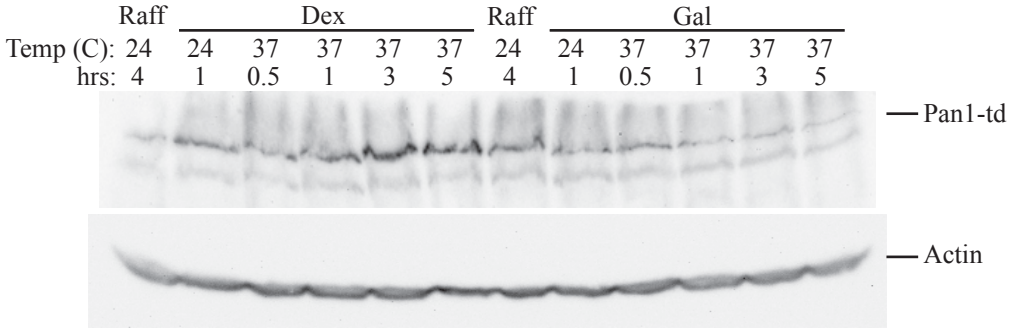


Figure AI.3. Optimization of DHFR tag does not increase efficiency of *pan1-td* degradation.

(A) Serial dilutions of indicated strains grown 3 d in labeled conditions. (B and C) Immunoblots detecting Pan1 and Actin of *pan1-td-ile* (B) and *pan1-td-leu* (C) cell lysates grown for the indicated times in the permissive or non-permissive conditions. Actin is used as a loading control.

B-estradiol induction of Ubr1 increases Pan1 degradation efficiency in *pan1-td*

The non-permissive conditions of *pan1-td* are high-temperature (37ºC) to induce the DHFR conformational change and galactose as the carbon source to induce overexpression of Ubr1. Thus, *pan1-td* cells must be grown at 30ºC with raffinose or dextrose as the carbon source. The abrupt carbon source switch necessary for Ubr1 expression may induce stress responses in the cells that can have off-target effects. To avoid the carbon source switch, a chimeric transcriptional activator, Gal4dbd.ER.V16 (GEV) can be used to recognize and activate transcription at Gal4 consensus sites upon addition of the hormone B-estradiol, instead of galactose, to the media. The pACT-GEV activator was constitutively expressed under the actin promoter in *pan1-td* cells and thus the new nonpermissive conditions are the addition of B-estradiol and the temperature shift. In the nonpermissive conditions, *pan1-td pACT-GEV* degraded Pan1 faster than *pan1-td* and resulted in a growth inhibition (Figure AI.4). Unfortunately, B-estradiol alone induced a growth defect in both *pan1-td* and WT cells containing pACT-GEV (Figure AI.4B). This suggests that pACT-GEV and/or B-estradiol may have other, unwanted, targets in the cells.

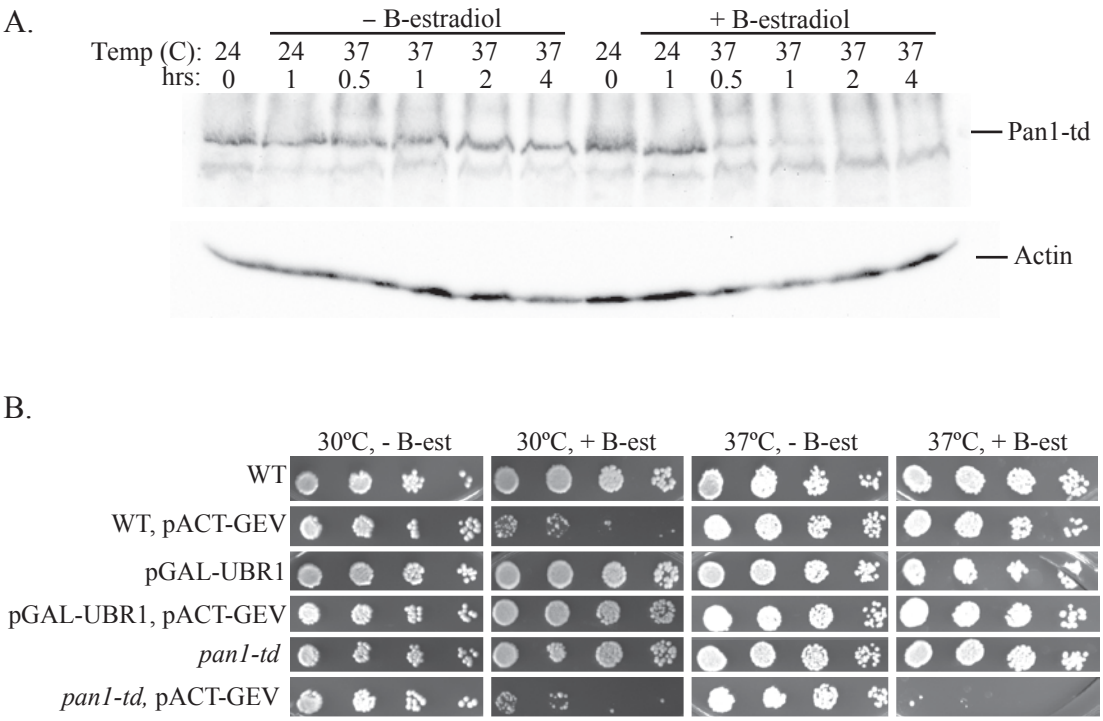


Figure AI.4. Using pACT-GEV and B-estradiol to induce Gal-expression of Ubr1 does not increase efficiency of *pan1-td* degradation.

(A and B) Immunoblots detecting Pan1 and Actin of *pan1-td,* pACT-GEV cell lysates grown for the indicated times in the permissive or non-permissive conditions. Actin is used as a loading control. (C) Serial dilutions of indicated strains grown 3 d in labeled conditions. .

Cell Wall Integrity pathway components restore growth of *pan1-td*, but not Pan1-AID cells

A preliminary candidate high-copy suppressor screen found that *pan1-td*'s lethality under nonpermissive conditions is suppressed by overexpression of Rom2, Rho2, and Pkc1 (K. Whitworth and B. Wendland, unpublished). All three are part of the complex CWI/nutrient-sensing pathway, which is activated during environmental stress to strengthen and repair the cell wall (Fuchs and Mylonakis, 2009). Stress sensors converge to activate Rom2, which is the guanine nucleotide exchange factor (GEF) of the GTPases Rho1 and Rho2 (Bickle *et al.,* 1998). Upon activation, Rho1/2 can activate several pathways to regulate actin cytoskeleton organization, cell wall biosynthesis, and transcription of other CWI genes (Levin, 2005). One of these pathways activates another high-copy suppressor of *pan1-td:* Pkc1, which triggers a MAP-kinase cascade that ultimately leads to transcription of genes involved in CWI (Figure AI.5) (Heinisch 1999). Activation of Rom2 is downstream of several environmental stress sensors. Of the two classic cell wall pH and heat sensors, Mid2 and Wsc1, only Mid2 can rescue *pan1-td* cells (Figure AI.5) (Levin, 2005). The nonpermissive conditions of *pan1-td* alone may induce stress pathways; thus, overexpression of these CWI pathway components could be rescuing *pan1-td* cells simply by priming the stress pathways prior to induction of cell wall stress versus inducing a Pan1-dependent essential pathway. To test this, the Pan1-AID system was used to test if high-copy expression of CWI pathway components could restore growth in the absence of Pan1 and stress induction. None of these proteins were able to restore growth of Pan1-AID cells in the presence of auxin (Figure AI.5). This confirms that Pan1-AID, instead of *pan1-td*, is a more ideal strain for high-copy

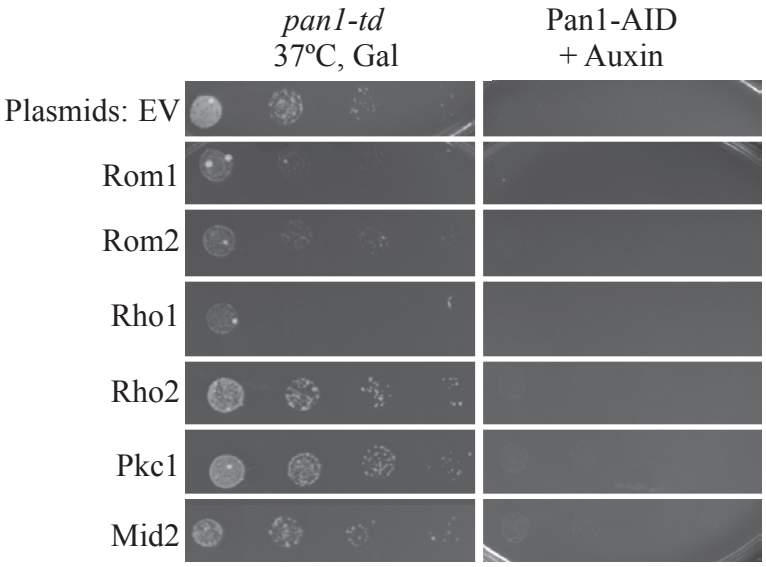


Figure AI.5. Overexpression of Cell Wall Integrity proteins rescues growth of *pan1-td*, but not Pan1-AID, in non-permissive conditions.
Serial dilutions of *pan1-td* and Pan1-AID cells containing indicated high-copy plasmids (on left) grown for 3 d in respective non-permissive conditions.

suppressor screens because it does not induce temperature or carbon source stress pathways.

DISCUSSION

Creating an efficient and robust system to deplete cells of a target protein in nonpermissive conditions without creating extra stress on the cell is critical for uncovering the function of essential proteins. The DHFR-galactose degron system, introduced in Sanchez-Diaz *et al*. (2004), does deplete the target protein upon the switch to nonpermissive conditions. However, these nonpermissive conditions are not innocuous and result in their own phenotypes, which can occlude phenotypes due to loss of the target protein. It is imperative to consider these caveats when evaluating results and include proper controls to validate all phenotypes found using the DHFR-degron system. This system was used to create the *pan1-td* strain, which targets Pan1 for degradation. Upon the switch to the nonpermissive conditions, Pan1 protein is degraded, cells cannot grow, and endocytosis is arrested (Figure AI.1 and AI.2). These results corroborate those from Pan1-AID to confirm that Pan1 is essential for growth and endocytosis (Chapter 2).

In the *pan1-td* cells, Pan1 protein is depleted between 3-4 hours after the switch to nonpermissive conditions; this is depletion is significantly slower than previously reported results for other proteins using the DHFR-degron system, which show complete degradation of target protein within 30 minutes (Sanchez-Diaz *et al.,* 2004). Attempts to increase the efficiency of degradation by optimizing recognition of the DHFR-tag by Ubr1 were not successful (Figure AI.3). This suggests that the slow rate of Pan1 degradation may be specific to Pan1 and not the DHFR system.

Yeast cells require a source of carbon in the media in order to thrive. Although there are several sugars that can be used for this carbon source, not all are optimal for robust growth and switching the carbon source can often activate cellular stress

pathways. In *pan1-td* experiments, cells must be grown in raffinose media, and then switched to media containing galactose to promote Ubr1 overexpression. Cells do not grow well in either condition and the carbon source switch activates stress pathways. To avoid this carbon source shift, the pACT-GEV system was utilized which uses the hormone B-estradiol to induce the galactose promoter through pACT-GEV. Using this system in *pan1-td* cells did result in faster degradation of Pan1 protein, but the presence of B-estradiol did slow growth at the permissive temperature, 30ºC (Figure AI.4). Therefore, although the B-estradiol system does remove the stress of the carbon source shift, it adds a new stress to the cells and is therefore not an ideal system for the study of Pan1 function.

A candidate screen that was conducted to uncover the essential function of Pan1 found that several components of the Cell Wall Integrity (CWI) pathway could restore growth of *pan1-td* cells in non-permissive conditions. The CWI pathway is activated upon cellular stress and upregulates pathways used to protect the cell from these harmful environments. However, these CWI proteins, Rom2, Rho2, Pkc1, and Mid2, when overexpressed were not able to restore growth of Pan1-AID cells in the presence of auxin (Figure AI.5). This means that CWI pathway rescue is specific to *pan1-td* cells and the DHFR degron system, not depletion of Pan1. Overexpression of these components may be priming *pan1-td* cells for the switch to the stressful, non-permissive conditions, allowing for cell growth. Therefore, Pan1 may not be involved in the CWI pathway for its essential function.

For future study of Pan1 functions, it is imperative to use a degron system that introduces the least amount of additional stress to the cells. The Pan1-AID system, as

opposed to the *pan1-td* system, only has one variable, addition of auxin, to induce degradation of Pan1 protein (Pan1-AID system explained in Chapter 2). Auxin and TIR1, as opposed to temperature and carbon source shifts and Ubr1, have no obvious off-target effects on cells. Therefore, Pan1-AID will be used for all further study of Pan1's endocytic and essential function(s).

APPENDIX 2: UNCOVERING THE ESSENTIAL FUNCTION OF PAN1

ABSTRACT

Pan1 regulates and interacts with several endocytic proteins; however, the current list may not be complete. By analogy to Pan1's mammalian homologue ITSN, Pan1 may function in other pathways; thus, learning more about these novel Pan1 interactions should provide insight into ITSN functions. The Pan1-AID strain provides an efficient way to screen for pathways that can suppress lethality in nonpermissive conditions. A high-copy expression library was transformed into the Pan1-AID strain and colonies were tested for rescue of growth of Pan1-AID in the nonpermissive conditions. A trial screen was completed and one candidate gene, Das1, was validated. Das1 is an F-box protein and might be expected to compete with TIR1 for binding to the SCF complex; however, it appears that in the presence of high-copy Das1, Pan1-AID was still degraded. Das1 restored growth of Pan1-AID cells, but it did not restore endocytosis nor did it restore growth of other AID-tagged strains or a *pan1Δ* strain. Further experimentation is required to confirm how Das1 is bypassing the loss of Pan1 to restore viability.

INTRODUCTION

There are currently over 60 proteins associated with the clathrin-mediated endocytic pathway and Pan1 is known to associate, either directly or indirectly, with a number of these. There are still a number of open questions regarding the endocytic process, so it is important to continue to search for novel proteins and interactions involved in the process. Pan1 is thought to have an additional, essential and non-endocytic role in the cells. CME is not an essential process in yeast and Pan1's mammalian homologue, Intersectin (ITSN), has non-endocytic functions, thus we hypothesized that Pan1 may have another, non-endocytic, essential role (Payne *et al.*, 1988; Chu *et al.*, 1996; Madania *et al.*, 1999; Kaksonen *et al.*, 2005; Newpher and Lemmon, 2006; Prosser *et al.*, 2011, Tysba *et al.,* 2011).

Genetic screens in yeast are an efficient and rapid method to uncover novel proteins involved in conserved cellular processes. In a high-copy suppressor screen, a genetic library of plasmids containing fragments of genomic DNA is transformed into a strain lacking a particular protein or pathway. Candidate high-copy suppressor proteins are selected by their ability to restore the targeted defect phenotype. The Pan1-AID strain provides a unique opportunity to discover, for the first time, proteins that can bypass the loss of Pan1 by restoring growth in the presence of auxin. Candidate high-copy suppressors could either upregulate an essential pathway that Pan1 is involved in or a parallel pathway that functions similarly. Alternatively, a high-copy suppressor could inhibit the death pathway that is induced in the absence of Pan1. Discovery of a protein in either of these groups would provide insight into Pan1's unknown essential function(s).

From the Pan1-AID high-copy suppressor screen, one high-copy suppressor was validated—Das1 (YJL149w). Although Das1 is an F-box protein, it did not appear to compete for binding with TIR1 to the SCF complex, as Pan1 protein was still depleted in the presence of high-copy Das1. Das1 was not able to restore the growth in a *pan1Δ* strain, indicating that Das1 may not be upregulating an essential pathway. The exact interaction between Das1 and Pan1 needs to be further characterized.

MATERIALS AND METHODS

Media and Growth Conditions

Yeast cells were grown in rich (yeast extract-peptone) or synthetic (yeast nitrogenous base with amino acids selection of plasmid maintenance) medium with 2% dextrose. For experiments using auxin in liquid cultures, 3-Indoleacetic acid (Sigma I2886) was added to synthetic media to a final concentration of 500μm. For experiments using auxin on solid media, 1-Naphthaleneacetic acid (Sigma N0640) was added to synthetic media to a final concentration of 1mM. For plasmid shuffles,

Strains and Plasmids

The yeast strains and plasmids used in this study are listed in Tables AII.1 and AII.2, respectively. Strains were constructed using PCR-based genomic integration as described previously (Longtine *et al.,* 1998; Goldstein and McCusker, 1999; Nishimura *et al.,* 2009). DNA manipulations for plasmid construction were preformed using standard techniques, employing either T4 DNA polymerase-mediated ligations in *Escherichia coli* or homologous recombination with overlapping DNA fragments followed by plasmid rescue in *S. cerevisiae*. All restrictions enzymes were purchased from New England Biolabs (Ispwich, MA).

TABLE AII.1 Yeast strains used in this chapter

Strain	Genotype	Source
BWY2618	*MATα ura3-1 ade2-1 his3-11 leu2,3112 trp1-1 can1-100 pan1::HIS Pan1.416 (pBW513)*	Laboratory Strain
BWY5393	MATα *ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ade2::ADE2 ADH1-OsTIR1-9myc::URA3 HisΔ::URA*	This Study

BWY5394	*MATα ura3-1 ade2-1 his3-11, 15 leu2-3,112 trp1-1 can1-100 ADH1-OsTIR1-9myc::URA3 ade2::ADE2 HisΔ::URA Pan1-AID::G418*	This Study
BWY6481	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 QRI1-TAP-AID::URA3*	Cunningham Lab
BWY6482	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 Las17-TAP-AID-Flag::URA3*	Cunningham Lab

TABLE AII.2 Plasmids used in this chapter

Plasmid	Details	Description	Source
pRS425	2μ, LEU2	Empty Vector (EV)	Laboratory plasmid
pRS426	2μ, URA3	Empty Vector (EV)	
pBW010	pRS415::PAN1 (CEN, LEU2)	pPan1.415	Emr Lab
pBW513	pRS416::PAN1 (CEN, URA3)	pPan1.416	Sachs et al., 1992
pBW2652	pRS426::DAS1 (2μ, URA3)	pDas1.426	This study
pBW2695	pRS425::DAS1 (2μ, LEU2)	pDas1.425	This study

High-copy suppressor screen

A uracil high-copy library expressing genomic fragments was transferred into Pan1-AID cells and plated onto YNB-uracil-auxin plates. Colonies were replicated plated onto YNB-uracil + 1mM NAA plates to select for colonies that restored growth in the presence of auxin. Positive colonies were screened for false positives by selecting for revertants (colonies that restore growth in the presence of auxin after plasmid curing on 5FOA) and did not contain Pan1 on the plasmid. Plasmids were rescued from candidate colonies and sequenced.

Protein Expression

TCA Precipitation**:**

Mid to late-log phase cells -/+ Auxin at indicated time points were harvested and resuspended in 1ml of 10% trichloroacetic acid (TCA) with 1mM 4-(2-

aminoethyl)benzenesulfonyl fluoride (AEBSF, a protease inhibitor), incubated on ice for 20 min, and precipitates were centrifuged at 14,000 rpm for 10 min at 4°C. Precipitates were washed twice with cold acetone, resuspended by sonication, and air-dried. Protein samples were processed for SDS-PAGE separation by adding 2x protein sample buffer with 1mM AEBSF and solubilized by bead disruption.

SDS-PAGE and Immunoblotting:

Proteins were separated on polyacrylamide mini gels (7.5%) at 27mA in SDS running buffer (3mM SDS, 25mM Tris base, 192mm glycine) and then transferred onto nitrocellulose membranes at 80V for 90 min in cold transfer buffer (20% methanol, 0.0375% SDS, 48mM Tris base, 30mM glycine). The membranes were blocked in 5% milk in TBST (10mM Tris, pH 7.5, 0.25M NaCl, 0.025% Tween-20). Blots were incubated in the specified primary antibody overnight at the following concentrations: Rabbit-anti-Pan1 was a gift from D.G. Drubin and was used at 1:10,000; Rabbit-anti-Act1 was a gift from D. Pruyne and was used at 1:5,000. The blots were washed 3 times in TBST, incubated with secondary antibodies conjugated to HRP (Pierce, Rockford, IL) diluted 1:2,000 in milk solution for 45 min. Blots were washed again 3 times in TBST, and then developed with chemiluminescent substrate (Solution 1:2.5mM Luminol, 400uM paracoumaric acid, 100mM Tris-HCl, pH 8.5. Solution 2: 5.4mM H_2O_2, 100mM Tris-HCl, pH 8.5) for 2 min at room temperature. Chemiluminescence images were acquired using a FluorChem M FM0455 imager.

Growth Assays

Serial Dilutions: Log phase cells were diluted to OD600 of 0.25, from which four 1:5 serial dilutions were plated on YNB-tryptophan minus or plus 1mm NAA and grown for three days at 30°C.

Plasmid Shuffle: *pan1Δ* cells expressing *PAN1* from a *URA3* plasmid were used for counterselection on 5-FOA plates. Cells cotransformed with *PAN1* or *DAS1 LEU2* plasmids were grown on control and 5-FOA plates at 30ºC for 3 d.

Fluorescence microscopy

Mup1-pHl:

Mup1-pHlourin images were collected using a Zeiss Axiovert 135TV inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a Sensicam QE CCD camera (Cooke, Romulus, MI), Zeiss 100x 1.4 NA Plan-Apochromat objective, motorized filter wheels, fluorescein isothiocynate (FITC) and Texas Red filter sets (Semrock, Rochester, NY), and Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO). Images were captured with 500ms exposure, identical binning, intensification and illumination intensity. For quantification of fluorescence intensity, 16-bit image files were analyzed in Image J v1.41n. Background subtraction was performed before measurement of integrated density, and values were corrected for cell size.

Image/Statistical Analysis

Statistical significance between populations was determined by one-way ANOVA followed by Tukey's Multiple Comparison post hoc analysis.

RESULTS

A high-copy suppressor screen identifies Das1 as a potential suppressor of Pan1-AID death

A high-copy expression library was transformed into the Pan1-AID strain and colonies were tested for rescue of growth of Pan1-AID in the presence of auxin. A trial screen was completed and one candidate gene, Das1, was validated (Figure AII.1A). Das1, Dst1-delta 6-Azauracil Sensitivity 1, was so named in 2012 based on its ability to suppress the sensitivity of Transcription Factor IIS-deficient cells to 6-Azauracil (Gomez-Herreros *et al.,* 2012). Das1/YJL149w was originally described as a putative F-box protein based on its physical association with other SCF complex members Skp1 and Cdc53 and its genetic interaction with Cdc34, the E2 ubiquitin-conjugating enzyme that functions with the SCF complex in ubiquitin-dependent proteolysis (Willems *et al*., 1999). Currently, the galactose-inducted transcriptional repressor Mig2 is the only known target of Das1 (Lim *et al*., 2011).

All screens have the potential to find false positive candidate proteins—either proteins that encode the depleted protein (in this case, Pan1) or proteins that disrupt the nonpermissive conditions. In the high-copy suppressor screen in Pan1-AID cells, any protein that directly or indirectly inhibits the degradation of Pan1 will allow growth of the cells in the presence of auxin. To rule out these proteins, it is critical to observe the Pan1 levels in the presence of the high-copy suppressor candidate in the presence of auxin to ensure Pan1 protein is depleted. Das1 is an F-box protein and might be expected to compete with TIR1 for binding to the SCF complex, thus potentially decreasing or inhibiting the degradation of Pan1-AID in the presence of auxin. However, in the

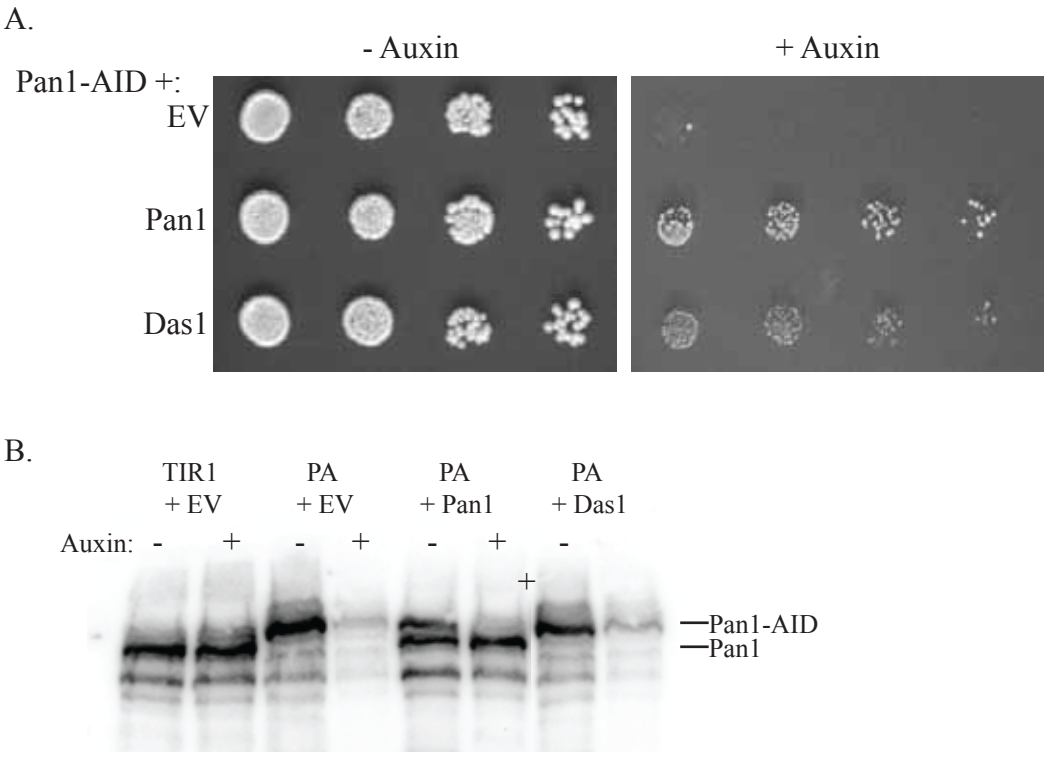


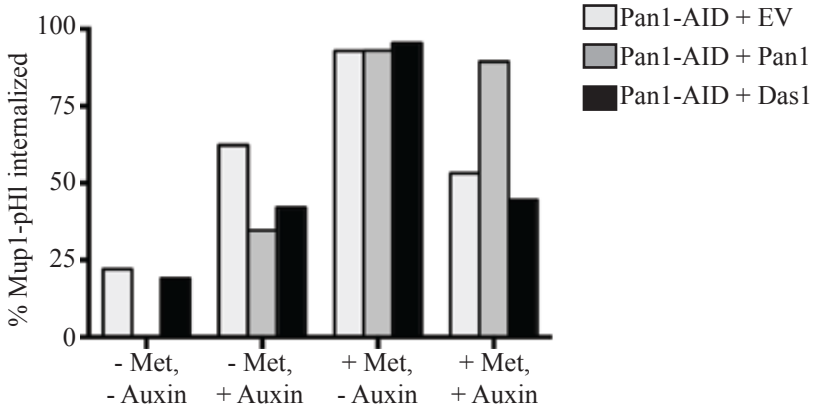
Figure AII.1: Das1 is a candidate high copy suppressor of Pan1-AID.
(A) Serial dilutions of Pan1-AID cells with indicated plasmids were grown on synthetic media ± auxin for 3 d at 30°C. EV = empty vector. (B) Immunoblots detecting Pan1 in TIR1 or Pan1-AID (PA) cell lysates plus indicated plasmids grown for 4 hr ± auxin.

presence of high-copy Das1, Pan1-AID was still degraded to similar levels to Pan1-AID containing an EV plasmid after 4 hours in the presence of auxin (Figure AII.1B).

High-copy Das1 does not restore endocytosis in Pan1-AID cells plus auxin

Das1 is an F-box protein that has no known roles in endocytosis. However, it may still have an indirect role in regulating the process through Pan1 and this function could be lost in the absence of Pan1. To determine if high-copy Das1 can restore Pan1-AID endocytic defects, a Mup1-pHl assay used to observe and quantify CME in the absence of Pan1 and presence of high-copy Das1 (Mup1-pHl assay explained in detail in Chapter 2). Mup1-pHl remained at the plasma membrane in presence of auxin in Pan1-AID cells expressing high-copy Das1, similar to empty vector plasmid (Figure AII.2). This suggests that Das1 was not able to rescue the endocytic arrest that occurs in the absence of Pan1. This indicates that Das1 does not restore growth in the absence of Pan1 by upregulating CME; could Das1 be functioning in Pan1's essential pathway? More experiments will need to be conducted to characterize Das1's function in relation to Pan1.

A.



B.

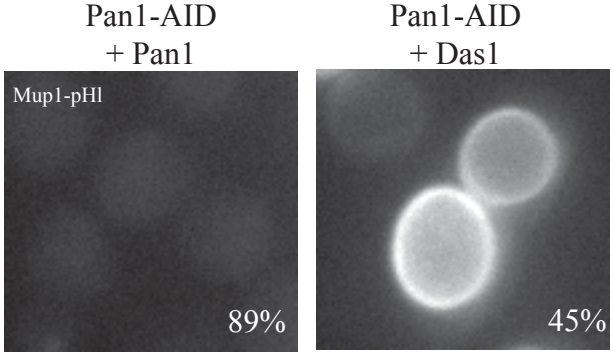


Figure AII.2 High-copy Das1 does not restore endocytosis in Pan1-AID cells plus auxin.
(A) Quantification of Mup1-pHl fluorescence internalized after 30 min ± auxin pre-treatment and 1 h ± methionine in Pan1-AID cells plus indicated plasmids. Percent Mup1-pHl internalized was calculated by measuring the amount of fluorescence at the end of the assay compared to the amount at the beginning. (B) Representative images of Pan1-AID cells plus indicated plasmid ± pre-treatment with auxin for 30 min followed by addition of methionine for 1 h. Percentage is amount of Mup1-pHl internalized over experiment.

High-copy Das1 does not promote viability of *pan1Δ* cells

Presumably, if high-copy Das1 upregulated a pathway that rescues Pan1-AID cells from death due to absence of Pan1, then high-copy Das1 should restore viability of *pan1Δ* cells as well. However, in a plasmid shuffle experiment high-copy Das1 did not rescue *pan1Δ* cells (Figure AII.3). This indicates that Das1 is acting as suppressor only in the AID background and, although Pan1 is being depleted, Das1 may be disrupting another AID specific pathway that is independent of Pan1.

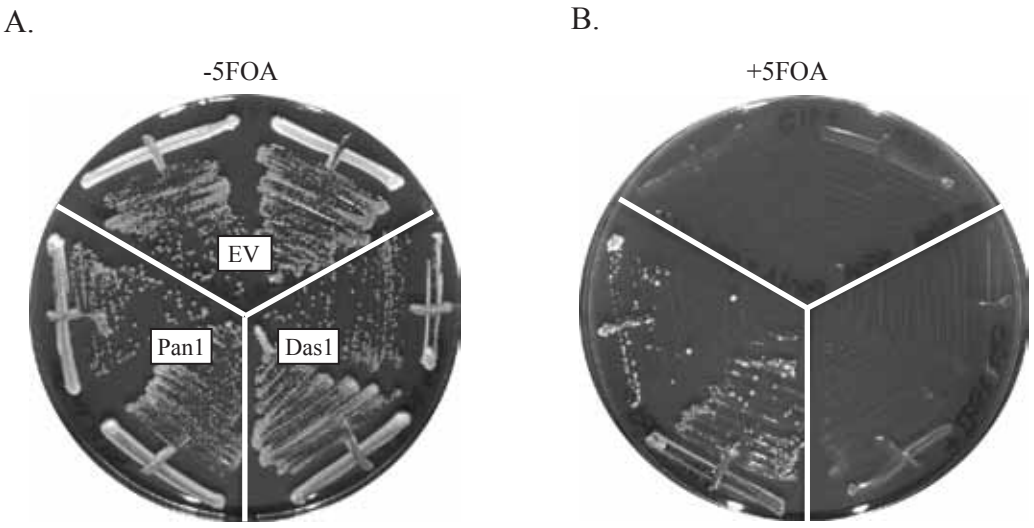


Figure AII.3: High-copy Das1 does not promote the viability of *pan1Δ* cells.
pan1Δ cells containing Pan1 on a uracil-selection plasmid plus indicated plasmids were streaked onto control (A) or 5FOA (B) plates and grown for 3 d at 30°C. EV = empty vector.

High-copy Das1 does not restore the growth of other AID strains in the presence of auxin

In order to test if Das1's restoration of growth of Pan1-AID was specific to Pan1 or to the AID system, it was necessary to test if Das1 could restore the growth of other AID-tagged strains. Qri1 is an essential protein and when tagged with AID, does not grow in the presence of auxin (Cunningham lab, unpublished data). Las17, while not an essential protein, does have a severe growth deficiency when deleted; when tagged with AID, the cells have a growth deficiency in the presence of auxin. High-copy Das1 did not restore the growth of Qri1-AID or Las17-AID (Figure AII.4). This indicates that Das1 is not a general high-copy suppressor of the AID system and is not competing with TIR1 to inhibit degradation of the AID-tagged target proteins.

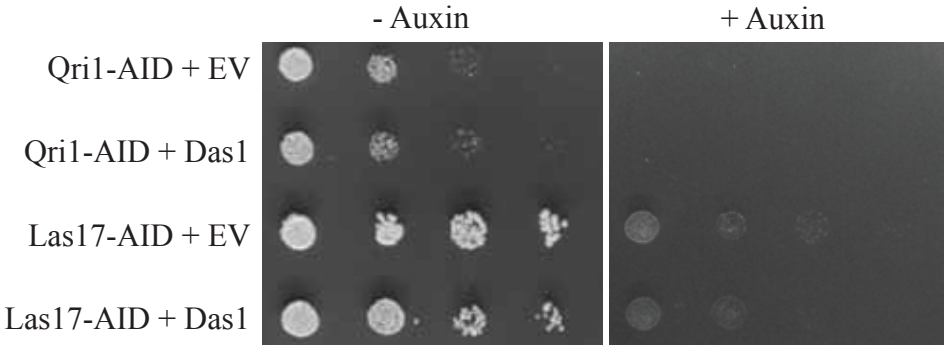


Figure AII.4 High-copy Das1 does not restore growth of other AID-tagged strains. Serial dilutions of Las17-AID and Qri1-AID cells with indicated plasmids were grown on synthetic media ± auxin for 3 d at 30°C. EV = empty vector.

DISCUSSION

The AID system is ideal for genetic screens because the nonpermissive conditions are fairly innocuous to the cells and should not induce a stress response or alter endocytic dynamics. In a high-copy suppressor screen of Pan1-AID, positive candidates will be selected for their ability to restore growth of Pan1-AID cells in the presence of auxin. In this system, three categories of candidate proteins should be found in the screen—Pan1, proteins that inhibit the degradation of Pan1, and proteins that bypass the loss of Pan1 to restore viability through a yet unknown pathway. Candidate library plasmids containing Pan1 were found in the screen, which validates that the screen worked as expected.

In this small pilot screen, only one candidate protein was validated to restore growth and not inhibit Pan1 degradation—Das1. Das1 is an F-box protein that one might expect, in theory, would compete with TIR1 for binding to the SCF complex and may inhibit degradation of Pan1. However, after 4 hours in the presence of auxin, in Pan1-AID cells with high-copy Das1, Pan1 protein was degraded to levels similar to EV (Figure AII.1B).

How is Das1 restoring growth? Pan1's main role in the cell is in CME, but high-copy Das1 does not restore endocytosis in Pan1-AID cells (Figure AII.2). Therefore, Das1 may be upregulating Pan1's unknown essential pathway. However, Das1 cannot restore growth of *pan1Δ* cells (Figure AII.3), which suggests that Das1 may require a trace amount of Pan1 protein that is present in the cell before it gets degraded in order to support viability. Is Das1 a general suppressor of the AID system? It appears not, as high-copy Das1 cannot restore growth of two other AID-tagged strains (Figure AII.4). The

data in Figures AII.3 and AII.4 seem contradictory, as one would predict that if a high-copy suppressor is not a general suppressor of the AID system, it is specific to the target protein and should restore viability it the delete strain. Further experiments will be conducted to clarify how Das1 is restoring viability and to observe the interactions between Pan1 and Das1. Do other F-box proteins restore viability? Preliminary results suggest that this rescue is specific to Das1 (data not shown). Does Das1 have an alternative role in the cell besides functioning as an F-box protein? Or, could Pan1's essential role be in assisting Das1 in its F-box function?

In the future, another high-copy suppressor screen will be conducted to find proteins that can complement the minimal essential region, Pan1 AA402-855, to restore growth. This screen can be done either in the AID-background or using a plasmid-shuffle based assay in a *pan1Δ* strain. We predict that candidate proteins would include proteins that may interact with this region directly or bind to one of the three flanking essential regions (reviewed in Chapter 4) in order to upregulate an essential pathway.

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CURRICULUM VITAE

Mary Katherine "Kate" Bradford

DOB: [redacted]

Department of Biology
The Johns Hopkins University
3400 N. Charles Street, Mudd Hall, Room 48
Baltimore, MD 21218-2685

Phone (cell): [redacted]
E-mail: mbradfo6@jhu.edu
[redacted]

Education

2015	Ph.D., Cellular, Molecular, and Developmental Biology and Biophysics Thesis Defense: April 24, 2015 The Johns Hopkins University
2009	B.S., Biology University of North Carolina at Chapel Hill
2008	Study Abroad University of New South Wales, Sydney, Australia

Research Experience

2010-present	Doctoral Thesis Research Department of Biology, The Johns Hopkins University Advisor: Dr. Beverly Wendland *Defining the essential and endocytic functions of Pan1 in Saccharomyces cerevisiae*
2009-2010	Postbaccalaureate Intramural Research Training Award Fellow *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health Advisor: Dr. Mary Lilly Developmental regulation of the cell cycle in Drosophila oogenesis
2007-2009	Undergraduate Research Assistant Lineberger Comprehensive Cancer Center, UNC-Chapel Hill Advisor: Dr. Norman Sharpless Developed a novel murine melanoma model to study the roles of various tumor suppressors and oncogenes
2006	Research and Development Intern Tri Tech Laboratories, Inc. Developed new products and re-designed older products for Bath and Body Works

Publications

1. **Bradford, MK**, Whitworth, K, Wendland, B. (2015). Pan1 regulates transitions between stages of clathrin-mediated endocytosis. *Mol. Biol. Cell.* 26*,* 1371-1385.

2. Whitworth, K, **Bradford MK**, Camara, N, Wendland B. (2014)**.** Targeted disruption of an EH-domain protein endocytic complex, Pan1-End3. *Traffic.* 15 (1), 43-59.

3. Carten, JD, **Bradford MK**, Farber, S. (2011)**.** Visualizing digestive organ morphology and function using differential fatty acid metabolism in live zebrafish. *Dev. Biol.* 360(2), 276-285.

4. Monahan, KB, Rozenberg, GI, Krishnamurthy, J, Johnson S, **Bradford MK**, Horner, J., DePinho, RA, Sharpless, NE. (2010) Somatic $p16^{INK4a}$ loss accelerates melanomagenesis. *Oncogene.* 29 (43), 5809-5817.

Posters

2014	Defining the endocytic function of Pan1 **American Society for Cell Biology Annual Meeting**, Philadelphia, PA
2013	Defining the essential function of Pan1 **American Society for Cell Biology Annual Meeting,** New Orleans, LA
2012	Defining the essential function of Pan1 **Yeast Genetics and Molecular Biology Meeting**, Princeton, NJ
2009	The Role of LKB1 in Melanoma **Celebration of Undergraduate Research Symposium**, UNC-Chapel Hill.

Honors and Awards

2015	Science Outreach Certificate Department of Biology, The Johns Hopkins University
2013	Elevator Speech Contest Winner American Society for Cell Biology Annual Meeting, New Orleans, LA
2012	Best Poster Award Department of Biology Retreat, The Johns Hopkins University
2009	Bachelor of Science with Distinction in Biology University of North Carolina at Chapel Hill
2005-2009	Dean's List, 3 semesters University of North Carolina at Chapel Hill

Teaching Experience

2014	Grading Teaching Assistant Cell Biology, The Johns Hopkins University
2014	Teaching Assistant Vaccines: Past, Present, and Future, The Johns Hopkins University
2012	Laboratory and Grading Teaching Assistant Cell Biology, The Johns Hopkins University
2011	Laboratory and Grading Teaching Assistant Genetics, The Johns Hopkins University

Training/Supervisory Experience

2012-present	2 graduate students, 1 summer undergraduate student

Leadership Experience

2013-2015	Cardio Kickboxing Instructor, JHU Recreation Center Aerobics & Fitness Association of America Group Fitness Instructor Certification
2012-2014	Southeast Regional Outreach Chair National Association for Graduate-Professional Students (NAGPS)
2011-2014	Legislative Chair (2013-2014) Biology Department Representative (2011-2013) Graduate Representative Organization, The Johns Hopkins University
2010-2015	Mentoring to Inspire Diversity in Science
2005-2014	Alumni Advisory Committee (2012-2014) New Member Coordinator (2007) Pi Beta Phi Fraternity for Women

Professional Development

2013	NAGPS National Conference, Western Michigan University
2013 (Sept)	NAGPS Legislative Action Days, Washington, DC
2013 (March)	NAGPS Advocacy Summit and Legislative Action Days, Washington, DC
2013	NAGPS Southeast Regional Conference, George Mason University
2012-present	Associate Faculty Member Faculty of 1000 (Cell Biology-Membrane and Sorting Division, 1 contributed evaluation)

Memberships

2013-present	American Association for the Advancement of Science
2013-present	American Society for Cell Biology
2012-present	Genetics Society of America